# Biological Research on Industrial Yeasts

## Volume III

Editors

**Graham G. Stewart**
Labatt Brewing Company
London, Ontario, Canada

**Inge Russell**
Labatt Brewing Company
London, Ontario, Canada

**Ronald D. Klein**
The Upjohn Company
Kalamazoo, Michigan

**Ronald R. Hiebsch**
The Upjohn Company
Kalamazoo, Michigan


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# FOREWORD

One of the major goals of the representatives from Upjohn and Labatt Brewing Company, Ltd. in planning this symposium* was to bring together, in one forum, industrial and academic scientists. It was concluded that both the topic and timing of the symposium were appropriate for disproving several "myths" surrounding the relationship between academic and industrial research. These myths range from industrial research being solely applied, to the widely held feeling that the focus of academic and industrial research differ significantly. The organizers of the symposium wished to address these misconceptions in several ways. First, it appeared that the topics of interest were common both to academic and industrial researchers, and that both the title and content of the symposium should reflect the attempt to bridge the usually presumed "academic-industrial gap" in research interests. Next, the participants and their contributions should reflect the full range of research interests, from studies of the basic mechanisms of yeast molecular biology to application of yeast expression technologies in industries as diverse as brewing, oil production and pharmaceuticals. Finally, it was concluded that the state-of-the-art was of interest, and not review-type papers, and the participants were requested to prepare their presentations accordingly.

The results of the experiment, at least as measured by the anecdotal observations of both participants and organizers, indicated that a successful experiment has been carried out in essentially all respects. International participants from both industrial and academic laboratories engaged in lively presentations and debate throughout the symposium, and new, unpublished research results were at the core of most presentations. Furthermore, research results presented as posters received equal weight to oral presentations and are included as full manuscripts in this current publication.

It is a great pleasure, therefore, to thank both the sponsors of the symposium, The Upjohn Company and Labatt Brewing Company, Ltd., and the participants for a stimulating and scientifically important symposium. It is our hope that the proceedings of this symposium will not only reflect the exciting synergism that occurred during the symposium, but will also contribute substantially to the scientific literature in this area.

**Ralph E. Christoffersen, Ph.D.**
Vice President
Biotechnology and Basic Research Support
The Upjohn Company

* *Symposium on the Biochemistry and Molecular Biology of Industrial Yeasts*, Brook Lodge, Kalamazoo, Mich., October 25 to 30, 1985, sponsored jointly the The Upjohn Company, Kalamazoo and Labatt Brewing Company, Ltd., London, Ontario, Canada.

## THE EDITORS

**Graham G. Stewart, Ph.D., D.Sc.,** is Director of Research and Quality Control for the Labatt Brewing Company, Ltd., London, Ontario, Canada. He received his B.Sc. Hons. Microbiology and B.Sc. Hons. Biochemistry degrees from the University of Wales, his Ph.D. in Microbial Biochemistry from Bath University, and a D.Sc. from Bath University in 1983. Between 1967 and 1969, Dr. Stewart lectured in Biochemistry at the Portsmouth College of Technology. He joined the Labatt Brewing Company in 1969 and has worked, published, and lectured extensively on the biochemistry of yeast.

Dr. Stewart, as Chairman of the International Commission for Yeasts, organized the 5th International Symposium for Yeasts held in London, Ontario in July, 1980. He is presently Vice-Chairman of the International Commission for Yeasts and Secretary of the Biotechnology Commission of IUPAC. He is co-editor of the CRC Press journal, *Critical Reviews in Biotechnology,* is on the editorial board of the *Journal of Food and Microbiology* and of *Biotechnology Letters,* as well as being on the advisory board of *Microbiological Sciences.* In addition to editing a number of books, Dr. Stewart has published over 100 original papers, patents, and reviews.

**Inge Russell, M.Sc.,** is Manager of the Microbiology/Genetics Section of the Research Department of the Labatt Brewing Company. She received her B.Sc. in Biology from the University of Western Ontario and her M.Sc. from the University of Strathclyde. She joined the Labatt Brewing Company in 1979 and has carried out research in the field of yeast genetics and biochemistry.

She is a co-editor of the CRC Press journal, *Critical Reviews in Biotechnology,* and is also on the editorial board of the *Journal of Industrial Microbiology.* In addition to editing a number of books, she has published over 40 original papers, patents, and reviews.

**Ronald Klein, Ph.D.,** is a Research Scientist in Molecular Biology Research at The Upjohn Company, Kalamazoo, Michigan. Dr. Klein began his scientific career after completion of his B.A. in Humanities and M.A. in International Studies at Western Michigan University. He majored in Biology and Chemistry at Kalamazoo College, completed his Senior Research Project under Dr. Oldrich K. Sebek at The Upjohn Company, and was a Graduate Fellow in the Biochemistry Department at Wayne State University. Dr. Klein received his Ph.D. in Molecular Biology from the University of Wisconsin-Madison followed by postdoctoral study at the Massachusetts Institute of Technology with Dr. H. Gobind Khorana. He was a Research Scientist in the Biotechnology Division of the Phillips Petroleum Company before joining The Upjohn Company in 1984. Dr. Klein is carrying out research in the molecular biology and biochemistry of industrial yeasts.

**Ronald R. Hiebsch, B.Sc.,** is a Research Associate in Molecular Biology Research at The Upjohn Company. Mr. Hiebsch completed his B.Sc. at Kansas State University where he worked on the mechanism of T-7 DNA replication. Mr. Hiebsch was a Research Assistant in the Department of Cell Biology and Anatomy at John Hopkins Medical School and later was a Research Associate in the Department of Biological Carcinogenesis at The Frederick Cancer Research Facility where he worked on the molecular biology of a C-typed retrovirus. His work at The Upjohn Company is focused on the biochemistry and molecular biology of industrial yeasts.

## ACKNOWLEDGMENTS

The editors wish to acknowledge the support of The Upjohn Company and Labatt Brewing Company, Ltd., in the organization of the symposium and in the compilation of this monograph. Particular gratitude is given to Janice Riddell who painstakingly assembled the program and then compiled the original manuscripts of this document. Gratitude is also given to Patrick J. Delehanty, Kathy J. Hiestand, Laurie A. Tuinstra, Gurnam S. Gill, and Phillip G. Zaworski of The Upjohn Company, and to Chandra J. Panchal and Carl A. Bilinski of Labatt Brewing Company whose advise and assistance in the organization of the symposium was invaluable.

# CONTRIBUTORS

**Vytas A. Bankaitis, Ph.D.**
Department of Biology
University of Illinois
Urbana, Illinois

**Hal Blumberg**
Department of Biochemistry
University of Washington
Seattle, Washington

**Cheryl Brazzell**
Molecular and Cellular Biology
Eli Lilly and Company
Indianapolis, Indiana

**Marian Carlson, Ph.D.**
Assistant Professor
Department of Genetics and Development
College of Physicians and Surgeons
Columbia University
New York, New York

**Christina Y. Chen, M.Sc.**
Research Associate
Department of Cell Genetics
Genentech, Inc.
South San Francisco, California

**Wim de Koning**
Department of Microbiology
State University of Groningen
Haren, The Netherlands

**Anneke C. Douma**
Department of Microbiology
State University of Groningen
Haren, The Netherlands

**K.S. Dumas**
Central Research and Development
Department
E.I. du Pont de Nemours and Company
Experimental Station
Wilmington, Delaware

**Scott D. Emr, Ph.D.**
Division of Biology
California Institute of Technology
Pasadena, California

**Mark C. Fagan**
Department of Microbiology
University of Illinois
Urbana, Illinois

**S.C. Falco, Ph.D.**
Central Research and Development
Department
E.I. du Pont de Nemours and Company
Experimental Station
Wilmington, Delaware

**D.J. Fantin**
Department of Biochemistry
The University of Western Ontario
and
Department of Clinical Pathology
Victoria Hospital
London, Ontario, Canada

**J.E. Golin, Ph.D.**
Central Research and Development
Department
E.I. du Pont de Nemours and Company
Experimental Station
Wilmington, Delaware

**Leonard Guarente, Ph.D.**
Associate Professor
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts

**William Hansen, Ph.D.**
Department of Biochemistry and
Biophysics
University of California, San Francisco
San Francisco, California

**Wim Harder, Ph.D.**
Professor
Department of Microbiology
State University of Groningen
Haren, The Netherlands

**Toinette Hartshorne**
Department of Biochemistry
University of Washington
Seattle, Washington

**Richard C. Heimsch, Ph.D.**
Professor
Department of Bacteriology and Biochemistry
University of Idaho
Moscow, Idaho

**Ronald A. Hitzeman, Ph.D.**
Senior Scientist
Department of Cell Genetics
Genentech, Inc.
South San Francisco, California

**Eduard C. Hurt**
European Molecular Biology Laboratory
Heidelberg, West Germany

**Meher H. Irani**
Senior Scientist
ZymoGenetics, Inc.
Seattle, Washington

**Sue Jinks-Robertson, Ph.D.**
Department of Biology
Emory University
Atlanta, Georgia

**Lianna M. Johnson**
Division of Biology
California Institute of Technology
Pasadena, California

**Helena Kopu, Ph.D.**
Biochemist
Research Laboratories
Finnish State Alcohol Company
Alko Ltd.
Helsinki, Finland

**Matti Korhola, Ph.D.**
Head, Department of Microbiology
Research Laboratories
Finnish State Alcohol Company
Alko Ltd.
Helsinki, Finland

**Pirkko L. Liljeström, M.Sc.**
Geneticist
Research Laboratories
Finnish State Alcohol Company
Alko Ltd.
Helsinki, Finland

**R.E. McDevitt**
Central Research and Development Department
E.I. du Pont de Nemours and Company
Experimental Station
Wilmington, Delaware

**Aaron P. Mitchell, Ph.D.**
Postdoctoral Fellow
Biochemistry and Biophysics Department
School of Medicine
University of California, San Francisco
San Francisco, California

**Lenore Neigeborn**
Graduate Student
Department of Genetics and Development
Columbia University
New York, New York

**Thomas D. Petes, Ph.D.**
Professor
Department of Molecular Genetics and Cell Biology
University of Chicago
Chicago, Illinois

**Hannele Ruohola**
Biochemist
Research Laboratories
Finnish State Alcohol Company
Alko Ltd.
Helsinki, Finland

**Laura Sarokin**
Graduate Student
Department of Genetics and Development
Columbia University
New York, New York

**Randy Schekman, Ph.D.**
Department of Biochemistry
University of California, Berkeley
Berkeley, California

**John F. Scott**
Department of Microbiology
University of Illinois
Urbana, Illinois

**Aileen Taguchi**
Department of Biochemistry
University of Washington
Seattle, Washington

**Wayne E. Taylor, Ph.D.**
Department of Biochemistry
University of Washington
Seattle, Washington

**Tuula Torkkeli, Ph.D.**
Biochemist
Research Laboratories
Finnish State Alcohol Company
Alko Ltd.
Helsinki, Finland

**Akhilesh Trivedi, Ph.D.**
Research Associate
Department of Biochemistry
The University of Western Ontario
and
Department of Clinical Pathology
Victoria Hospital
London, Ontario, Canada

**Juerg F. Tschopp, Ph.D.**
The Salk Institute Biotechnology/
Industrial Associates, Inc.
La Jolla, California

**E. Reno Tustanoff, Ph.D.**
Professor
Department of Biochemistry
The University of Western Ontario
and
Department of Clinical Pathology
Victoria Hospital
London, Ontario, Canada

**Adolphus P.G.M. van Loon**
Department of Biochemistry
Central Research Department (ZFE)
Hoffman-La Roche and Co. AG
Basel, Switzerland

**Marten Veenhuis**
Laboratory for Electron Microscopy
State University of Groningen
Haren, The Netherlands

**John S. Wood, Ph.D.**
Research Scientist
Department of Molecular Genetics
Eli Lilly and Company
Indianapolis, Indiana

**Rogers Yocum, Ph.D.**
Program Director
Yeast Molecular Biology
Biotechnica International
Cambridge, Massachusetts

**Elton T. Young**
Department of Biochemistry and Genetics
University of Washington
Seattle, Washington

**Josephine Yu, B.S.**
Department of Biochemistry
University of Washington
Seattle, Washington

**Phillip G. Zaworski**
Molecular Biology Research
The Upjohn Company
Kalamazoo, Michigan

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Chapter 1

# REGULATION OF ALCOHOL DEHYDROGENASE GENE EXPRESSION IN *SACCHAROMYCES CEREVISIAE*

**Hal Blumberg, Toinette Hartshorne, Meher H. Irani, Aileen Taguchi, Wayne E. Taylor, Josephine Yu, and Elton T. Young**

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## I. SUMMARY

The two cytoplasmic alcohol dehydrogenase (ADH) isoenzymes are regulated in a manner that insures an approximately equal amount of total ADH activity in the cell irrespective of carbon source in the medium. In the presence of a fermentable carbon source ADHI is the major isoenzyme present and ADHII activity is absent. During respiratory growth on a nonfermentable carbon source ADHI activity declines and ADHII activity appears.

The regulation of ADHII involves both *cis*- and *trans*- acting factors that influence expression of *ADH2*, the structural gene for ADHII. Sequences lying both upstream and downstream of the RNA start site have been implicated in the synthesis of stable *ADH2* mRNA. A regulatory sequence is present 215 bp 5′ to the TATA box and consists of a perfect 22-bp dyad. Deletion of this dyad results in a repressed and nonderepressible phenotype.

Gene loci that apparently act in an *ADH2*-specific manner have been identified and characterized in some detail. *ADR1* encodes an *ADH2*-specific activator of transcription whose function is mediated through the 22-bp dyad. Deletion analysis of the protein coding domain of *ADR1* indicates that the amino terminal 284 amino acids of this putative 1323 amino acid polypeptide are sufficient to activate *ADH2*. Fusion of *ADR1* to *Escherichia coli LacZ* produces a bifunctional protein which is expressed in equal abundance during repressing and derepressing growth conditions and is found predominantly in the nucleus of yeast cells. Amplification of *ADR1* on multicopy plasmids allows *ADH2* to escape glucose repression and to be hyperderepressed on a nonfermentable carbon source.

*ADR6* is another gene whose function is required for synthesis of stable *ADH2* mRNA. A gene fusion containing the *ADH2* promoter, the first 15 amino acids of ADH II, and most of *E. coli LacZ* does not require *ADR6* for β-galactosidase activity, suggesting that *ADR6* acts 3′ to the ATG of *ADH2*.

Evidence for positive activation of *ADH2*, rather than negative regulation mediated by a protein repressor, is suggested by three lines of evidence: (1) regulatory mutants clearly define loci specifying positive activators, but not negative repressors; (2) deletion analysis identifies a *cis*-acting site required for derepression, but not repression; and (3) in vivo competition experiments suggest that positive diffusible factors, but not negative ones, can be titrated by introducing a plasmid carrying the *ADH2* promoter. In the presence of such a plasmid, derepression of the chromosomal *ADH2* locus is inhibited. This inhibition requires the 22-bp dyad and the TATA box and CAP regions of the *ADH2* promoter.

The coordinate regulation of *ADH1* and *ADH2* appears to require *ADR1*. The derepression of *ADH2* expression is ADR1 dependent and the concomitant decrease in ADHI activity also requires *ADR1*. In the absence of *ADR1* function, *ADH2* is not expressed and the decrease in ADHI activity does not occur. Overproduction of *ADR1* results in overproduction of *ADH2* mRNA and a reduction in *ADH1* mRNA levels during derepressed growth conditions. Positively correlated with the reduction in *ADH1* mRNA levels is the appearance of a longer *ADH1* transcript that starts about 1.5 kb upstream of *ADH1* and proceeds through *ADH1*.

## II. INTRODUCTION

The three ADH isozymes in *Saccaromyces cerevisiae* constitute an interesting gene family, disparate in metabolic function, regulation, and cellular location, but very closely related in an evolutionary sense.[1,5] The two cytoplasmic isozymes, ADHI and ADHII are responsible for the final step in fermentation and the first step in gluconeogenesis from ethanol, respectively. They are believed to be responsible for maintaining the redox balance in the cell by the oxidation and reduction of NADH and NAD.[6] The third ADH isozyme, ADHIII, is encoded by a nuclear gene, but is a mitochondrial protein. It contains a leader whose

nucleotide sequence appears to be evolutionarily related to the nontranslated leader regions of *ADH1* and *ADH2* mRNAs.[5]

The ADH genes are regulated in distinctive fashions. *ADH1* (encoding ADHI) is present at high levels (about 1 to 2% of cellular protein) during growth on a fermentable carbon source such as glucose, and both enzyme and mRNA levels decrease in abundance five- to ten-fold during growth on a fermentable substrate.[7] No regulatory genes affecting *ADH1* expression were revealed by the initial genetic analysis.[4] The sequences upstream of the *ADH1* promoter appear to be responsible for the regulation, since removal of sequences upstream of position +377 (all numbering is with respect to the most 5′ RNA start site) led to high level expression during growth on both fermentable *and* nonfermentable substrates.[8] More precise deletion analysis, needed to identify the sequences responsible for *ADH1* regulation, were not performed. The fact that removal of 5′ sequences led to enhanced expression suggests that *ADH1* is normally *repressed* five- to ten-fold by growth on a nonfermentable carbon source rather than being glucose induced. Thus, *ADH1* appears to be negatively regulated.

*ADH2* is highly glucose repressed. Its mRNA and the enzyme activity are not detectable during exponential phase growth on media containing glucose as a carbon source. After removal of glucose from the media, *ADH2* mRNA and ADHII enzyme activity appear and reach high levels, up to several percent of total cellular protein.[9] Its absence during the fermentative phase of growth allowed Ciriacy[10] to isolate a collection of *cis*- and *trans*-acting mutations that altered *ADH2* regulation so that its activity became partially constitutive; that is, the activity was present on glucose. These mutants were isolated in a strain lacking ADHI activity and restored the ability of the cell to grow fermentatively.

The mutants generated in vivo consisted of *cis*-acting mutations that were due to *Ty* insertions 5′ of *ADH2* and alterations of a $(dA)_{20}$ tract (extension to $[dA]_{55}$) 5′ of the promoter.[11,12] The *trans*-acting mutations were shown to be allelic to a locus identified previously. The initial alleles at this locus, called *ADR1* (Alcohol Dehydrogenase Regulator), were ADHII-defective, later shown to be ADHII $mRNA^-$.[9] The regulation-defective alleles were thus called $ADR1^c$, since they expressed ADHII constitutively.

The relationship between the *cis*-acting *Ty* and $(dA)_{20}$ mutations and the *trans*-acting *ADR1* alleles was revealing. The ADH2-*Ty* alleles did not respond to the $ADR1^c$ or *adr1* alleles, whereas the $(dA)_{55,54}$ ($ADH2\text{-}4^c$, $ADH2\text{-}5^c$) did respond.[10] As it turned out, all the *Ty* insertions (with one exception) were located between the *ADH2* TATA box and its UAS, and *ADH2* was converted from a glucose-regulated gene to one regulated by the *Ty* elements regulatory circuitry.[13] The one exception was mutation $ADH2\text{-}Ty8^c$ in which the *Ty* element inserted 3′ to the *ADH2* TATA box. The fact is that the *Ty* elements have no sequence specificity for insertion, yet all (except one) *Ty* mutations that gave ADHII-constitutivity inserted within a 100-bp "window" between the TATA box and UAS suggest that *Ty* insertion 5′ to the UAS would not produce constitutive expression.

*ADH3* is also regulated by catabolite repression, but only to a small extent compared to *ADH2*. Its activity is present on both glucose- and ethanol-containing media, but it is increased about three-fold on the latter, in concert with many other mitochondrial activities.[5] Interestingly, mitochondrial ADH activity does not support fermentation,[14] even when overproduced severalfold (unpublished data), suggesting that there is some compartmentalization of substrates and/or cofactors, most likely NAD and NADH which cannot penetrate the mitochondrial inner membrane. The inability of an *adh1 adh2 ADH3* strain to grow by fermentation provides a strong selection scheme for isolating mutants altered in ADHIII cellular location and mitochondrial function.

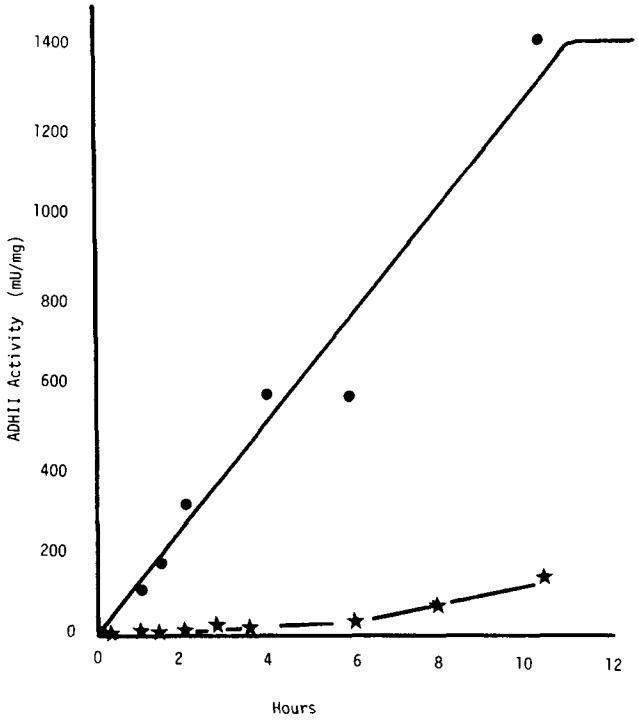
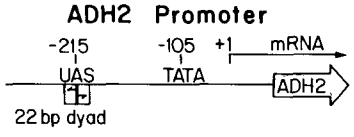



FIGURE 1. Kinetics of *ADH2* derepression in strains containing or lacking the *ADH2* UAS.

**Table 1**
**RESPONSE OF THE *ADH2* PROMOTER TO *ADR1***




## III. IDENTIFICATION OF AN *ADH2* UPSTREAM ACTIVATION SITE (UAS)

The site of *Ty* insertions and a comparison of *ADH1* and *ADH2* sequences 5′ of the TATA box led to deletion studies that identified the upstream activation site (UAS) responsible for *ADH2* derepression.[15,16] This sequence contains a 22-bp perfect inverted repeat whose deletion from the *ADH2* chromosomal locus leads to a 10- to 20-fold reduction is *ADH2* expression (Figure 1). The mutant allele, ADH2-11a, also fails to respond to mutant alleles of *ADR1* or excess copies of *ADR1* (Table 1), suggesting that *ADR1* acts through this region, presumably by binding to it. Current studies with a hybrid gene containing the *ADH2* UAS upstream of a *CYC1* promoter fused to *Lac*Z suggest that the 22-bp dyad is sufficient to activate the *CYC1-Lac*Z fusion gene.[25]

Extensive deletion analysis both 5′ and 3′ of the UAS failed to uncover a site that mediated repression. Extensive 5′ deletions that brought pBR322 sequences close to the UAS or TATA box alone caused constitutive *ADH2* expression, but yeast sequences in the same position did not.[15] We interpreted the results obtained when pBR322 sequences were near the promoter

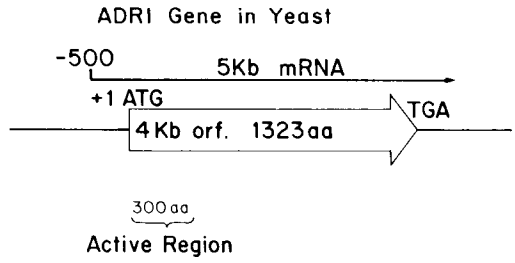



FIGURE 2. Schematic diagram of *ADR1*, a transcriptional activator of *ADH2*.

as meaningless with respect to normal regulation of *ADH2*, since the same deletions, but with yeast sequences replacing the vector sequences, gave contrasting results. Moreover, no internal deletions of smaller size gave high constitutive *ADH2* expression, never more than 10% of the derepressed level. Thus, we concluded that *ADH2* is regulated primarily by positive, rather than negative control,[15,16] as is consistent with extensive genetic analyses.[4,17]

## IV. CHARACTERIZATION OF *ADH2* "TRANSCRIPTION FACTORS"

The quotation marks above are a necessary caveat until a yeast in vitro transcription system is available. By current indirect tests, two genes, apparently specific for *ADH2*, or at least very restricted in their range of action, are candidates for transcription factors.

### A. *ADR1*

*ADR1* and a constitutive allele *ADR1*$^c$ have been cloned,[18] and the wild-type nucleotide sequence has been determined.[19] *ADR1* encodes a protein of 1323 amino acids, is expressed constitutively, and is found in the nucleus[19] (unpublished data). A null mutation is haploid viable and ADHII negative, indicating that it does not have a repressive function on glucose-containing media.[19] Surprisingly, the amino-terminal 302 amino acids are sufficient for *ADR1* function: *ADH2* is efficiently derepressed by an *ADR1* gene truncated at a Sau 3A site located 910 bp from the met codon.[19] Since this activity is present in a strain with a chormosomal null mutation, it is not due to complementation between two defective polypeptides. A schematic view of the *ADR1* gene is shown in Figure 2.

*ADR1* protein is also functional when synthesized as an *ADR1*-β-galactosidase fusion protein. This fusion protein is found in the nucleus, indicating that *ADR1* contains a nuclear entry domain as well as a (presumed) DNA binding domain.[20] As far as we know, this is the first example of a positive transcription factor that can function as a β-galactosidase fusion; yeast repressors (*α2* at least) have been shown to function as DNA binding proteins when fused to β-galactosidase.[21] As in the case of α2-*Lac*Z fusions, this should facilitate identification and purification of *ADR1* protein.

We can only speculate on why *ADR1* is so large. Presumably, the carboxyl-terminal 1000 amino acids perform some function independent of *ADH2* activation that we have not yet identified; one possible function will be described later. A computer-aided search of other DNA sequences did not reveal any significant homologies between other proteins and the C-terminal two thirds of *ADR1*.

### B. *ADR6*

A second *ADH2* "transcription factor" is identified by mutations at the *ADR6* locus. A null mutation at this locus is defective in accumulating *ADH2* mRNA.[22] Unlike *ADR1*, *ADR6*

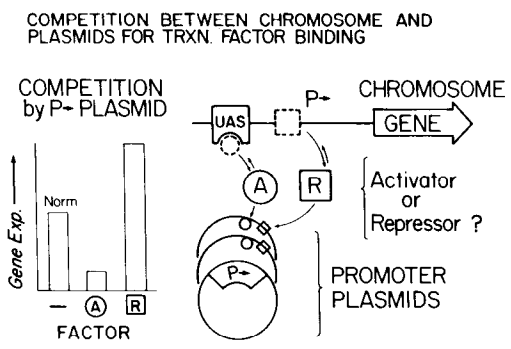



FIGURE 3. Consequences of titrating a positive vs. a negative effector of transcription on gene expression.

affects transcription from *ADH2-Ty* alleles. This suggests that *ADR6* is required for transcription per se, that is, it is not an *ADH2* regulatory factor. Analysis of various hybrid genes suggests that *ADR6* might function via sequences within the gene itself. This could mean that *ADR6* affects mRNA stability or processing rather than transcription. *ADR6* also affects *ADH1* expression, but only during growth on a derepressing carbon source, when the requirement for *ADR6* function in activating *ADH2* transcription is most stringent.[22]

Another interesting aspect of *ADR6* is that an adr6 homozygous diploid is sporulation defective.[22] This is not an indirect effect due to lack of ADH activity, since other ADH-defective mutants can sporulate. Thus, *ADR6* must affect the activity of other genes or their products.

## V. IN VIVO TITRATION STUDIES

Studies of *E. coli* gene expression have made use of the ability of multiple copies of Lac or gal operators to bind their cognate proteins. We have used a similar situation to ask whether *ADH2* is positively or negatively regulated, and to study mutant or truncated *ADH2* promoters. As shown in the diagram in Figure 3, the predicted results are very different in the presence of excess copies of the control region, depending on whether the gene is regulated positively or negatively. If *ADH2* were positively controlled, excess copies of the *ADH2* 5′ flanking sequence would be expected to decrease the maximum level of gene expression seen during derepression. If *ADH2* were negatively controlled by binding of a repressor protein, the repressed level of expression would be increased by extra copies of the 5′ flanking region. As shown in Figure 4, *ADH2* behaves as if it were positively controlled: when an *ADH2* promoter fragment is present on a high copy number plasmid, there is no effect on chromosomal *ADH2* expression during repressed growth conditions, but the rate and final level of derepressed gene expression is reduced four- to fivefold. Studies with other promoter fragments lacking the UAS, the TATA box and CAP site, or lacking just the initiation region indicate that all three regions are necessary to see this "titration" effect.[23] One interpretation of this result is that stable binding by the limiting factor(s) requires that all three regions be present on the same DNA molecule.

Since a positive factor is apparently "titrated" by excess copies of a functional *ADH2* promoter, we asked whether it was *ADR1* that was limiting *ADH2* chromosomal gene expression. *ADR1* was cloned on the high copy vector pC1/1 in the presence or absence of the *ADH2* promoter fragment. *ADR1* alone greatly stimulated *ADH2* gene expression as shown in Table 2. Excess *ADR1* allows *ADH2* to be expressed to fully derepressed levels

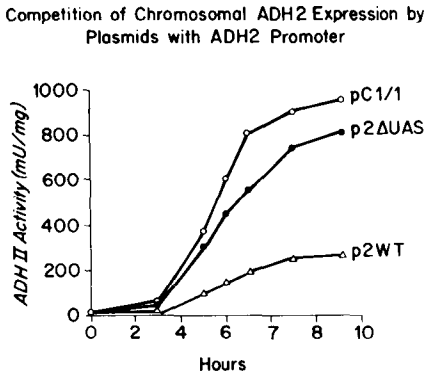



FIGURE 4. *ADH2* expression in the presence and absence of a competing *ADH2* promoter present on a multicopy plasmid.

## Table 2
## ENHANCED *ADH2* EXPRESSION: EFFECT OF INCREASED GENE DOSAGE OF *ADR1*, THE *ADH2* PROMOTER, OR THE *ADH3* GENE

| Plasmid | ADR1 gene | ADH2 promoter | ADHII enzyme activity | |
|---|---|---|---|---|
| | | | Glucose | Ethanol |
| C1/1 | − | − | 4 | 1000 |
| C1/1 | + | − | 2,400 | 12,000 |
| C1/1 | + | WT | 34 | 1,800 |
| C1/1 | + | UAS$^-$ | 460 | 5,500 |
| — | − | − | 8 | 2,100 |
| YEpADH2B/X | ADR1 | − | 300 | 65,000 |
| YEpADH2B/B | adr1$^-$ | | 160 | 2,000 |

during growth on glucose, and to be expressed at 10- to 20-fold higher than normal levels during growth on a drepressing carbon source. Thus, *ADR1* is clearly limiting during both growth conditions, and excess *ADR1* can apparently overcome whatever mechanism usually keeps it turned off on glucose medium. The amount of activity stimulated by excess *ADR1* from a single copy chromosomal *ADH2* locus is one fourth to one half of that observed if the *ADH2* gene is present on a multicopy plasmid, and there is a single copy of the *ADR1* gene.

If both *ADR1* and the *ADH2* promoter (P2) are present on the same plasmid, several interesting results are observed. Chromosomal *ADH2* expression is reduced about 40-fold in repressing medium and about 5-fold in derepressing medium (Table 2). Thus, although *ADR1* is limiting, increasing the amount present does not abolish the "titration" caused by the *ADH2* promoter; in fact, its presence reveals that some factor is even more limiting for *ADH2* expression during repression than during derepression. Whether the same factor is limiting in both media is unclear, and whether that factor is *ADR1* itself is also uncertain. The "titration" on glucose can even be observed on plates, because the cells with both *ADR1* and the *ADH2* promoter on pC1/1 ferment poorly compared to pC1/1-ADR1 alone. This might provide a genetic means to determine what the limiting factor is.