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Expression and in vitro phosphorylation of the yeast transcriptional activator ADR1

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EXPRESSION AND *IN VITRO* PHOSPHORYLATION OF THE YEAST TRANSCRIPTIONAL ACTIVATOR ADR1

BY

Joel R. Cherry

B.A. Carleton College, 1982

DISSERTATION

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Doctor of Philosophy

in

Biochemistry

September, 1988

For my brother Jim and my wife Jennifer.

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ABSTRACT

EXPRESSION AND *IN VITRO* PHOSPHORYLATION OF THE YEAST TRANSCRIPTIONAL ACTIVATOR ADR1

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The ADR1 protein activates transcription of the gene coding for the glucose repressible alcohol dehydrogenase II (ADH2) in *Saccharomyces cerevisiae*. Previous characterization of $ADR1^{C}$ mutations, which allow ADH2 to escape glucose repression, led to the hypothesis that ADR1 is inactivated post-translationally by a cAMP-dependent phosphorylation mechanism. This hypothesis was tested by investigating the *in vitro* phosphorylation of ADR1 and $ADR1^{C}$ proteins in which $ADR1^{C}$ proteins were predicted to display a diminished level of phosphorylation. A fragment of the ADR1 gene coding for the N-terminal 658 amino acids was fused to the lacZ gene of *E. coli* to allow for identification and assay of the ADR1 protein. The fused gene was placed under the control of the *E. coli* tac promoter to ensure efficient production. Beta-galactosidase activities in plasmid-bearing *E. coli* indicated that ADR1/ß-galactosidase fusion proteins were being expressed. SDS-polyacrylamide gel analysis of *E. coli* extracts revealed discrete plasmid-encoded fusion proteins.

Fusion proteins contained in crude *E. coli* extracts were phosphorylated *in*

vitro using purified catalytic subunit of mammalian cAMP-dependent protein kinase (cAPK). Peptide mapping results indicated that ADR1 was phosphorylated at two discrete sites *in vitro*. The primary phosphorylation site was identified as serine-230. A secondary site to the N-terminal side of serine-230 was also identified.

ADR1 DNA sequences were replaced in the expression plasmids with three sequences containing ADR1^{C} mutations, each of which encodes a single amino acid substitution within the phosphorylation sequence located between residues 227-231 (Arg-Arg-Ala-Ser-Phe). The *in vitro* phosphorylation of ADR1 and ADR1^{C} containing fusion proteins were compared using both bovine cAPK and the yeast cAPK catalytic subunit encoded by the TPK1 gene. Addition of purified yeast regulatory subunit completely blocked fusion protein phosphorylation by the yeast kinase in the absence of cAMP. The ADR1-2^{C} and ADR1-5^{C} mutations were found to diminish phosphorylation at serine-230. The ADR1-7^{C} mutation, which substitutes a leucine residue for serine-230, completely eliminated phosphorylation.

The results of this work support the hypothesis that the ADR1^{C} proteins bypass glucose repression by avoiding phosphorylation and suggest that ADR1 is regulated by a cAMP-dependent phosphorylation. Possible mechanisms by which phosphorylation may regulate ADR1 activity are discussed.

I. INTRODUCTION

The ability of an organism to respond physiologically to changes in its environment is often dependent on the precise and timely control of gene expression. In the yeast *Saccharomyces cerevisiae* proteins of the respiratory chain, glyoxylate cycle and gluconeogenesis are repressed when the organism is grown in medium containing a fermentable carbon source such as glucose[1-11]. When the fermentable carbon source is depleted, however, synthesis of proteins such as iso-1-cytochrome c[2], citrate synthase[10], and alcohol dehydrogenase II[11] is derepressed. Thus by altering its pattern of gene expression, yeast is able to metabolically adjust to changes in the availability of glucose. While the expression of many proteins is known to be modulated in response to changes in the extracellular environment, little is known about the molecular mechanisms responsible for these changes. Because yeast are easily manipulated using both the techniques of classical genetics and molecular biology, it represents an ideal organism in which to study the control of eukaryotic gene expression. The present work investigates the mechanism by which the positive regulator ADR1 controls alcohol dehydrogenase II expression in yeast.

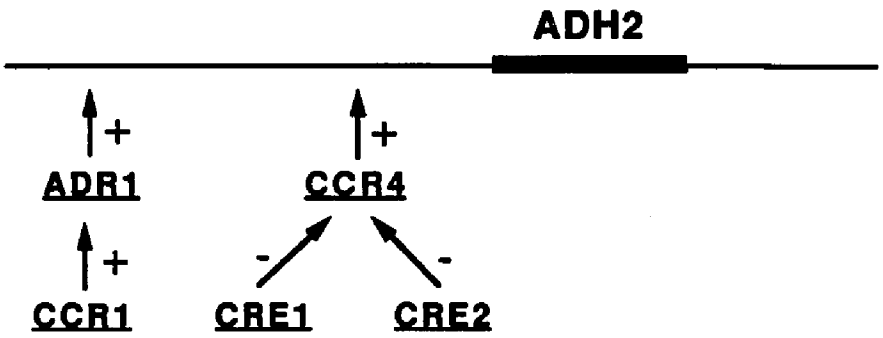
Alcohol dehydrogenase II (ADH II) is one of three active ADH isozymes found in *S. cerevisiae.* The metabolic function of two of the isozymes, ADH I and ADH II, was elucidated by the studies of Lutsdorf and Megnet[11]. The classical fermentative isozyme, ADH I, is a cytoplasmic enzyme which catalyzes the NADH-linked reduction of acetaldehyde to ethanol[13]. ADH I plays a vital role in the cell during anaerobic growth by regenerating NAD^+ for use by the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. The ADH II isozyme is also localized to the cytoplasm, but appears to kinetically favor catalysis of the opposite reaction - the NAD^+ linked oxidation of ethanol to acetaldehyde[14]. ADH II allows yeast to utilize ethanol as an energy and carbon source when glucose is unavailable. Cells unable to produce the fermentative isozyme due to mutation of the ADH1 gene* are viable under glucose growth conditions, but grow slowly due to the need to metabolize glucose aerobically. The other isozyme, ADH III, is expressed at very low levels[14]. Cells lacking this mitochondrially-associated isozyme are viable and appear not to be defective in any cellular functions, indicating that ADH III performs no essential metabolic function.

Transcription of the the classical fermentative isozyme, ADH I, is activated

*Throughout this work three letter gene designations will be underlined as is customary in yeast genetics. Three letter designations not underlined refer to the protein products i.e., ADR1 gene, ADR1 protein.

during growth in the presence of glucose and decreases approximately six-fold when cells are transferred to medium containing a nonfermentable carbon source such as ethanol[15]. ADH II expression, on the other hand, is repressed at the level of transcription when cells are grown in the presence of glucose[16]. Magasanik, who demonstrated in bacteria that the repression of enzyme synthesis in response to glucose is mediated by some glucose catabolite rather than glucose itself, termed this catabolite repression[17]. ADH2 has come to be referred to as a catabolite repressed gene, and the term may be apt since fermentable sugars other than glucose have been demonstrated to repress ADH II expression[12].

When glucose is depleted from the growth medium or cells are grown in medium containing nonfermentable carbon sources, ADH II enzyme activity increases approximately 500-fold as ADH2 mRNA production is derepressed[16]. Derepression of ADH2 was first determined by Ciracy[18-20] to be dependent upon the presence of the two trans-acting regulatory genes ADR1 and CCR1. Denis[21] later demonstrated that there are two regulatory pathways controlling ADH2 expression: one consisting of the previously characterized positive activators ADR1 and CCR1, and the other consisting of a positive effector, CCR4, whose activity is controlled by two negative effectors CRE1 and CRE2 as illustrated below:



This model was based on the epistatic relations of the various regulatory loci and does not necessarily imply that the proteins they encode interact directly. Because ADR1^{C} mutations could be isolated which activated ADH2 expression in the absence of a functional CCR1 gene, Denis concluded that ADR1 more directly affects ADH2 than does CCR1. ADR1 appears to be specific for the regulation of ADH2 expression since it does not affect the activities of several other enzymes subject to catabolite repression[21,22]. A recent report by Bemis and Denis[23] concludes that ADR1 is required for growth in medium containing glycerol as the sole carbon source, although did not found affect the activity of some enzymes involved in glycerol metabolism. ADR1 may therefore control a process required for the growth of cells in medium containing nonfermentable carbon sources other than glucose, but does not appear to be a general activator of catabolite-repressed genes. In contrast, CCR1 and CCR4 are pleiotropic activators which have been shown to affect the expression of several glucose-repressed enzymes[19,21]. They may therefore encode proteins which

play a more universal role in transcription than does ADR1.

The ADR1 gene has been cloned[24] and sequenced[25] and is predicted to encode a protein 1323 residues in length with a calculated molecular weight of 151 kilodaltons (KDa). ADR1 has been shown to be localized to the yeast nucleus via a targeting sequence within its N-terminal 642 amino acids[26], indicating that the protein may be intimately involved with the transcription of ADH2.

ADR1 function has been elucidated largely through the characterization of recessive adr1 mutations and dominant $ADR1^{C}$ mutations. Strains carrying recessive adr1 mutations are unable to derepress transcription of ADH2 normally, while strains carrying an $ADR1^{C}$ allele allow ADH2 transcription to occur in medium containing glucose.

The first of the adr1 mutations to be characterized, adr1-1, was found to contain an umber mutation at the eleventh codon position which blocks translation of the ADR1 protein (Lynne Bemis, personal communication). Strains carrying this allele have virtually no ADH II enzyme activity[18], indicating that the ADR1 protein is essential to derepress transcription of ADH2 (Table 1a).

Strains carrying other adr1-type mutations fail to derepress ADH2 transcription to varying extents (Figure 1a). These mutations were found to encode ADR1 proteins containing single amino acid substitutions within a region which shares homology with the "zinc finger" DNA-binding sequence

Table 1.

a. ADH II Enzyme Activities

Relevant Genotype	ADH II Specific Activity (mU/mg) Glucose	Ethanol
adr1-1	2	10
ADR1	5	2500
ADR1-2^{C},4^{C}	200	3500
ADR1-5^{C}	370	3400
ADR1-7^{C}	250	2800

ADH II activities were taken from Cherry et al.[27] with the exception of the values for the adr1-1 allele, which were taken from Bemis and Denis[23]. ADH II activities were determined as described[16] following growth of cultures overnight at 30^{o} C. Culture mediums contained YEP medium (2% yeast extract, 1% Bacto-peptone, 20 mg/L adenine and uracil) supplemented with either 8% glucose or 3% ethanol.

b. Identity of the Constitutive ADR1 Mutations

ADR1 allele	base pair change	nucleotide number	amino acid change	residue
ADR1-2^{C},4^{C}	T to C	692	Phe to Ser	231
ADR1-5^{C}	G to A	683	Arg to Lys	228
ADR1-7^{C}	C to T	689	Ser to Leu	230

The identity of the ADR1-5^{C} mutation is presented as reported by Denis and Gallo[32]. The identities of the ADR1-2^{C}, ADR1-4^{C}, and ADR1-7^{C} mutations are presented as reported by Cherry et al.[27]

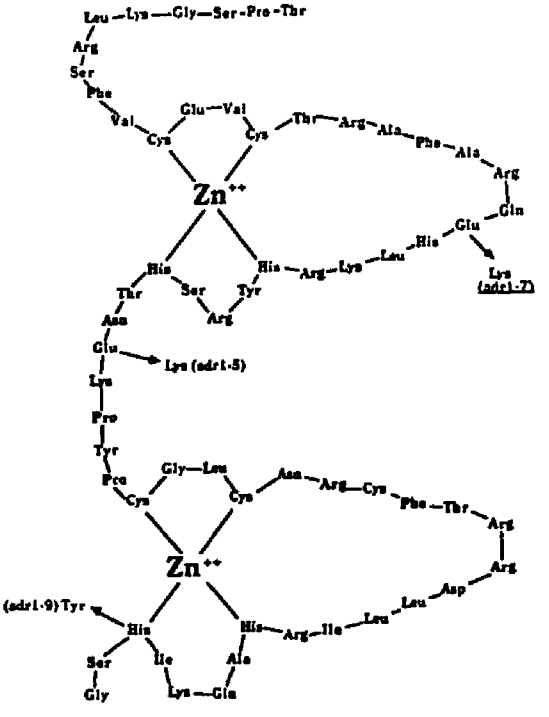
Figure 1

a. ADH II Activities of Strains Bearing adr1-5^c-x Mutations

Relevant Genotype	ADH II Specific Activity (mU/mg) Glucose	Ethanol
adr1-5^c-5	16	1100
adr1-5^c-7	1	11
adr1-5^c-9	0	137
ADR1-5^c	370	3400

ADH II activities were taken from Ref. 97 with permission from the author. ADH II activities were determined as described[16] following growth of cultures overnight at 30° C. Culture mediums contained YEP medium (2% yeast extract, 1% Bacto-peptone, 20 mg/L adenine and uracil) supplemented with either 8% glucose or 3% ethanol.

b. Location of adr1-x Amino Acid Substitutions in the Putative Zinc Finger



Taken with permission from ref. 98. Drawn by D. Mullaney as adapted from Hartshorne et al. (ref. 25).

motif first noted in the 5S rRNA transcriptional activator TFIIIA of *Xenopus laevis*[26,27]. These fingers, which are repeated nine times in TFIIIA[28], are repeated twice in ADR1 and occur between residues 98-126 and 127-155. Hartshorne et al.[25] have proposed, by analogy to TFIIIA, that these regions of ADR1 form functional DNA-binding structures by coordinately binding Zn^{2+} ions as illustrated in Figure 1b.

This proposal is consistent with numerous studies which suggest that the ADR1 protein activates transcription of the ADH2 gene by binding directly to a region of DNA centered approximately 230 base pairs (bp) upstream of the ADH2 transcription start site. This region, termed an upstream activating sequence or UAS, contains a 22 bp perfect inverted repeat. Deletion of the UAS from the ADH2 upstream region eliminates ADR1-mediated transcription[29,30]. Conversely, transcription of ADH2 was inhibited by placement of upstream sequences on multi-copy plasmids[31]. Inhibition was relieved by placing a copy of the ADR1 gene on the same plasmid[31], presumably as a result of increased ADR1 expression. These results suggest that ADR1 interacts directly with the ADH2 UAS and that this interaction is essential to transcription.

The ADR1 protein may well require the zinc finger structures for binding to the ADH2 UAS. While metal binding has not yet been demonstrated for the

intact ADR1 protein, a synthetic peptide comprising amino acids of the C-terminal finger have been demonstrated to bind Zn^{2+} ions. Metal-dependent DNA binding was observed, but the peptide:Zn^{2+} complex failed to bind specifically to ADH2 upstream sequences (G. Parraga, personal communication). Therefore it appears that the fingers are necessary but not sufficient for sequence-specific binding of ADR1 to DNA. They may simply stabilize general ADR1-DNA interactions while the sequence specificity of DNA-binding is conferred by other sequences on the ADR1 protein. Sequences just N-terminal to the fingers have been implicated in such a role (T. Young, personal communication), but the mechanism of interaction has yet to be elucidated. Regardless of the role the finger regions play in ADR1 function, it is evident from the ADH II activities in strains carrying this second class of adr1 mutations that these regions are essential to ADR1 activity.

Like the second class of adr1 mutations, the $ADR1^{c}$ mutations were found to encode single amino acid substitutions within the ADR1 protein (Table 1b), but with a different effect on ADH2 expression[27,32] (Table 1a). These mutations allow ADH2 expression to partially overcome glucose repression, in some cases increasing ADH II activity by 70-fold (Table 1a). Furthermore, since ADR1 mRNA has been shown to be constitutively expressed and is independent of the ADR1 allele[32], this data implies that the ADR1 protein is present but inactive

under glucose repressed conditions. The ADR1^{C} mutations must encode mutant proteins which can partially bypass the ADR1-inactivation mechanism.

Such a system of post-translational control of ADR1 is supported by studies which examined the effect of increased ADR1 gene dosage on ADH2 expression[33]. Previous studies had shown that ADH II activity is directly related to the level of ADH2 mRNA transcribed[16], therefore ADH II activities were used to provide a measure of ADR1 activity. ADH II activities in glucose-grown cells were observed to increase linearly as the ADR1 gene dosage was increased. A similar effect was observed in strains with increased ADR1-5^{C} dosages, indicating that the linear increase in ADH2 expression was the result of increased levels of ADR1 protein. In contrast, under derepressed growth conditions ADH II activities increased with increased ADR1 dosage until a maximum ADH II activity was approached asymptotically. Strains carrying two to three copies of ADR1 had ADH II activities approximately one-half that of strains carrying as many as 75 copies of ADR1. ADH2 expression in strains with high ADR1 dosage was observed to increase when the dosage of ADH2 was increased, indicating that during derepression ADH2 expression was limited by the dosage of the ADH2 gene. These results imply that during glucose repression transcription of ADH2 is limited by the level of ADR1 protein, but that under derepressed conditions ADR1 protein can nearly activate ADH2

transcription maximally. Increased levels of ADR1 protein may therefore override some mechanism which normally inactivates ADR1 during glucose repression. Because strains with increased ADR1 dosage are phenotypically similar to strains with an $ADR1^{C}$ allele, the same ADR1 protein inactivation mechanism may be overridden or bypassed in both cases.

Characterization of the $ADR1\text{-}5^{C}$ mutation prompted Denis and Gallo[32] to propose that the post-translational modification which renders ADR1 inactive during glucose growth is a cAMP-dependent phosphorylation of the ADR1 protein. The sequence Arg-Arg-Ala-Ser-Phe lying between residues 227-231 of ADR1 is homologous to sequences recognized by type II cyclic AMP-dependent protein kinases (cAPK), Arg-Arg-X-Ser-Y, where Y tends to be a hydrophobic residue, X is not conserved, and the serine serves as the phosphoacceptor[34,35]. Four of the $ADR1^{C}$ constitutive mutations were cloned and sequenced, and all four were found to alter residues within this cAPK phosphorylation consensus site (Table 1b). $ADR1\text{-}5^{C}$, the first of the constitutive mutations to be characterized, was found to encode a protein in which the arginine residue at position 228 was replaced by a lysine[32]. A synthetic peptide containing an $ADR1\text{-}5^{C}$-like alteration in the phosphorylation consensus sequence was determined by Kemp et al.[35] to increase the K_m of phosphorylation 16 fold relative to an ADR1-like sequence (see Figure 2).

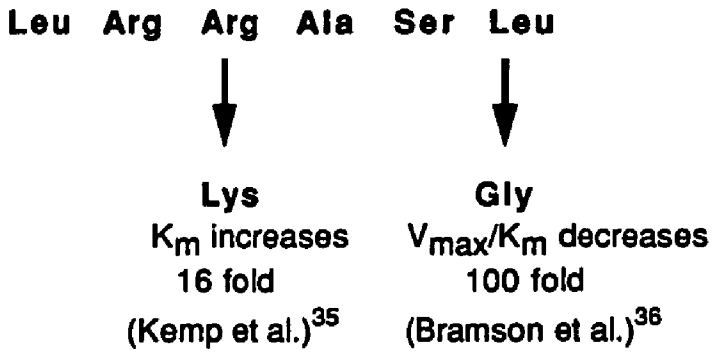
Similarly, the ADR1-2^{C} and ADR1-4^{C} mutations, which were found to be identical (hence only ADR1-2^{C} will be discussed), cause phenylalanine 231 to be replaced by a serine[27]. Alteration of this amino acid, which is adjacent to the phosphoacceptor serine and normally occurs as a hydrophobic residue, might also be expected to diminish phosphorylation by analogy to peptide studies carried out by Bramson et al.[36] (Figure 2). The fourth constitutive mutation characterized, ADR1-7^{C}, alters the putative phosphoacceptor serine to a leucine[27]. Such a change would of course be expected to completely block phosphorylation at this site. Therefore all four of the constitutive mutants of ADR1 isolated thus far would be expected to diminish or eliminate phosphorylation of ADR1 at serine 230. Because the ADR1^{C} proteins are able to cause a significant escape from glucose repression but only moderately elevate the level of ADH II expression in the absence of glucose, reversible phosphorylation of ADR1 was suggested by Denis and Gallo[32] to be involved in the glucose repression of ADH2.

cAMP may therefore be one of the cellular signals which mediates glucose repression of ADH2. cAMP has been suggested to be involved in the inactivation of numerous enzymes necessary to nonfermentative growth in yeast including malate dehydrogenase[37,39], fructose-1,6-bisphosphatase[38-41], and phosphoenolpyruvate carboxykinase[39]. In the case of fructose-1,6-bisphosphatase Tortora et al.[45]

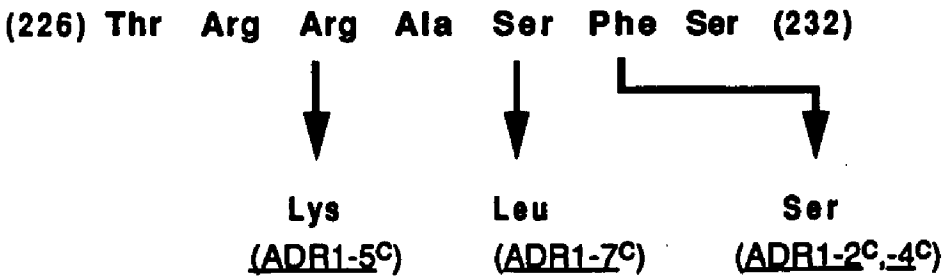
Figure 2.

The Effect of Amino Acid Substitutions on the Kinetics of Synthetic Peptide Phosphorylation by Bovine cAPK

Kemp synthetic peptide ("Kemptide"):



ADR1 protein sequence (residues 226-232):



demonstrated that cAMP-dependent phosphorylation of the yeast enzyme reduces its catalytic activity and is a necessary step in its proteolytic degradation. cAMP-dependent phosphorylation is also known to be involved in the regulation of trehalose and glycogen metabolism in yeast. The enzymes responsible for the synthesis and breakdown of the disaccharide trehalose, trehalose synthase and trehalase, are controlled coordinately by cAMP-dependent phosphorylation. Phosphorylation increases the enzymatic activity of trehalase[43], while phosphorylation decreases the activity of the trehalose synthase complex[42]. A similar coordinate control system has also been determined to regulate enzymes involved in glycogen metabolism[44]. In all of these cases the cAMP signal alters enzyme activity through direct phosphorylation of the protein. Interestingly, fructose-1,6-bisphosphatase contains significantly more phosphorylated serine residues when isolated from cells grown in medium containing glucose than in cells grown in medium containing a nonfermentable carbon source[41]. This implies that cAMP-dependent phosphorylation is stimulated by the presence of glucose. Glucose has been observed to cause a dramatic, transient increase in the intracellular cAMP concentration in yeast[46-48]. In sum, the evidence suggests that in yeast glucose causes increased cAMP-dependent phosphorylation of many proteins necessary for nonfermentative growth.

The enzymes presumably responsible for these phosphorylations, the yeast cAMP-dependent protein kinases (cAPK) are encoded by four genes, three of which encode catalytic subunits (TPK1, TPK2, and TPK3)[49] while one codes for a regulatory subunit (BCY1)[50]. The yeast cAPK, like its mammalian counterparts, exists as an inactive complex of regulatory (R) and a catalytic (C) subunits in the absence of cAMP. When cAMP levels are increased the R subunits bind cAMP, causing dissociation of the complex and freeing active C subunits. *In vitro* studies indicate the yeast cAPK responds to cAMP in a manner analogous to the mammalian cAPK[51], but the subunit stoichiometry in the active and inactive complexes has yet to be determined.

Of the three yeast catalytic subunits isolated thus far, only C1 (encoded by TPK1) has been characterized biochemically[52]. In studies using synthetic peptides modeled after the mammalian cAPK consensus sequence ("Kemptide"), C1 was shown to be capable of phosphorylating this ADR1-like sequence with a K_m approximately 20-fold higher than the mammalian cAPK[52]. Yeast strains deficient in any two of the three TPK genes are viable, while strains lacking all three TPK genes grow very slowly[49]. This indicates that the C subunits which these genes encode recognize sufficiently similar sequences that they can functionally substitute for one another in the cell. One of the C subunits may therefore be responsible for a glucose-dependent

phosphorylation of ADR1.

The objectives of this work were to answer the following questions concerning the phosphorylation of ADR1: 1) Is ADR1 phosphorylated by cAMP-dependent protein kinase *in vitro*? 2) if so, at what site(s) is it phosphorylated? and 3) do the characterized ADR1 constitutive mutations (ADR1-2^{C},ADR1-5^{C}, and ADR1-7^{C}) eliminate or diminish phosphorylation of ADR1 by cAPK?

To study the phosphorylation of the ADR1 protein it was necessary to create a fusion protein which would allow both qualitative and quantitative detection of ADR1. To this end various fragments of ADR1 or ADR1^{C} were fused to the lacZ gene of *E. coli*and placed under the control of suitable yeast or *E. coli* promoters. Gene fusions on appropriate plasmid vectors were used to express ADR1/ß-Galactosidase fusion protein in either organism, depending on the promoter contained in the plasmid constructs. The presence of such fusion proteins were then detected by published assay techniques in both cells extracts and whole cells, be they yeast or *E. coli.* In yeast, plasmids were expected to express fusion protein with two determinable functions- ß-galactosidase and ADR1. ADR1 activity would be measured indirectly by assaying ADH II activity in strains in which the only functional ADR1 would be encoded by the plasmids.

Fusion proteins detectable in crude extracts were phosphorylated *in vitro*

using purified bovine or yeast cAPK in conjunction with radiolabeled ATP. Phosphorylated fusion proteins were examined by immunoprecipitation with anti-ADR1 or anti-ß-galactosidase antibodies followed by fractionation on SDS-polyacrylamide gels. Determination of the site(s) of phosphorylation in ADR1 was determined by peptide mapping of chymotryptic peptides derived from the phosphorylated fusion proteins.

II. MATERIALS AND METHODS

E. coli and Yeast Strains. *E. coli* strains M182 (F^-, Δ(lac IOPZYA)X74, gal U, gal K, $strA^r$) and RR1 (F^-, hsds20 (r_B^-, m_B^-), supE44, ara-14, proA2, lacY1, galK2, rpsL20 (str^r), xyl-5, mtl-1, λ^-) were used. Yeast strain 500-16 (MAT **a** adh1-11 adh3 adr1-1 trp1 ura1 his4) contains a nonsense mutation in ADR1 (L. Bemis, personal communication).

Growth Conditions. *E. coli* were grown overnight at 37° C in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5) containing ampicillin at a concentration of 50 μg/ml. Twenty milliliter cultures were used for protein analysis and 1.5 ml cultures were routinely used for plasmid DNA isolations. ß-galactosidase expression was screened for using MacConkey-lactose indicator plates containing ampicillin (50 g/L BBL MacConkey Agar (BBL Microbiology Systems, Cockysville, MD), 25 mg/L ampilcillin). Colonies which appeared bright red after overnight growth indicated the presence of cells expressing levels of ß-galactosidase activity in excess of 500 U/mg soluble protein.

Yeast were grown in 2 ml cultures overnight at 30° C with constant shaking . YEP medium (2% bactopeptone, 1% yeast extract, 20 mg/L adenine and uracil) containing 8% (w/v) glucose (YEP-Glc) or 3% ethanol (YEP-Et) was used for non-selective growth. Where indicated, the respiratory inhibitor antimycin A

was added to a concentration of 0.6 µg/L. Trp^- medium contained 6.7 g/l yeast nitrogen base, 20 mg/ml adenine, uracil, and tyrosine, 5 g/L casamino acids, and 10 ml/L Trp dropout solution (0.5 g valine, 0.5 g threonine, 0.6 g phenylalanine, 0.2 g methionine, 0.4 g lysine, 0.6 g leucine, 0.6 g isoleucine, 0.2 g histidine, and 0.2 g arginine in 100 ml water) and was supplemented with either 8% glucose (Trp-Glc) or 3% ethanol (Trp-EtOH). Selective plates used to screen for ß-galactosidase expression consisted of Trp-Glc medium buffered to pH 7 with 0.1 M potassium phosphate (5.32 g/L KH_2PO_4, 10.63 g/L K_2HPO_4) and supplemented with 25 g/L bactoagar. Just prior to use, 100 µl of X-Gal (5-bromo-4-chloro-3-indoyl-ß-D-galactoside, 2% in dimethyl formamide) was spread on the plate surface. Cells expressing ß-galactosidase activity in excess of 0.5 U/mg soluble protein appeared dark blue after two days growth at 30° C.

Plasmids. Plasmids used in this work are listed in Table 2. The 5' ADR1 DNA sequences used in the construction of plasmids JC200Z and JC25 were derived from plasmid JS119 constructed by Jeff Schuster at Chiron Inc., Emeryville, CA. Plasmid JS119 contains an Nco I site at the start ATG of ADR1 resulting from the ligation of a synthetic 56 bp oligonucleotide to the 5' Hinc II site at bp +48 of ADR1. This oligonucleotide conservatively replaces the 5' end of ADR1 while providing a new Nco I site at bp -1, and a new Bgl II site at bp -7 (Table 2b).

Table 2a. Plasmids Used in Constructions

Plasmid	Fragment of interest	Source
pBR322	(*E. coli* vector)	BRL
pUC19	(*E. coli* vector)	BRL
YRp7-ADR1-23A-6-5	ADR1	C. Denis[27]
YRp7-ADR1(ΔBcl)	ADR1	A. Rovelli*
JS119	ADR1 and G3PDH promoter	J. Shuster*
YRp7-ADR1-5^{C}-23A	ADR1-5^{C}	C. Denis[24]
YRp7-ADR1-2^{C}	ADR1-2^{C}	C. Denis[27]
YRp7-ADR1-7^{C}	ADR1-7^{C}	C. Denis[27]
pKK240-11	tac promoter	M. Ptashne[55]
pRB45	lacZYA	M. Rose[54]
pSKS105	lacZYA	M. Casadaban[56]

*These plasmids are described in Materials and Methods.

Table 2b. Oligonucleotide Sequence Used in the Construction of JS119

Met Ala Asn Val Glu Lys Pro Asn Asp Cys Ser Gly Phe Pro Val
AGA TCT ATT ACC ATG GCT AAC GTT GAA AAG CCA AAC GAT TGT TCT GGT TTT CCA GTT
TCT AGA TAA TGG TAC CGA TTG CAA CTT TTC GGT TTG CTA ACA AGA CCA AAA GGT CAA

Bgl II^ Nco I^ Hinc II^

Plasmid YRp7-ADR1-311ΔBcl used in the construction of plasmid JC1Z was constructed by Ann Rovelli in the Denis Laboratory by digestion of plasmid YRp7-ADR1-311[24] with Bcl I and subsequent religation. The resulting plasmid is identical to plasmid YRp7-ADR1-311 except that it lacks ADR1 sequences from bp -1600 to bp +440.

Plasmid Constructions. Restriction enzymes, bacterial alkaline phosphatase, and DNA ligase were purchased from New England Biolabs (Beverly, MA) or Bethesda Research Laboratories (Bethesda, MD). Plasmid purification from bacterial cells was performed essentially according to the method of Birnboim and Doly[53] and is described in Appendix A. Restriction digestions were performed according to the manufacturers' specification using the LS, MS, HS or KS buffers described in Appendix B. DNA fragments were separated by agarose gel electrophoresis in TAE buffer and visualized under UV light after staining with ethidium bromide as described by Maniatis et al.[57] DNA ligations were performed as described in Appendix C. *E. coli* transformations were performed using competent RR1 or M182 cells either purchased from BRL (Bethesda, MD) or cells prepared by the $CaCl_2$ procedure described by Maniatis et al.[57] When necessary, transformants were screened by colony hybridization using an appropriate ^{32}P-labeled ADR1 probe as has been described[58].

Preparation of Soluble Protein Extracts from Yeast Cells were recovered by centrifugation of overnight yeast cultures and cell pellets were washed once using 2 ml buffer A:200 (25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM ß-mercaptoethanol, 1 mM phenylmethylsulfonyl chloride, 2 µM pepstatin, 0.6 µM leupeptin, 200 mM KCl). Washed cells were resuspended in 200 µl buffer A:200 and glass beads (0.5 mm in diameter) were then added at a ratio of approximately 1g per 100 µl cell suspension. Cells were then lysed at 4° C by vigorous mixing for approximately five minutes. Extracts were separated from the glass beads, spun in a microcentrifuge for five minutes to remove insoluble debris, and stored on ice for immediate assay.

Preparation of Soluble Protein Extracts from *E. coli.* 20 ml cultures were seeded with a single red colony off of a Mac-AMP plate and grown overnight at 37° C. The cells were collected by centrifugation at 3000 X g, transferred to 1.5 ml microcentrifuge tubes, and washed twice with 500 µl buffer A:200. The washed cells were resuspended in 500 µl of buffer A:200 and lysed by sonication at 0° C. Sonication was performed for 15-20 seconds at 80% power using the 4 mm probe of a Biosoniks Sonicator (VWR scientific, Brunswick, NJ). Extracts appearing translucent after sonication indicated efficient lysis as judged by protein determination and ß-galactosidase activity measurements (data not shown). Sonication of extracts for time periods in excess of 30 seconds resulted in loss of measurable enzyme activity. Extracts appearing

opaque after after initial sonication were cooled on ice and sonicated for 5 to 10 additional seconds. Translucent extracts were centrifuged at full speed (13,000 X g) in a microcentrifuge for 15 minutes before removal of the supernatant fraction containing soluble proteins. Extracts were then placed on ice for immediate assay or frozen in a dry-ice/ethanol bath for long-term storage. Extracts could be stored for up to one month at -70° C with no appreciable loss in ß-galactosidase enzyme activity.

Native Polyacrylamide Gel Electrophoresis. Native polyacrylamide gels were run as described[59].

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Electrophoresis was performed according to the method of Laemmli[60] with minor changes. Samples were mixed 1:4 with 5X SDS sample buffer (made by mixing a warm solution containing 5 ml glycerol, 1 g SDS, and 2.5 ml 0.125 M Tris, pH 6.8 with 14.6 M ß-mercaptoethanol in a 3:1 ratio just prior to use) and placed in a boiling water bath for 3 minutes just before application to the polyacrylamide gel. Bromophenol blue was added to the upper buffer chamber as a tracker dye.

Gels were stained for protein using Kodak Kodavue (Rochester, NY), the Gelcode silver stain of Pierce Chemical Co., Rockford, IL., or using the silver stain protocol of Wray et al.[61]

In Situ Staining of Protein Extracts with ß-Galactosidase Activity. Native polyacrylamide gels were stained for ß-galactosidase activity by washing for 15 minutes at 37° C with Z buffer[62] (0.1 M sodium phosphate pH7.0, 10 mM KCl, 1mM $MgSO_4$) supplemented with 10 mM $MnCl_2$. Gels were then placed on a glass plate with a white background and coated with 200 µl Bluogal (BRL, 2% solution in dimethylformamide). After incubation at room temperature for 15-20 minutes, proteins having ß-galactosidase activity appeared as blue bands. Extracts containing as low as 50 to 100 units of ß-galactosidase activity routinely yielded visible, albeit diffuse, bands.

ß-galactosidase and ADR1/ß-galactosidase fusion proteins present in extracts fractionated on SDS-PAGE were detected by activity staining . Electrophoresis was performed as described[60] except that samples were not boiled after addition of sample buffer. After electrophoresis gels were first washed with water and then washed twice for 30 minutes in 250 ml 10 mM Tris, pH 9, with water rinses between washes. After an overnight wash in Tris, gels were rinsed in water and washed for an additional 2 hours in Z buffer supplemented with 10 mM $MnCl_2$ to activate the enzyme. Gels were then placed on a white-backed glass plate and stained as was described above for native gels. Gels were covered with plastic wrap to prevent them from drying out and were allowed to develop for approximately two hours. Extracts with less

than 300 units of ß-galactosidase activity did not yield visible bands.

ADH Assays. Assays of ADH activity in yeast extracts were performed as previously described[16] by mixing 0.89 ml ADH assay buffer (18 mM sodium pyrophosphate pH 8.8, 2.5 mM NAD^+) , 10 µl extract, and 100 µl 3 M ethanol in a 1 ml cuvette and measuring the change in absorbance at 340 nm. An extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ was used for NADH. Activities are reported as milliUnits ADH activity per mg protein where one Unit of ADH activity is defined as µmoles NAD^+ reduced min^{-1} at 22° C. Protein assays were performed by the method of Lowry[63] using bovine serum albumin as the standard.

ß-Galactosidase Enzyme Assays. *E. coli* and yeast extracts were assayed essentially as described by Miller[62]. Assay reaction mixes generally contained 0.99 ml Z buffer, 10 µl soluble protein extract, and 0.2 ml *o*-nitrophenyl-ß-D-galactoside (4 mg/ml in 0.1 M phosphate buffer, pH 7.0). Reactions were monitored by measuring the change in absorbance at 420 nm at 22° C. Specific activity was defined as nmoles ONPG hydrolysed per minute per mg soluble protein using the formula:

$$\text{nmoles ONPG/min} \cdot \text{mg} = \frac{(\Delta OD_{420}/\text{min})\,(VT)}{((0.0045\ cm^{-1}\ ml\ nmol^{-1})(V_A)([protein])(1\ cm)}$$

Where: ΔOD_{420}/min = change in absorbance at 420 nm/ minute

V_T = total assay volume (ml)

0.0045 cm^{-1} ml $nmol^{-1}$ is the extinction coefficient of ONPG[62]

V_A = volume of soluble protein extract (µl)

[protein] = protein concentration in mg/µl

Protein assays were performed by the method of Lowry[63] or using the BCA reagents of Pierce Chemical using bovine serum albumin as the standard.

Affinity Purification of ß-Galactosidase Fusion Proteins. The synthesis and use of Sepharose-p-aminophenyl-1-thio-ß-D-galactopyranoside in the purification of ß-galactosidase fusion proteins was conducted according to the method of Ullmann[64]. *E. coli* extracts were prepared as described above except that lysis was conducted in column application buffer (20 mM Tris, pH 7.4, 1.6 M NaCl, 10 mM $MgCl_2$).

Antibodies. Polyclonal rabbit anti-ß-galactosidase antibodies were purchased from Cooper Biomedical, Inc. Malvern, PA. Anti-ADR1 polyclonal rabbit antibodies were kindly produced by Michael Morgan in the Denis laboratory by immunization of rabbits with a synthetic peptide corresponding to amino acid residues 208-231 of ADR1 and will be described elsewhere.

Immunoprecipitations. ADR1/ß-galactosidase fusion proteins were precipitated from *E. coli* extracts using anti-ß-galactosidase antibodies under native conditions as previously described[16]. Precipitation of fusion proteins

under denaturing conditions using anti-ADR1 antibodies was performed essentially as described[65].

In Vitro Phosphorylation Using Bovine cAMP-Dependent Protein Kinase. *E. coli* extracts were phosphorylated using the catalytic subunit of bovine cAMP-dependent protein kinase (Sigma P2645) in 10 mM Tris, pH 7.4, 4 mM $MgCl_2$, 3 mM ß-mercaptoethanol, 2 mM $MnCl_2$, 1 mM EDTA, 40 nM ATP, 2.6 X 10^6 cpm/pmol [γ-^{32}P] ATP. The amount of kinase used is given in picomolar units (pmol U) as defined by Sigma (1 pmol U is defined as the amount of enzyme required to transfer 1.0 pmol of phosphate from [γ -^{32}P] ATP to hydrolyzed and partially dephosphorylated casein per minute at pH 6.5 at 30° C.) In experiments where the kinase concentration was varied the ATP concentration was increased to 0.1 mM (2500 cpm/pmol [γ -^{32}P] ATP) such that it would not be limiting. Reactions were allowed to proceed for 10 minutes at 22° C. and were terminated by the addition of 5X SDS sample buffer followed immediately by immersion in a boiling water bath for three minutes. Autoradiography of SDS polyacrylamide gels was as previously described[33].

In Vitro Phosphorylation Using Yeast cAMP-Dependent Protein Kinase C1. Purified catalytic subunit of the yeast cAMP-dependent protein kinase encoded by the TPK1 gene was the kind gift of Mark Zoller of Cold Spring Harbor Laboratory. Reactions were carried out using the protocol of

Roskowski[66]. Reaction mixtures contained *E. coli* extracts, 50 mM MOPS (pH 7.0), 10 mM $MgCl_2$, 0.1 mM ATP (1000 cpm/pmol [γ -^{32}P] ATP), and 1.9 µg/ml purified C1 kinase in 50 µl reaction volumes. Reactions were carried out and analyzed in a manner analogous to reactions using the bovine kinase described above.

Peptide Mapping of the ADR1 Phosphorylation Site Protein extracts containing the ADR1/ and ADR1^C/ß-galactosidase fusion proteins were phosphorylated *in vitro* and separated by SDS-polyacrylamide gel electrophoresis as described above. The phosphoproteins of interest were localized by autoradiography and excised from the dried gel. Chymotryptic digestion of the 177, 175 KDa doublet and the 164 KDa single fusion protein was performed essentially as described by Karess and Hanafuss[67]. Gel slices containing either the 177, 175 KDa doublet or the 164 KDa fusion protein species were washed 4X for 15 minutes in 10% methanol at room temperature and lyophilized. Dried gel slices were treated with performic acid (performic acid was prepared by mixing one part 30% H_2O_2 with nine parts 90% formic acid followed by incubation on ice for one hour) for one hour on ice, washed twice with 1 ml water, and lyophilized. Dried performic acid-treated gel slices were then neutralized by washing 2X for five minutes with 1 ml 50 mM NH_4HCO_3 after which slices were washed with 1 ml water and lyophilized. The

resulting dried gel slices were incubated with 0.75 ml chymotrypsin (Sigma, 0.1 mg/ml in 50 mM NH_4HCO_3) at 37° C for 15 hours. Peptides were eluted from the gel slices with 0.9 ml of water by shaking end-over-end for 12 hours at 4° C. The elution procedure was repeated once and the combined supernatants were lyophilized. The resulting samples were then redissolved in 0.1 ml 50 mM NH_4HCO_3 and redigested with 0.1 ml chymotrypsin for 6 hours. NH_4HCO_3 was once again removed by lyophilization and the dried samples were resuspended in peptide-mapping sample buffer (1 M acetic acid, 0.05 M NH_4OH, 8 M urea).

The chemically synthesized peptide Leu-Lys-Lys-Leu-Thr-Arg-Arg-Ala-Ser-Phe, representing ADR1 amino acid sequence 222-231, was used as a standard in the peptide mapping experiment. This peptide was phosphorylated *in vitro* using bovine cAMP-dependent protein kinase in a manner analogous to the method described for labeling the *E. coli* extracts. The labeled peptide was purified on a Sephadex G-15 column in 0.05 M NH_4HCO_3. Fractions containing the phosphopeptide were identified by electrophoresis on a 50% polyacrylamide gel as described by West and Bonner[68]. Fractions containing phosphorylated peptide were lyophilized and an aliquot was removed for treatment with chymotrypsin. Chymotrypsin digestion of the synthetic peptide was performed in parallel with digestions of proteins contained in the gel slices

as described above. A phosphorylation mix lacking a peptide or ADR1/ß-galactosidase fusion protein substrate was also digested in parallel as a control.

Final analysis of the phosphorylated peptides was conducted using the 50% pH 3 polyacrylamide gel electrophoresis system described by West and Bonner[68]. Gels were dried and phosphorylated peptides were visualized by autoradiography.

III. Results

Expression of ADR1/ß-Galactosidase fusion protein in Yeast. Expression of ADR1 in *S. cerevisiae* was accomplished using plasmids in which fragments of the ADR1 gene were ligated to the lacZ gene which codes for ß-galactosidase. The ADR1/lacZ fragments were placed under the control of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) promoter[69] and inserted into the yeast plasmid vector YRp7. The G3PDH promoter was chosen for its efficiency in promoting transcription; G3PDH mRNA normally accounts for 2-5% of the total yeast poly A mRNA[70]. These expression plasmids were therefore expected to produce high levels of ADR1/ß-galactosidase fusion protein. Fusion proteins were predicted to be bifunctional, having both ADR1 acivity and ß-galactosidase activity. ß-Galactosidase activities were to be a measure of the amount of ADR1/ß-galactosidase fusion protein, while ADH II enzyme activity was predicted to provide an accurate measure of the amount of functional ADR1 protein present. Denis has shown that under glucose growth conditions the level of ADH II activity is directly proportional to the ADR1 or ADR1-5^{C} gene dosage[33]. Due to possible problems with degradation or stability of the ADR1 portion of the fusion proteins, ß-galactosidase activities may be a less reliable measure of the amount of functional ADR1 produced than is ADH II enzyme activity.

Two yeast ADR1/ß-galactosidase expression plasmids were used. The first plasmid, JC1Z, contains DNA sequences encoding the N-terminal 304 amino acids of ADR1 fused in frame to eigth codon of lacZ (Figure 3). The second yeast expression plasmid, JC25, contains sequences coding for a larger fusion protein containing 658 ADR1 amino acids fused to the sixth codon of lacZ (Figure 4).

These plasmids were transformed into yeast strain 500-16 (adr1-1, trp1) and the expression of plasmid-encoded fusion proteins was tested by growing cells on complete medium lacking tryptophan which had been supplemented with the chromogenic ß-galactosidase substrate X-Gal. The formation of small colonies that were deep blue in color.confirmed that fusion proteins with ß-galactosidase activity were being expressed. Plasmid-bearing cells grew very slowly relative to plasmid-free controls. In addition, expression plasmids were poorly maintained, even under selective growth conditions. This suggests that ADR1/ß-galactosidase overexpression may be toxic to the cell.

The level of ADR1/ß-galactosidase fusion protein expression was measured by assaying transformants for ß-galactosidase and ADH II under various selective growth conditions (Table 3). Cells containing plasmid were selected for by growth in rich medium lacking tryptophan, by growth in the presence of the respiratory inhibitor antimycin A, or by using cells which had spontaneously

Figure 3

Construction of Yeast ADR1/ß-Galactosidase Expression Plasmid JC1Z

ADR1 sequences used in the construction of plasmid JC1Z were derived from plasmids YRp7-ADR1-311ΔBcl and JS119, both of which are described in Materials and Methods. The Bam HI-Bcl I fragment of JS119 containing the G3PDH promoter and the first 440 nucleotides of the ADR1 coding sequence was ligated into the unique Bcl I site of YRp7-ADR1-311ΔBcl to create plasmid JC1. JC1 was cut at the unique Bam HI site at +907 and ligated into plasmid pSKS105[56] at the Bam HI site in the polylinker. The resulting plasmid, JC1Z, encodes a fusion protein containing the 304 N-terminal ADR1 residues fused to the seventh amino acid of ß-galactosidase. Expression of the ADR1/lacZ gene fusion is controlled by the G3PDH promoter and the plasmid is selectable on medium lacking tryptophan when present in a yeast strain containing the trp1 allele.

Figure 3: Plasmid JC1Z Construction

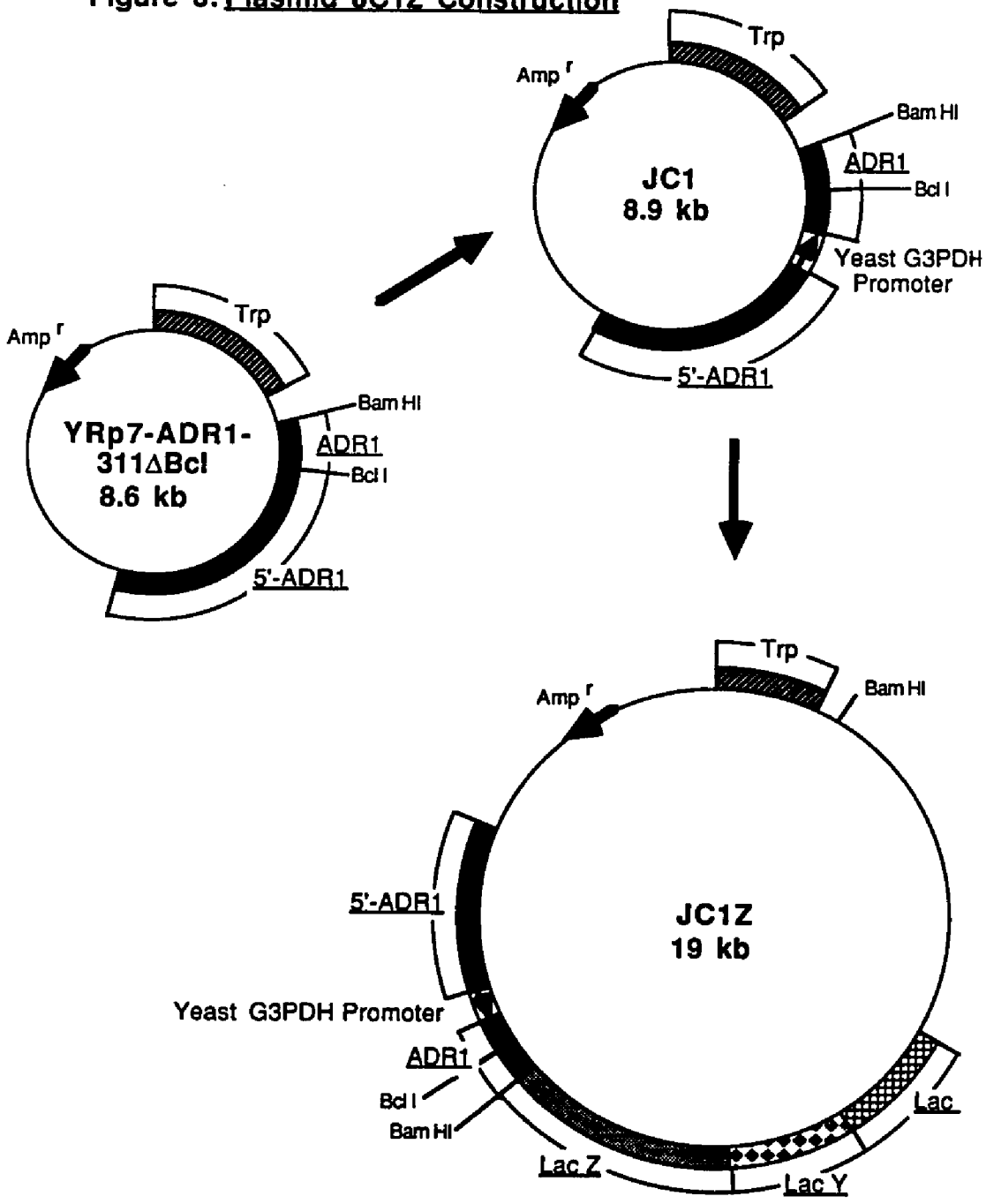


Figure 4

Construction of Yeast ADR1/ß-Galactosidase Expression Plasmid JC25

Plasmid JC25 was constructed by inserting the 8.6 kb Bam HI-Sal I fragment from JC100Z (described in Figure 5) into yeast vector YRp7. The final JC25 construct contains codons for amino acids 1 to 658 of ADR1 fused in frame to the sixth codon of the lacZ' gene.

Figure 4: Plasmid JC25 Construction

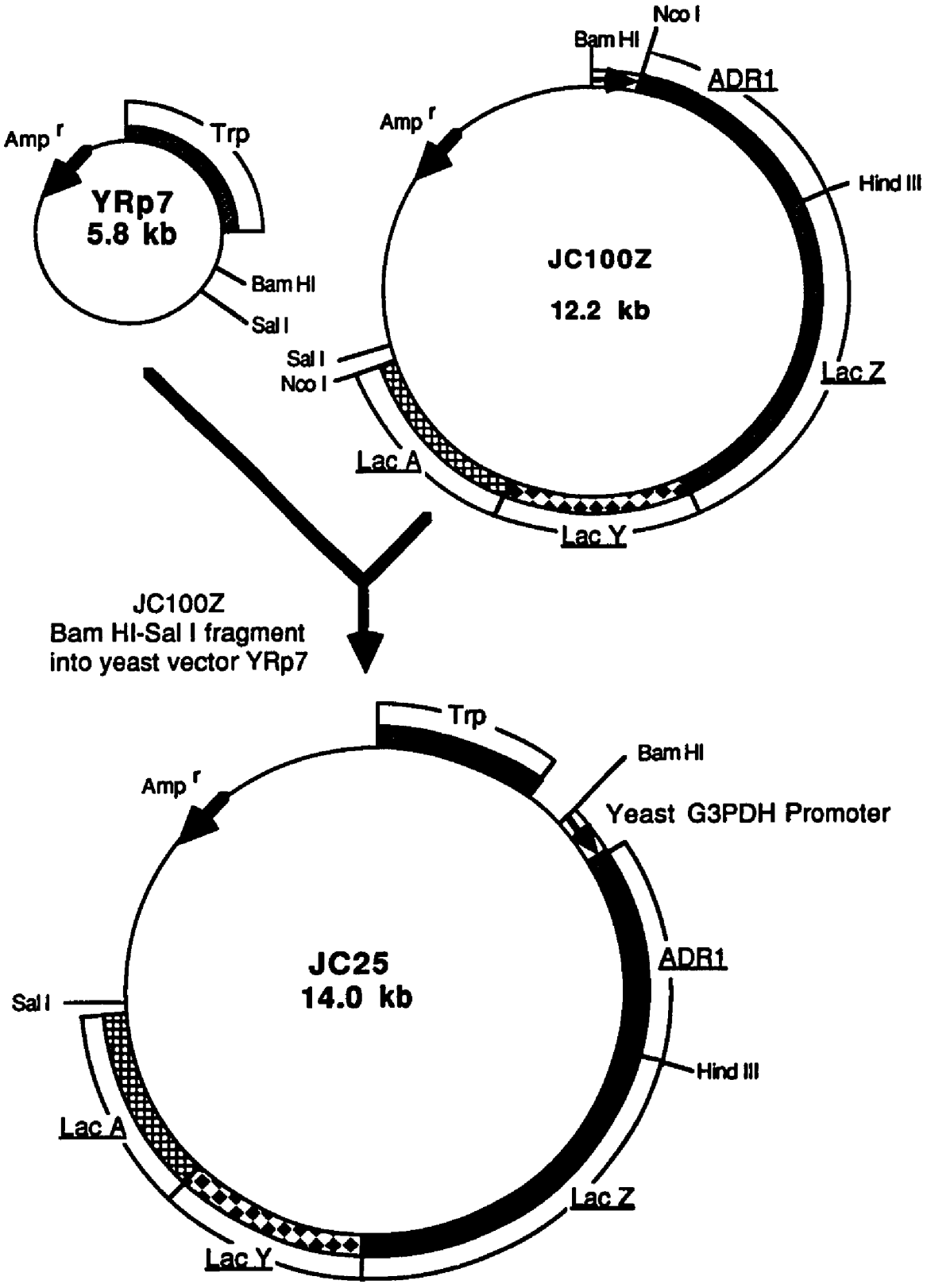


Table 3. ADR1/ß-Galactosidase Fusion Protein Expression In Yeast

a. nmoles ONPG hydrolysed per minute per mg soluble protein. All assay values are the average of a minimum of three separate determinations.

b. µmoles NADH oxidized per minute per mg soluble protein.

c. Yeast strain 500-16 (adr1-1) containing no plasmid as determined by Bemis and Denis[23]. All strains assayed except ADR1-304 are isogenic to strain 500-16.

d. YEP medium contains 2% w/v bactopeptone, 1% w/v yeast extract, 20 mg/L adenine and uracil and was supplemented with either 8% glucose (YEP-Glc) or 3% ethanol (YEP-EtOH).

e. Trp^- medium contained 6.7 g/l yeast nitrogen base, 20 mg/ml adenine, uracil, and tyrosine, 5 g/L casamino acids, and 10 ml/L Trp dropout solution (0.5 g valine, 0.5 g threonine, 0.6 g phenylalanine, 0.2 g methionine, 0.4 g lysine, 0.6 g leucine, 0.6 g isoleucine, 0.2 g histidine, and 0.2 g arginine in 100 ml water) and was supplemented with either 8% glucose (Trp-Glc) or 3% ethanol (Trp-EtOH).

f. YEP medium containing 8% glucose and 0.6 µg/ml antimycin A.

g. Assay values as determined by Bemis and Denis[23] for strain 500-16 carrying a plasmid which encodes the N-terminal 642 amino acids of ADR1 under the control of the ADR1 promoter. Values are shown for strains carrying either a single integrated plasmid copy or free plasmid as indicated.

h. Assay values as determined by Denis and Young[24] for strain 500-16 carrying a plasmid which encodes the N-terminal 304 amino acids of ADR1 under the control of the ADR1 promoter. Values are shown for strains carrying either a single integrated plasmid copy or free plasmid as indicated.

Table 3. ADR1/ß-Galactosidase Fusion Protein Expression in Yeast

plasmid	ß-galactosidase activity (U/mg)[a]		ADH II activity (mU/mg)[b]		culture conditions
	Glc	EtOH	Glc	EtOH	
none[c]	--	--	2	10	YEP[d]
JC25	1.4	4.1	170	350	Trp-[e]
JC25	13	--	1600	--	YEP-Glc+At[f]
JC25p	13	--	1470	--	YEP-Glc
ADR1-642 (integrant)[g]	--	--	8	1400	YEP
ADR1-642 (plasmid)[g]	--	--	530	--	YEP-Glc+At
JC1Z	2.3	1.4	30	390	Trp-
JC1Z	4.5	--	70	--	YEP-Glc+At
ADR1-304 (integrant)[h]	--	--	5	800	YEP
ADR1-304 (plasmid)[h]	--	--	100	--	YEP-Glc+At

become respiration deficient due to irreversible mitochondrial petite mutation. Petite cells and cells grown in the presence of antimycin A must ferment glucose to obtain energy. ADH II is required for fermentation in these cells since the yeast strain transformed lacks the fermentative isozyme ADH I. Because ADR1 is required for ADH II production, petite cells or cells grown in the presence of antimycin A must maintain ADR1-expressing plasmid to survive. Respiration deficient cells bearing plasmid JC25, whether petite or grown in the presence of antimycin A, had ADH II and ß-galactosidase activities 9-fold greater than found in cells grown in tryptophan-deficient medium. Plasmid JC1Z-induced ADH II and ß-galactosidase activities were 2-fold higher in cells grown in the presence of antimycin A than found in cells grown in tryptophan-deficient medium. ß-galactosidase and ADH II activities therefore indicate that respiration deficient cells selected more strongly for plasmids JC25 and JC1Z than did cells grown on medium lacking tryptophan.

It should also be noted in Table 3 that under glucose growth conditions there was a direct correlation between ß-galactosidase activity and ADH II activity. In strains carrying JC25, the ratio of ADH II activity to ß-galactosidase activity (mU/U) is approximately 120 mU/U, while in JC1Z-strains the ratio is 14 mU/U. These data indicate that under glucose growth conditions ß-galactosidase activity provides a good measure of the amount of functional ADR1/ß-

galactosidase activity. Furthermore, it also indicates that the smaller JC1Z-encoded fusion protein has approximately one-ninth the transcriptional activation activity (or ADR1 activity) of the JC25-encoded fusion protein, assuming that the respective fusion proteins have similar ß-galactosidase activities and are not differentially degraded by the cell. The efficiency of fusion protein expression was therefore measured by comparison of the ADH II activities of cells bearing plasmids JC25 or JC1Z to cells bearing plasmids with similar-sized ADR1 truncations under the control of the ADR1 promoter.

The ADH II activity of strain 500-16 carrying plasmid JC25 can be compared to the ADH II activity of the same strain carrying ADR1-642, a plasmid constructed by Bemis and Denis[23], when plasmids are maintained by growth in the presence of the respiratory inhibitor antimycin A (At). Plasmid ADR1-642 contains a truncated ADR1 gene encoding the N-terminal 642 amino acids of ADR1 under the control of the ADR1 promoter. JC25 was designed to produce an ADR1/ß-galactosidase fusion protein containing 659 N-terminal residues of ADR1. Comparison of ADH II activities in cells rendered respiration deficient indicates that JC25 produces about 3-fold more ADR1 activity than does the ADR1-642 plasmid. Similarly,the ADH II activities of cells carrying plasmid ADR1-304[24] can be compared to cells carrying JC1Z since both plasmids contain ADR1 sequences coding for the N-terminal 304 amino acids of ADR1.

500-16/JC1Z ADH II activities were found to be 0.7-fold that of the ADH II activity of 500-16/ADR1-304 when cells are grown in the presence of antimycin A. Therefore under conditions which maximize expression, the JC1Z plasmid expresses slightly less active ADR1 protein than comparable plasmids with ADR1 promoters, while the JC25 plasmid expresses more. This indicated that the expression or stability of the JC1Z protein, unlike its JC25 counterpart, is adversely affected by the fusion with ß-galactosidase under glucose growth conditions.

Under ethanol growth conditions the ADH II activity in strains carrying plasmid JC25 rose 90 milliunits per unit of ß-galactosidase, while the ADH II activity in strains carrying plasmid JC1Z rose 280 milliunits per unit of ß-galactosidase. Thus the JC1Z was a more efficient activator of ADH2 transcription under ethanol growth conditions than under glucose growth conditions (mU ADH II/U ß-gal = 13 for glucose, 280 for ethanol). JC25-encoded fusion protein, on the other hand, was slightly less efficient on ethanol than on glucose (mU ADH II/U ß-gal = 120 for glucose, 90 for ethanol). Furthermore, derepressed ADH II activites in strains carrying plasmids were significantly less than strains expected to express similar sized ADR1 proteins from truncated ADR1 genes integrated in the genome (compare JC25 and JC1Z activities to ADR1-642 and ADR1-304 integrant activities under ethanol growth conditions).

Despite the fact that the ADH II activities of strains bearing plasmid JC25 or JC1Z grown under derepressing conditions were much lower than expected, ADH2 was still significantly derepressed. In the case of JC1Z, ß-galactosidase activities indicated that approximately three-fifths as much fusion protein was made under ethanol growth conditions as under glucose growth conditions, yet ADH II activity was derepressed 13-fold. JC25 gave about a 3-fold derepression of ADH2 and ß-galactosidase activities indicated that there was 2.9-fold more fusion protein present under ethanol growth conditions. This discrepancy in correlating ß-galactosidase activities with ADH II derepression is thought to be an artifact which arose due to the extremely poor growth of cells bearing plasmid JC25 when grown in ethanol containing medium.

Attempts to detect plasmid-encoded ADR1/ß-galactosidase fusion protein in yeast extracts were unsuccessful (data not shown). Silver staining of SDS-polyacrylamide gel lanes containing anti-ß-galactosidase antibody precipitates and yeast extracts failed to detect the presence of any proteins unique to plasmid-bearing cells. Assuming that ADR1/ß-galactosidase fusion proteins have specific activities comparable to purified ß-galactosidase (purified ß-galactosidase has a specific activity of 3×10^5 U/mg[62]), fusion proteins were estimated to account for approximately 0.004% of the soluble protein in respiratory deficient strains bearing plasmid JC25. This estimate may be low as

numerous ß-galactosidase fusion proteins have been shown to have specific activities significantly lower than wild-type ß-galactosidase[64].

Expression of ADR1/ and ADR1^{C}/ß-Galactosidase Fusion Proteins in *E. coli.*

Plasmids containing portions of the ADR1 coding sequence were ligated in frame to the lacZ' gene and placed under the control of the tac promoter. The tac promoter was constructed by de Boer and coworkers[71] by fusion of the *E. coli* trp "-35" (T-T-G-A-C-A) and lac UV-5 Pribnow box (T-A-T-A-A-T) promoter regions. Transcription from this extremely efficient synthetic hybrid promoter is repressed by the lac repressor and can be derepressed by the addition of the fortuitous inducer isopropyl thiogalactoside (IPTG).

Two constructs, JC200Z and p6a45 (Figures 5 and 6), were used to produce ADR1/ß-galactosidase fusion proteins in *E. coli.* Plasmid JC200Z was predicted to produce a fusion protein of 191 KDa containing the N-terminal 659 amino acids of ADR1 fused to the eighth amino acid of ß-galactosidase . Plasmid p6a45 was designed to produce a slightly smaller fusion protein of 187 KDa and containing ADR1-5^{C} protein sequences from amino acid 17 to 642.

E. coli strains M182 (lac^{-}) and RR1 (lac^{+}) were transformed with plasmid 6a45 or JC200Z. Cells bearing either expression plasmid appeared red and were distinguishable from yellow control strains when examined 12 to 20 hours after plating on MacConkey-lactose indicator plates containing ampicillin.

Figure 5

Construction of *E. coli* ADR1/ and ADR1^{C}/ß-Galactosidase Expression Plasmids in the JC200Z Series

ADR1 sequence derived from plasmid JS119 was used in the construction of JC200Z. A 2.4 kb BamHI- HindII fragment of JS119 encompassing ADR1 sequences from +1 to +1971 was inserted into the polylinker of *E. coli* vector pUC19 to create plasmid JC100. A 1.9 kb Hinc II-Hind III fragment containing ADR1 coding sequences from +48 to +1971 was removed from JC100 and replaced with a 622 bp Hinc II-Hind III fragment from pBR322 to create plasmid JC0. This "stuffer" DNA was inserted to eliminate contamination of ligation reactions with ADR1 sequences during subsequent introduction of ADR1^{C} sequences. JC102, JC105, and JC107 were then created by replacing the stuffer DNA in JC0 with 1.9 kb Hinc II-Hind III fragments from plasmids YRp7-ADR1-2^{C}, YRp7-ADR1-5^{C}, and YRp7-ADR1-7^{C}, respectively. A 2.4 kb BamHI-HindII fragment of JC100, JC102, JC105, or JC107 encompassing ADR1 or ADR1^{C} sequences from +1 to +1971 was inserted into the polylinker of pSKS105[56] to create in-frame ADR1-lac Z' fusions in JC100Z, JC102Z, JC105Z, or JC107Z. An 8 kb Nco I-Nco I fragment from the start of ADR1 to the end of the lac operon was isolated from these plasmids and inserted into pKK240-11[55] at a unique Nco I site downstream from the tac promoter. The final JC200Z series constructs contain codons for amino acids 1 to 658 of ADR1 or ADR1^{C} fused in frame to the sixth codon of the lac Z' gene.

Figure 5: JC200Z Series Plasmid Constructions

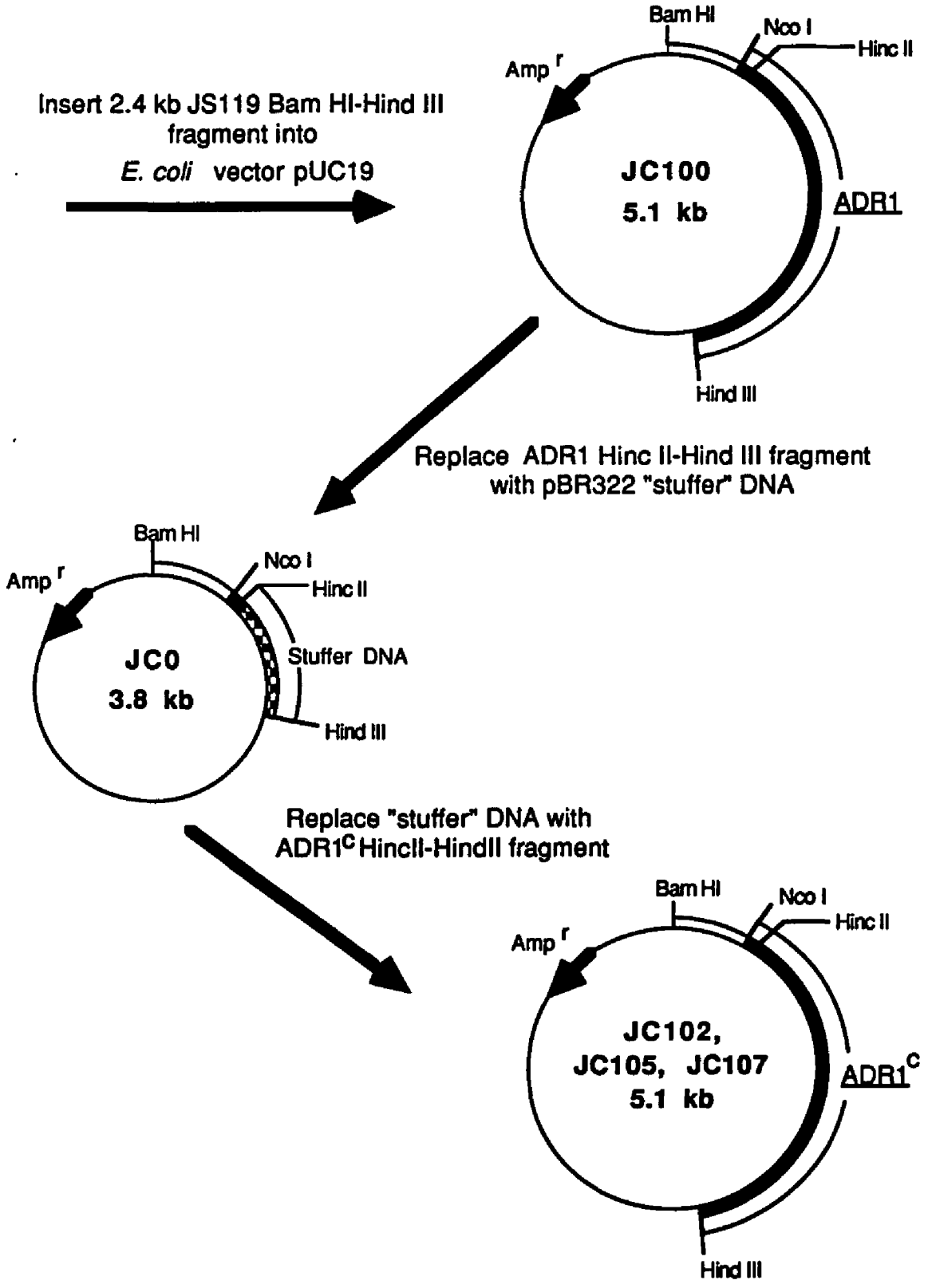


Figure 5 (continued)

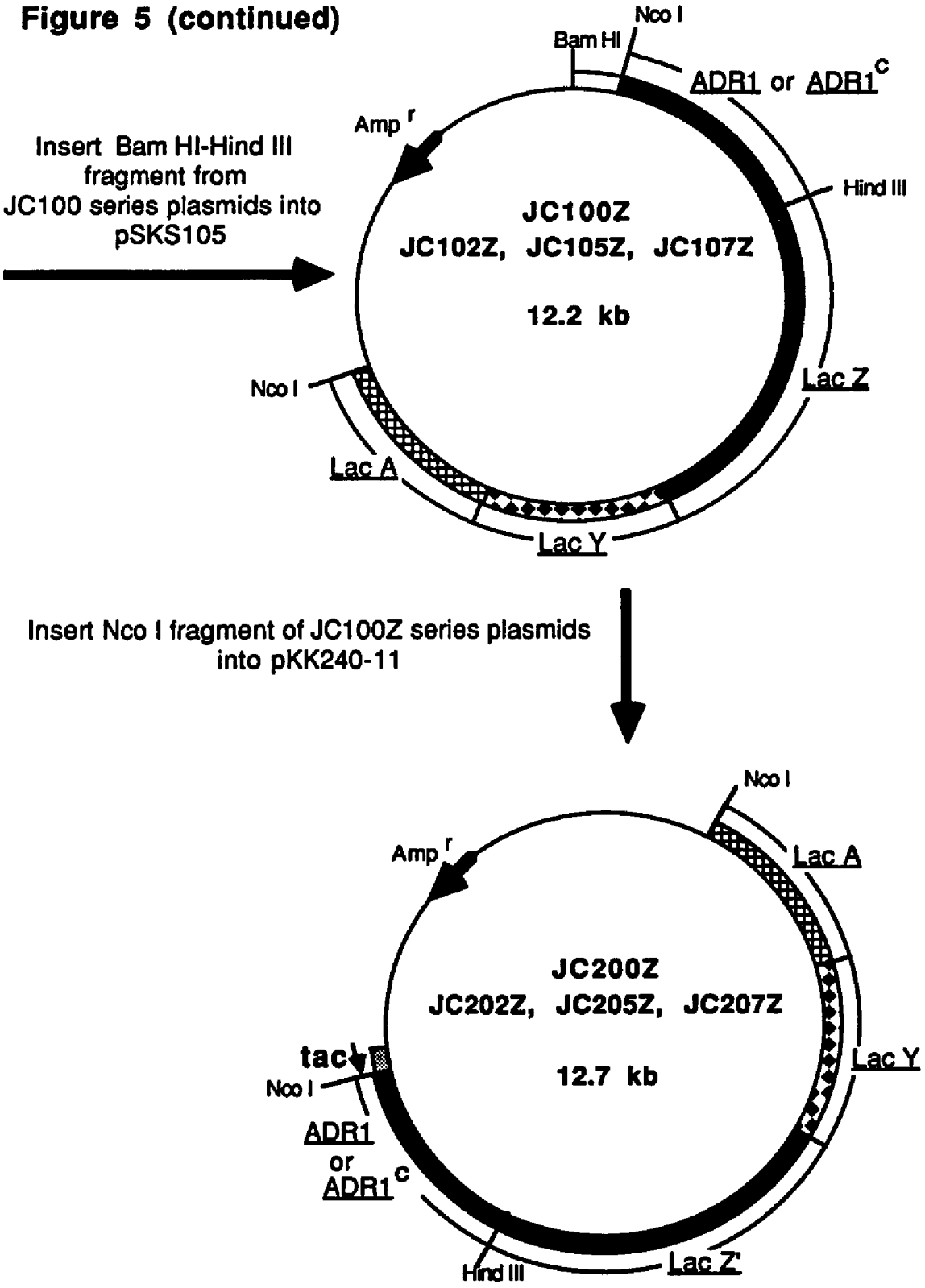
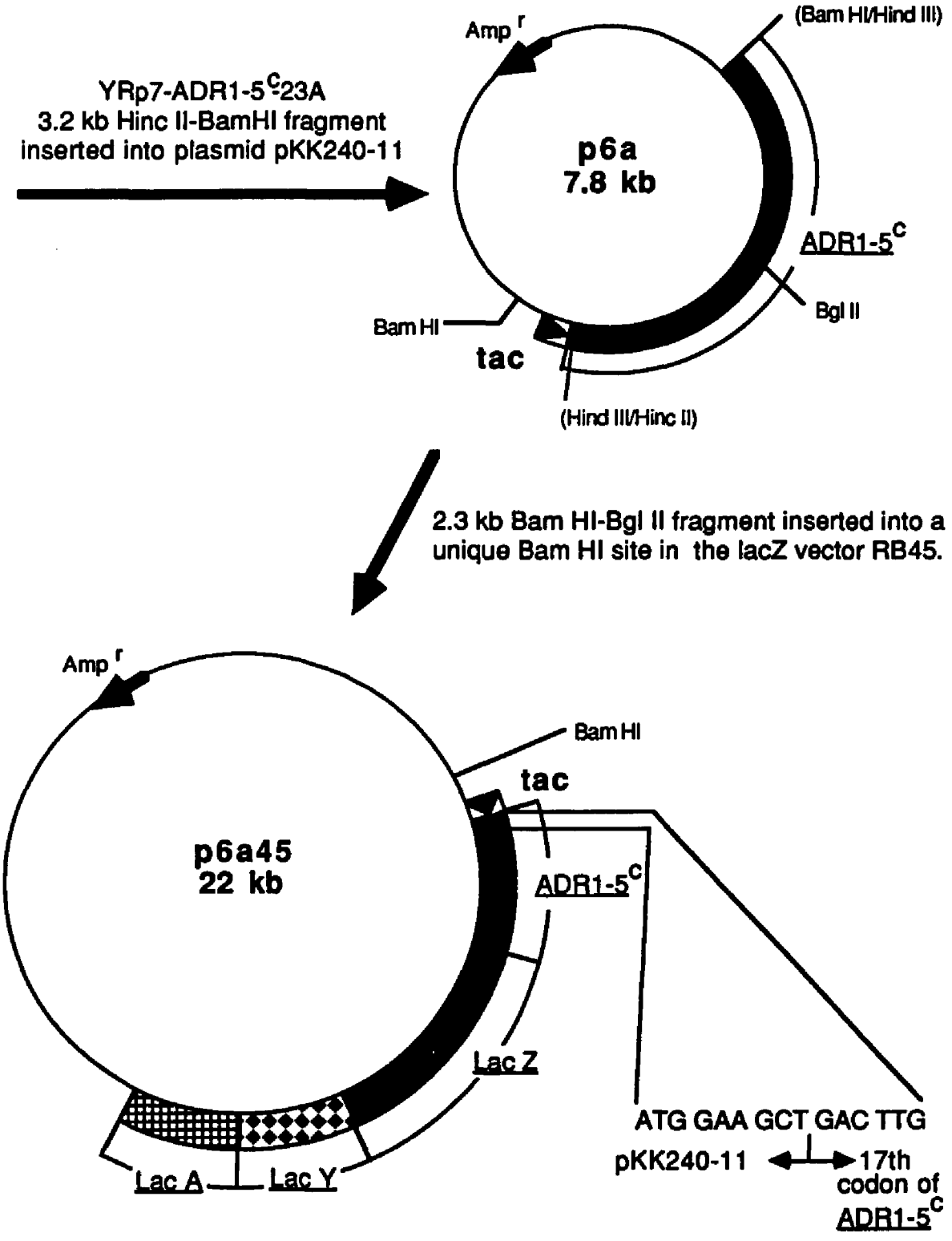


Figure 6

Construction of *E. coli* ADR1-5^{C}/ß-Galactosidase Expression Plasmid 6a45

For plasmid p6a45 the Hinc II-Bam HI 3.2 kb fragment of plasmid YRp7-ADR1-5^{C}-23A[16] was ligated into plasmid pKK240-11[55] at the Hind III site after both fragments had been rendered blunt-ended using the Klenow fragment of *E. coli* polymerase I. The unique Hind III site in plasmid pKK240-11 is situated such that a tac promoter, an ATG start codon, and two additional codons were fused in frame to the 17th codon of ADR1-5^{C} upon ligation. This plasmid, designated p6a, was then digested with Bgl II and Bam HI and the resulting 2.1 kb fragment containing the tac promoter and a portion of the ADR1-5^{C} gene was ligated into the Bam HI site of lacZ vector pRB45[54]. The final construct, p6a45, contains codons for amino acids 17-642 of ADR1-5c fused in-frame to the lac Z' gene at codon eight. The nucleotide sequence of the pKK240-11/ ADR1-5^{C} junction is shown the lower right corner.

Figure 6: Construction of plasmid 6a45



Some cells were observed to lose the ability to express ß-galactosidase after repeated plating as indicated by their inability to produce the characteristic red bile salts. This result was interpreted to be indicative of plasmid loss or mutation; cells which had lost their ability to yield red colonies were discarded.

Extracts of cells containing plasmid p6a45 and JC200Z were prepared and assayed for ß-galactosidase activity as described in Materials and Methods. *E. coli* plasmids were found to express significantly more ß-galactosidase activity than comparable yeast plasmids (Table 4). JC200Z, which is predicted to produce a fusion protein identical to that produced by the yeast vector JC25, expressed approximately 300 fold more ß-galactosidase activity than its yeast counterpart. *E. coli* fusion proteins were estimated to account for 1-2% of the total soluble protein in the *E. coli* cell extracts. Fusion protein production was found to be relatively insensitive to the strain differences.

Detection of Fusion Proteins Produced by Plasmids JC200Z and 6a45.

Protein extracts from plasmid-bearing cells were fractionated on SDS-polyacrylamide gels and stained for protein (Figure 7a). Protein extracts derived from *E. coli* carrying plasmid JC200Z contained five discrete fusion proteins which were unique to plasmid-bearing cells. These proteins had relative molecular weights of 182, 177, 175, 164, and 122 KDa. The size of the largest fusion protein detected, 182 KDa, is in approximate agreement with the

Table 4

ß-Galactosidase Activities of ADR1/ß-Galactosidase Fusion Proteins Expressed in *E. coli*

plasmid	strain	ß-galactosidase activity (U/mg)[a]
----	RR1(lac+)	5
p6a45	RR1(lac+)	5000
JC200Z	RR1(lac+)	3500
----	4562(lac-)	0
p6a45	4562(lac-)	4900
JC200Z	4562(lac-)	4400

[a] nmoles ONPG hydrolysed per minute per mg soluble protein.

Assays were performed essentially as described by Miller[62]. All assay values are the average of a minimum of three separate determinations. Cells to be assayed were grown at 37° C overnight in L-broth supplemented with 50 µg/ml ampicillin and were lysed at 0° C by sonication in buffer A:200 (25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM ß-mercaptoethanol, 1 mM phenylmethylsulfonyl chloride, 2 µM pepstatin, 0.6 µM leupeptin, 200 mM KCl).

predicted size of 190 KDa for the full-length JC200Z fusion protein. The 182, 177, 175, and 164 KDa fusions appear to be present at approximately equal concentrations, while the 122 KDa species is significantly more abundant than the other fusions. Densitometric quantitation of the 122 KDa species relative to the higher molecular weight fusions was impeded by negative staining of this protein on silver-stained gels.

The four truncated fusion proteins smaller than 182 KDa could have been the result of transcription or translation initiation from within the ADR1 coding sequence or of specific proteolysis of the full-length fusion protein. Searches for prokaryotic promoter elements[72] or ribosome binding sites (Shine-Delgarno sequences)[73] within ADR1 revealed no such homologies. If the truncations were the result of specific proteolysis, the degradation must have occurred within the cell prior to lysis since cells lysed by boiling in SDS-sample buffer produced the same gel pattern as cells lysed by sonication in the absence of protease inhibitors (data not shown).

Plasmid p6a45 was observed to produce a pattern of five discrete fusion protein products similar to that obtained with JC200Z (Figure 7a). The largest of these, presumably the full-length fusion protein, had a slightly greater mobility than the full-length JC200Z fusion protein. This was consistent with the predicted difference in molecular weight between the two proteins , as JC200Z

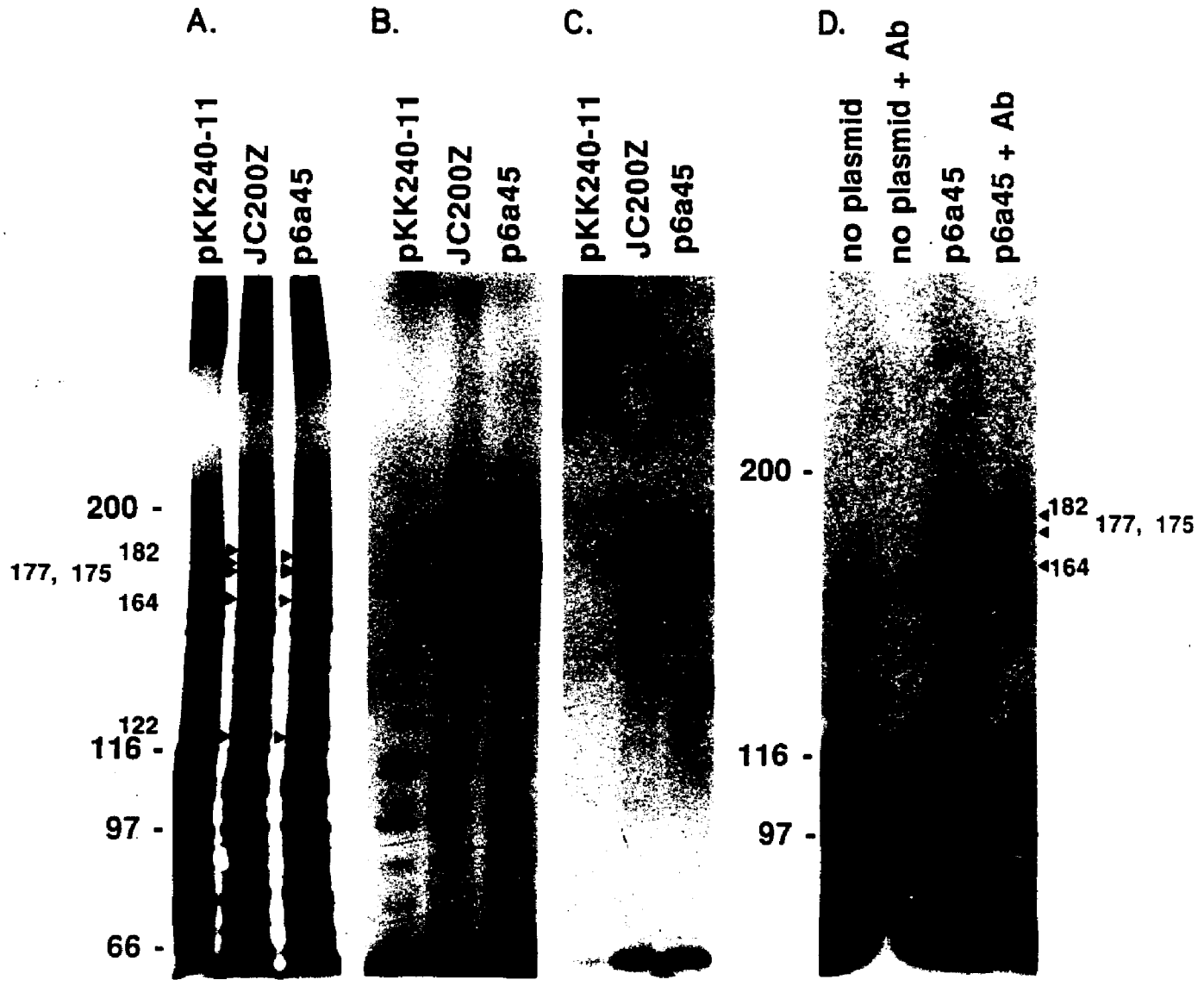
Figure 7. Detection of ADR1/ß-Galactosidase Fusion Proteins in *E. coli*

a. Identification of ADR1/ß-galactosidase fusion proteins. Silver-stained SDS-polyacrylamide gel of extracts from *E. coli* containing either the control plasmid pKK240-11, plasmid JC200Z, or plasmid p6a45. Numbers at the left of the gel refer to molecular weights of marker proteins in KDa, arrowheads indicate plasmid-encoded fusion proteins with calculated molecular weights of 182, 177, 175, 164, and 122 KDa for JC200Z and 180, 175 (two comigrating proteins), 162, and 120 KDa for p6a45. The gel was stained with Gelcode silver stain (Pierce Chemical Co., Rockford, IL)

b. *In vitro* phosphorylation of ADR1/ß-galactosidase fusion proteins. Autoradiogram of the gel pictured in **a.** showing fusion proteins phosphorylated by bovine cAMP-dependent protein kinase *in vitro.* Reactions were performed as described in Materials and Methods with bovine cAPK at a concentration of 1 pmol U/µl.

d. Precipitation of phosphorylated fusion proteins using anti-ADR1 antibodies. Antibodies raised against a synthetic peptide containing ADR1 amino acids 208-231 were used to precipitate ADR1-containing fusion proteins from denatured extracts of *E. coli* strain M182 carrying the plasmid indicated at the top of each lane.

d. Precipitation of phosphorylated fusion proteins using anti-ß-galactosidase antibodies. Autoradiogram of whole extracts and anti-ß-galactosidase antibody precipitates of extracts of *E. coli* strain RR1 carrying no plasmid (controls) or plasmid p6a45.


A.
B.
C.
D.
pKK240-11
JC200Z
p6a45
pKK240-11
JC200Z
p6a45
pKK240-11
JC200Z
p6a45
no plasmid
no plasmid + Ab
p6a45
p6a45 + Ab
200 -
116 -
97 -
66 -
182
177, 175
164
122
200 -
116 -
97 -
182
177, 175
164

was predicted to produce a 190 KDa protein while p6a45 was predicted to produce a 186 KDa protein. With the possible exception of the 122 KDa species, the truncated products of p6a45 also appear to be slightly reduced in size when compared to their JC200Z counterparts.

The fusion proteins in extracts of *E.coli* strain M182 (lac$^-$) carrying plasmid p6a45 were partially purified on a sepharose-p-aminophenyl-1-thio-ß-D-galactopyranoside ß-galactosidase affinity column. Peak fractions were inspected for fusion proteins using SDS-PAGE (Figure 8). The elution fraction having the highest ß-galactosidase activity also contained numerous proteins which were presumably bound non-specifically to the affinity resin despite the use of the high-volume, high-stringency washing procedure of Ullmann (100 column volumes with 1.6 M NaCl in phosphate buffer). Of the identified fusion proteins, only the 122 KDa fusion protein species appeared to be significantly enriched (Figure 8; compare p6a45 lane to ACF lane). This fusion protein, which is in the greatest abundance of all the fusion proteins in crude extracts, must therefore have ß-galactosidase activity. While the other fusion species may also have activity, their affinity to the column may be detrimentally affected by the presence of the N-terminal ADR1 sequences. It is unlikely that the abundant 122 KDa species sterically excluded the other fusion proteins from binding to the resin as the column was significantly under-loaded (see Figure 8

Figure 8

SDS-PAGE of ß-Galactosidase Affinity Column Peak Fraction Proteins

Silver stained SDS-polyacrylamide gel analysis of affinity purified fusion proteins encoded by plasmid p6a45 in *E. coli* strain M182 (lane designated ACF for Affinity Column Fraction). Reference lanes contain extracts of strain M182 bearing no plasmid or plasmid p6a45, as indicated. Numbers to the left of the gel indicate the mobilities of marker proteins with molecular weights in KDa. The arrowhead on the right indicates the 122 KDa fusion protein, as discussed in the text.

Approximately 2 mg of soluble protein containing 8800 units of ß-galactosidase activity was applied to a 1 ml ß-galactosidase affinity column (capacity estimated to be 84,000 units) prepared according to Ullmann[64]. The column was washed with 100 column volumes of high-salt wash buffer to remove non-specifically bound proteins. Proteins having ß-galactosidase activity were eluted from the column with 100 mM sodium borate, pH 10, 10 mM ß-mercaptoethanol. 0.5 ml fractions were collected and assayed essentially as described[62]. The second and third fractions were found to contain ~80% of the activity loaded and represented an approximately 7-fold purification. Peak fractions were pooled and concentrated and an aliquot containing 15 µg of protein was applied to the gel. Reference lanes contain 100 µg of protein in each lane.

66
97
116
200
122
no plasmid
p6a45
ACF

legend).

Extracts of *E. coli* bearing p6a45 were also analysed using native PAGE followed by *in situ* activity staining for ß-galactosidase (Figure 9). Only a single diffuse band of ß-galactosidase activity was observed. This band was excised and re-fractionated using SDS-PAGE. The only active band observed was identified as the 122 KDa fusion protein (Figure 9). Therefore, although all the fusion proteins could have ß-galactosidase activity, ß-galactosidase activity was only detected in the 122 KDa species.

In Vitro Phosphorylation of ADR1/ß-Galactosidase Fusions Using Bovine cAMP-Dependent Protein Kinase. Extracts of *E. coli* bearing either plasmid 6a45 or JC200Z were phospho-labeled *in vitro* with excess bovine cAMP-dependent protein kinase and [γ-^{32}P] ATP. The resultant radioactive proteins were subjected to SDS-PAGE and detected by autoradiography (Figure 7b). The JC200Z-encoded proteins of 182, 177, 175, and 164 KDa were all phosphorylated, as were the corresponding p6a45 proteins. The 122 KDa species, although the most abundant of the fusion proteins, was not phosphorylated. This species therefore appears to lack sufficient ADR1 residues to act as a substrate for the protein kinase, as discussed below.

Polyclonal antibodies directed against a synthetic peptide containing ADR1 residues 208-231 were used to precipitate the phosphorylated fusion proteins

Figure 9

a. *In Situ* Activity Staining of Proteins Displaying ß-Galactosidase Activity.

Lanes contain approximately 300 µg of soluble protein from *E. coli* strain M182 (lac$^-$) bearing no plasmid, plasmid p6a, or p6a45 as indicated. Purified ß-galactosidase was purchased from Sigma; approximately 700 units were applied (3 µg). Native PAGE and subsequent staining for ß-galactosidase were performed as described in Materials and Methods.

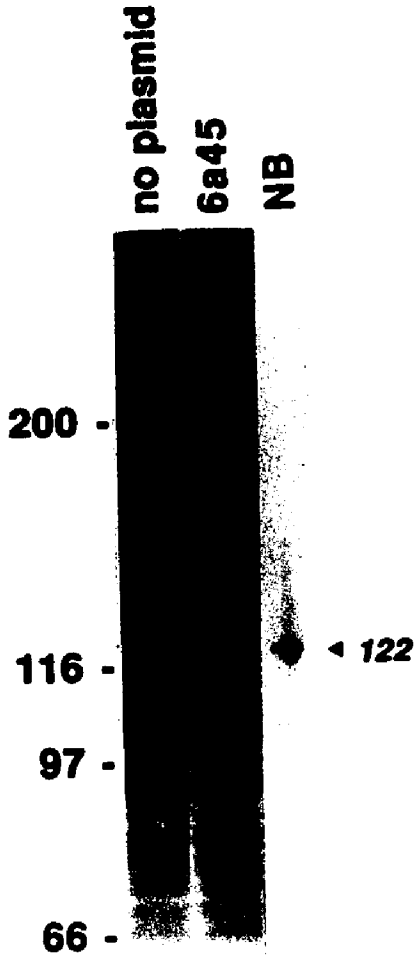
b. SDS-PAGE of ß-Galactosidase Activity-Containing Fusion Proteins Isolated from a Native Polyacrylamide Gel

Crude extracts of E. coli M182 bearing no plasmid or plasmid p6a45 were run for reference in the first two lanes. The third lane contains the major band of ß-galactosidase activity in **a.** (NB). Numbers to the left of the gel refer to the molecular weights of marker proteins in KDa. The 122 KDa fusion protein is indicated by the arrowhead on the right.

After excision from the native gel, the band containing the p6a45 active band was soaked overnight in 10% acetic acid. The gel slice was then washed twice for one hour in 1X SDS sample buffer, decanted, and the gel slice was placed in a boiling water bath for 5 minutes prior to insertion into the sample well. The SDS gel was stained with the Gelcode stain of Pierce Chemical Co. (Rockford, IL).


purified B-gal
no plasmid
p6a
p6a45

a.


no plasmid
6a45
NB
200 -
116 -
97 -
66 -
122

b.

encoded by plasmids p6a45 and JC200Z (Figure 7c). The 182, 177, and 175 KDa fusion proteins were all specifically precipitated by this antibody. The analogous proteins from the p6a45 extract were also observed to be immunoprecipitated. The 164 KDa fusion protein produced by either JC200Z or p6a45 was not immunoprecipitated by this antibody and therefore either does not contain sufficient ADR1 sequences to be recognized by the antibody or folds in such a way that interaction with the antibody is blocked. The 122 KDa protein, which is not phosphorylated, was also not visible in protein stains of the anti-ADR1 antibody immunoprecipitates. These results indicate that the 122 KDa fusion protein does not contain the ADR1 protein sequences recognized by cAPK or the anti-ADR1 antibodies.

A similar immunoprecipitation experiment was conducted using a polyclonal anti-ß-galactosidase antibody. All fusion proteins expressed by p6a45, as evidenced by protein staining (Figure 10) and autoradiography (Figure 7d), were observed to be immunoprecipitated. These results indicate that although only the 182, 177, 175 and 164 KDa species were phosphorylated (Figure 7a), all five fusion proteins contain sufficient ß-galactosidase sequence to be recognized by the anti-ß-galactosidase antibody.

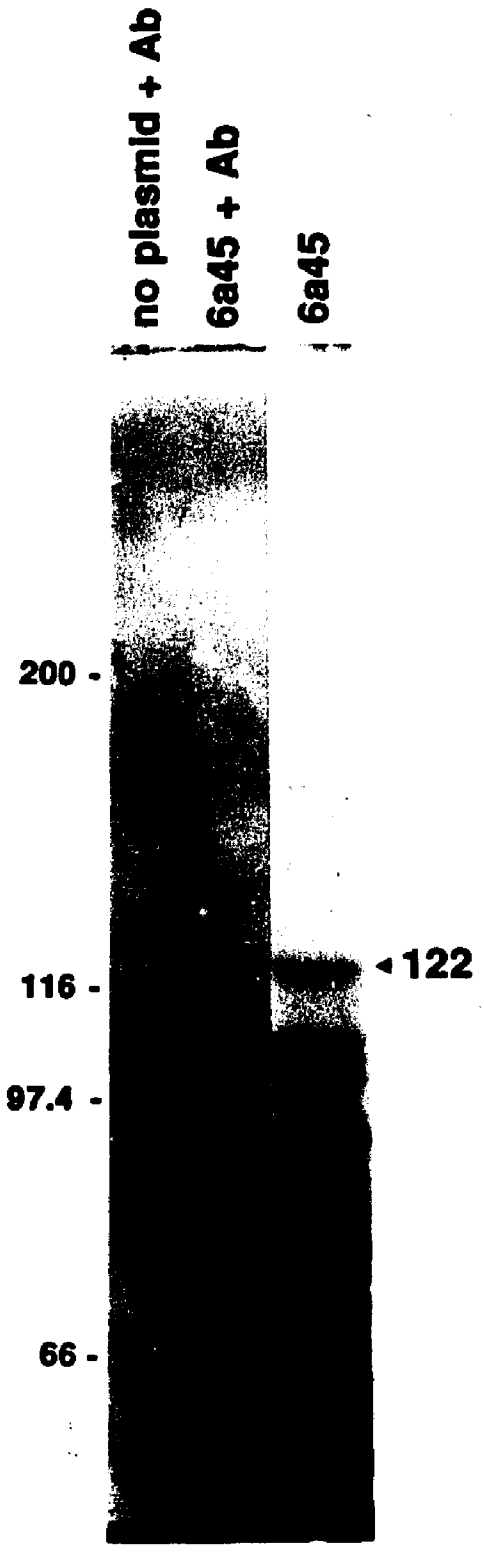
The approximate locations of the truncation sites resulting in the 122 KDa and 164 KDa JC200Z-encoded fusion protein products were postulated by the

Figure 10

SDS-PAGE Analysis of p6a45-Encoded Proteins Precipitated by Anti-ß-Galactosidase Antibody

Silver stained SDS-polyacrylamide gel of anti-ß-galactosidase antibody precipitates from *E. coli* RR1 bearing no plasmid or plasmid p6a45. The right hand lane contains crude p6a45/RR1 extract. Numbers to the left of the gel indicate migration of molecular weight standards in KDa. The p6a45-encoded 122 KDa fusion protein is indicated on the right.

Antibody precipitations were carried out under native conditions using polyclonal anti-ß-galactosidase antibodies as described in Materials and Methods. The gel was stained with the Kodavue system (Eastman Kodak, Rochester, NY).


no plasmid + Ab
6a45 + Ab
6a45
200 -
116 -
97.4 -
66 -
122

following reasoning: The smallest and most abundant fusion protein (122 KDa) was not phosphorylated nor was it precipitated by the anti-ADR1 antibody. It was, however, precipitated by the anti-ß-galactosidase antibody and *in situ* activity staining indicated that it did have ß-galactosidase activity. Based on the molecular weight derived from SDS-PAGE, the 122 KDa fusion protein was estimated to contain 550 fewer amino acids than the full length fusion protein which contains 659 ADR1 residues and 1017 ß-galactosidase residues. The 122 KDa species therefore most likely contains only a small portion of ADR1 lacking the phosphorylation site at amino acid 230 fused to a whole or near whole functional ß-galactosidase protein. The 164 KDa protein was phosphorylated, was precipitated by anti-ß-galactosidase antibody, but was not precipitated by the anti-ADR1 antibody. The truncation site in the 164 KDa ADR1/ß-galactosidase fusion protein may lie between residues 208 and 227 of ADR1 in order for it not to be recognized by the anti-ADR1 antibody which is directed specifically against the 208-231 ADR1 peptide, yet still retain the ability to be phosphorylated at serine-230. Results of peptide mapping experiments discussed below indicate that phosphorylation of the 164 KDa fusion protein occurs solely at residue 230. Alternatively, the SDS-PAGE derived molecular weight indicates that the 164 KDa species lacks approximately 170 residues indicating that cleavage may occur at a site to the N-terminal side of residue

208, yet alter the folding of the resulting protein such that interaction with the anti-ADR1 antibody is blocked.

Relative Phosphorylation of ADR1/ and ADR1^{C}/ß-Galactosidase Fusion Proteins. Previous investigations hypothesized that ADR1 mutant proteins which allow ADH2 expression to partially escape glucose repression are not phosphorylated as efficiently as wild-type ADR1[32]. This hypothesis was tested using plasmids JC202Z, JC205Z, and JC207Z. These plasmids are identical to plasmid JC200Z except they contain the ADR1-2^{C}, ADR1-5^{C}, and ADR1-7^{C} mutations, respectively (Figure 5). Extracts of *E. coli* bearing these plasmids were phosphorylated using varying concentrations of bovine cAMP-dependent protein kinase and analyzed by SDS-PAGE (Figure 11).

The ADR1-2^{C}, ADR1-5^{C}, and ADR1-7^{C} mutations were found to significantly decrease the level of fusion protein phosphorylation relative to wild-type ADR1. This trend became progressively clearer as the level of kinase in the phosphorylation mixes decreased (Figure 12). In other words, at low kinase concentrations the ADR1 protein was a better substrate for cAPK and was phosphorylated to a greater extent than were the ADR1-2^{C}, ADR1-5^{C}, and ADR1-7^{C} proteins. At higher kinase concentrations the ADR1-2^{C} and ADR1-5^{C} proteins appeared to be phosphorylated to nearly the same extent as the wild-type protein. Thus ADR1 mutations which render ADH2 expression constitutive

Figure 11

Relative Phosphorylation of ADR1/, ADR1-2^C/, ADR1-5^C/, and ADR1-7^C/ß-Galactosidase Fusion Proteins Using Bovine cAMP-Dependent Protein Kinase

Autoradiogram of plasmid-bearing E. coli strain M182 extracts phosphorylated *in vitro* using bovine cAMP-dependent protein kinase and separated on a 5% SDS-polyacrylamide gel. Extracts of E. coli strain M182 bearing plasmids JC200Z (ADR1), JC202Z (ADR1-2^C), JC205Z (ADR1-5^C), and JC207Z (ADR1-7^C) were prepared, phospho-labeled and fractionated on an SDS-polyacrylamide gel as described in Materials and Methods. The ATP concentration was 0.1 mM with a specific activity of 2.5 X 10^3 cpm/pmol and the concentration of the catalytic subunit of bovine cAMP-dependent protein kinase was varied as indicated.

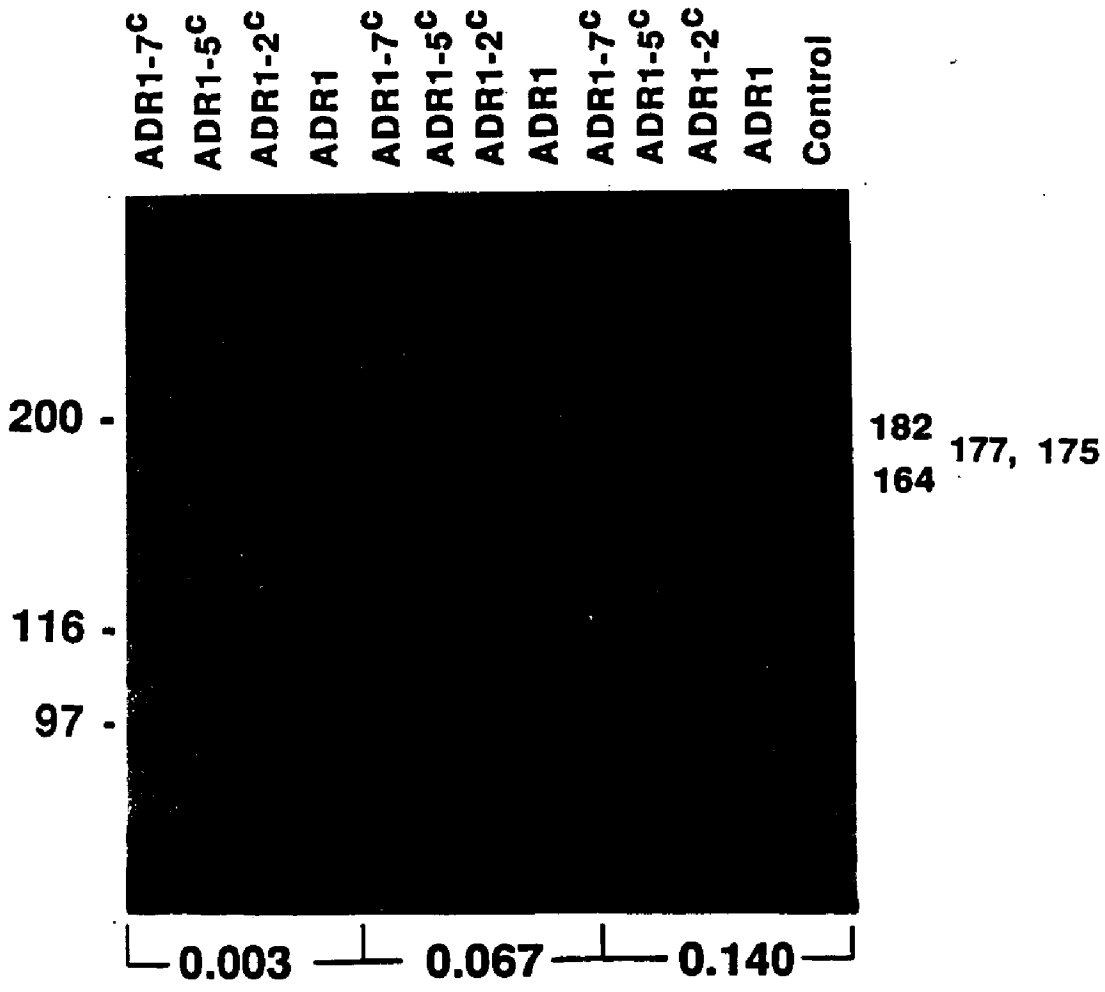

ADR1-7C
ADR1-5C
ADR1-2C
ADR1
ADR1-7C
ADR1-5C
ADR1-2C
ADR1
ADR1-7C
ADR1-5C
ADR1-2C
ADR1
Control
200
116
97
182
164
177, 175
0.003
0.067
0.140
[kinase], pmol U/µl

Figure 12

Graphical Representation of Phosphate Incorporation in the ADR1^{C} Fusion Proteins Relative to the ADR1 Fusion Proteins

Phosphate incorporation was measured by densitometric scanning and integration of the 164 KDa fusion protein bands appearing on the autoradiogram in Figure 11. Phosphate incorporation in each fusion protein was scaled to the amount of fusion protein present in each labeled band as determined by densitometric analysis of the silver stained polyacrylamide gel. The total amount of phosphate incorporated into the ADR1/ß-galactosidase fusion proteins at the highest kinase concentration (0.14 pmol U/ µl) was arbitrarily scaled to 1. Each value for the indicated fusion protein species is presented as the fraction of that total.

Figure 12a. 182 KDa Fusion Phosphorylation

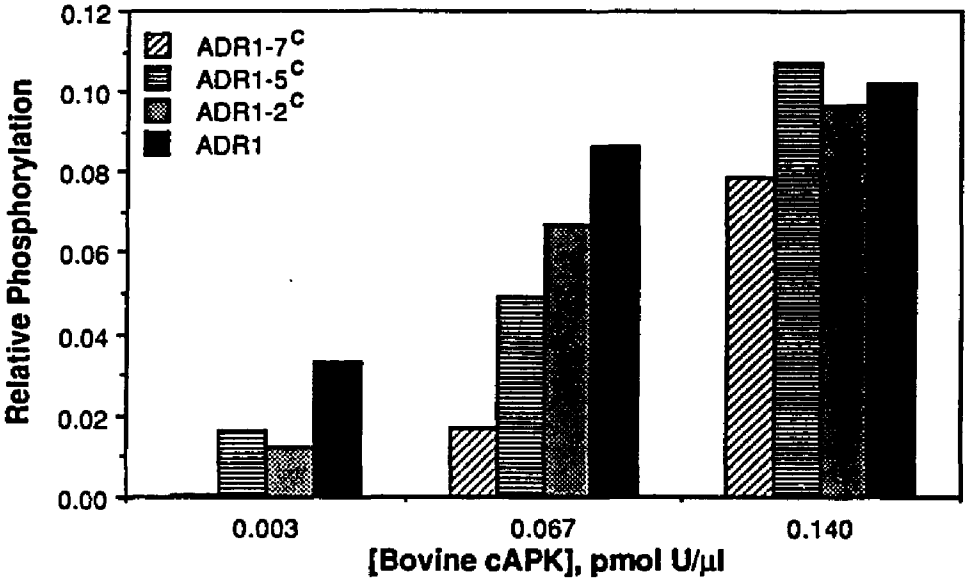


Figure 12b. 177,175 KDa Fusion Phosphorylation

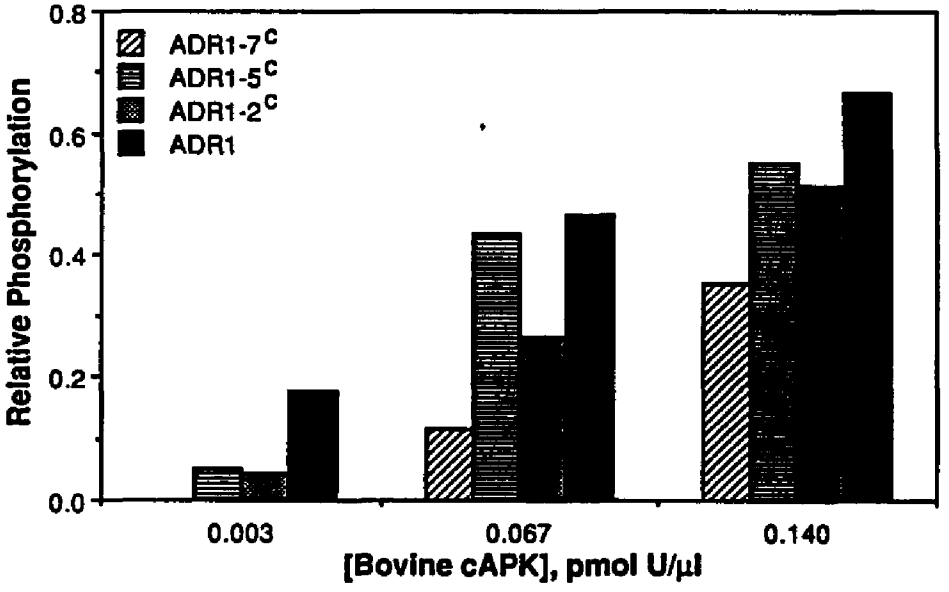


Figure 12c. 164 KDa Fusion Phosphorylation

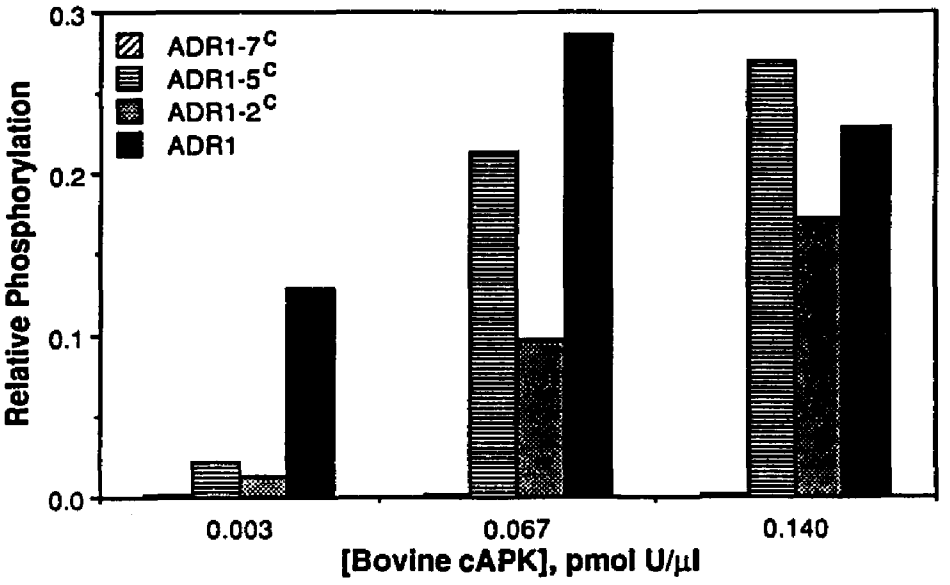
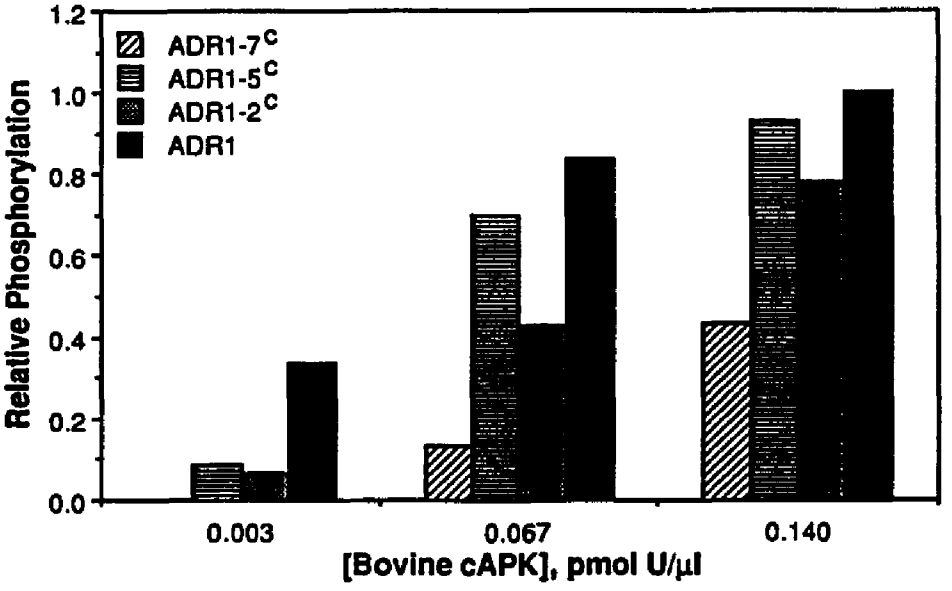


Figure 12d. Total Fusion Phosphorylation



in yeast also decrease *in vitro* phosphorylation of ADR1 by bovine cAMP-dependent protein kinase. Since these mutations alter residues within the phosphorylation consensus region found between amino acids 227 and 230, the serine residue at amino acid 230 most likely serves as the primary phosphate acceptor of ADR1 cAMP-dependent phosphorylation.

The presence of a second ADR1 phosphorylation site was indicated by the phosphorylation of fusion proteins produced by plasmid JC207Z. This plasmid carries the ADR1-7^{C} mutation and therefore encodes a fusion protein in which the phosphoacceptor serine at amino acid 230 is replaced by a leucine. Although such a mutation would be expected to eliminate phosphorylation at the primary site, it did not completely block phosphorylation of the fusion proteins (Figure 11). The 182, 177, and 175 KDa ADR1-7^{C}/ß-galactosidase fusion proteins were all slightly but significantly phosphorylated (Figs. 11 & 12). However, even at high kinase concentrations, the 164 KDa fusion protein was not phosphorylated. It therefore appears that a secondary phosphorylation site exists to the N-terminal side of the primary site at amino acid 230 which is not present in the truncated 164 KDa protein. Phosphorylation of this secondary site was only detectable at high enzyme concentrations indicating that it is a less "preferred" substrate for the cAMP-dependent protein kinase than the primary site.

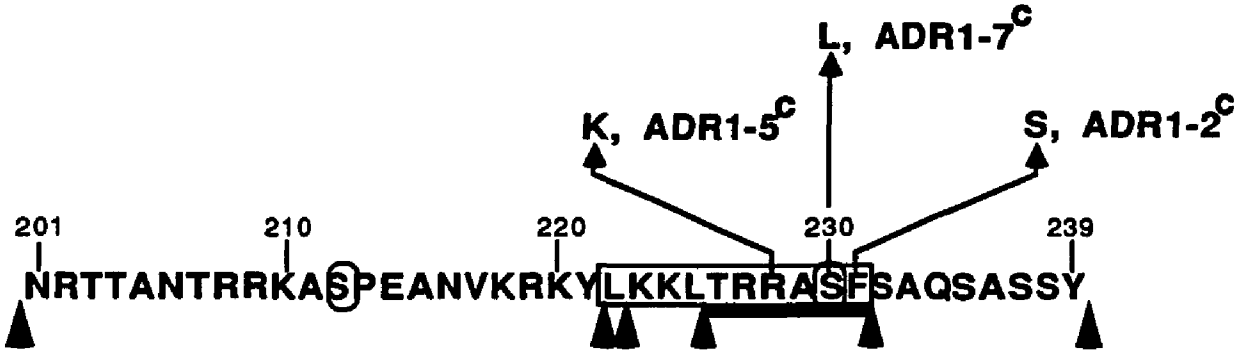
Peptide Mapping of the ADR1 Phosphorylation Sites. Phosphorylated ADR1/ and ADR1^{C}/ß-galactosidase fusion proteins were isolated from SDS-polyacrylamide gels and digested with chymotrypsin. The resulting peptides were analyzed using native PAGE as described in Materials and Methods (Figure 14).

The 164 KDa fusion protein, the doublet containing the 177 and 175 KDa species, and the 182 KDa fusion protein were each excised from lanes containing JC200Z (ADR1), JC202Z (ADR1-2^{C}), JC205Z (ADR1-5^{C}) and JC207Z (ADR1-7^{C}) extracts. The proteins contained in the 182 KDa and 177, 175 KDa doublet were thought to contain two phosphorylation sites, a primary site at amino acid 230 and an unidentified secondary site, while the 164 KDa protein apparently contained only the primary site. Comparison of labeled peptides produced by digestion of these two protein species thus allowed differentiation between those chymotryptic peptides containing the primary site of phosphorylation and those containing the secondary site of phosphorylation.

A synthetic peptide spanning amino acids 222 to 231 of ADR1 was phosphorylated and electrophoresed on the mapping gel in both chymotrypsin-digested and undigested form as indicated in Figure 13. This peptide contained the putative primary phosphorylation site at amino acid 230 and thus served as a standard for comparison to the ADR1 fusion protein digest. The undigested

Figure 13

ADR1 Amino Acid Sequence Surrounding the Putative Phosphorylation Site at Amino Acid 230.



The sequence of the undigested synthetic peptide is boxed. Bold arrows beneath the sequence indicate possible chymotryptic cleavage sites, circles represent serine residues likely to serve as phosphoacceptors based on homology to known sites of cAMP-dependent protein kinase recognition sequences. Predicted alterations in primary structure caused by the ADR1^{C} mutations are indicated above the single letter amino acid abbreviations. The bold line beneath residues 226-231 indicates the presumed limit digest product of the ADR1/ß-galactosidase fusion containing the primary phosphorylation site. The ADR1-2^{C} limit digest product was predicted to contain residues 226-239 due to loss of the chymotryptic site after phenylalanine^{231}. Calculated charge to frictional coefficient ratios of the putative chymotryptic digest products are approximated in calculations presented in Appendix D.

Figure 14

Peptide Mapping of the Primary Phosphorylation Site in ADR1

Autoradiogram of a 50% polyacrylamide gel (pH 3) containing phosphorylated peptides produced by chymotryptic digest of ADR1/ß-galactosidase fusion proteins as described in Materials and Methods. Digests of the 182 KDa fusions, the 177 and 175 KDa fusions, and the 164 KDa fusions appear in the lanes indicated. The top of each lane is labelled according to the ADR1 mutation it contains. Lanes containing digested and undigested synthetic ADR1 peptide standards are so indicated and are described in Materials and Methods. The Control lane contains a chymotrypsin-digested phosphorylation mix which lacked a peptide or E. coli extract substrate.

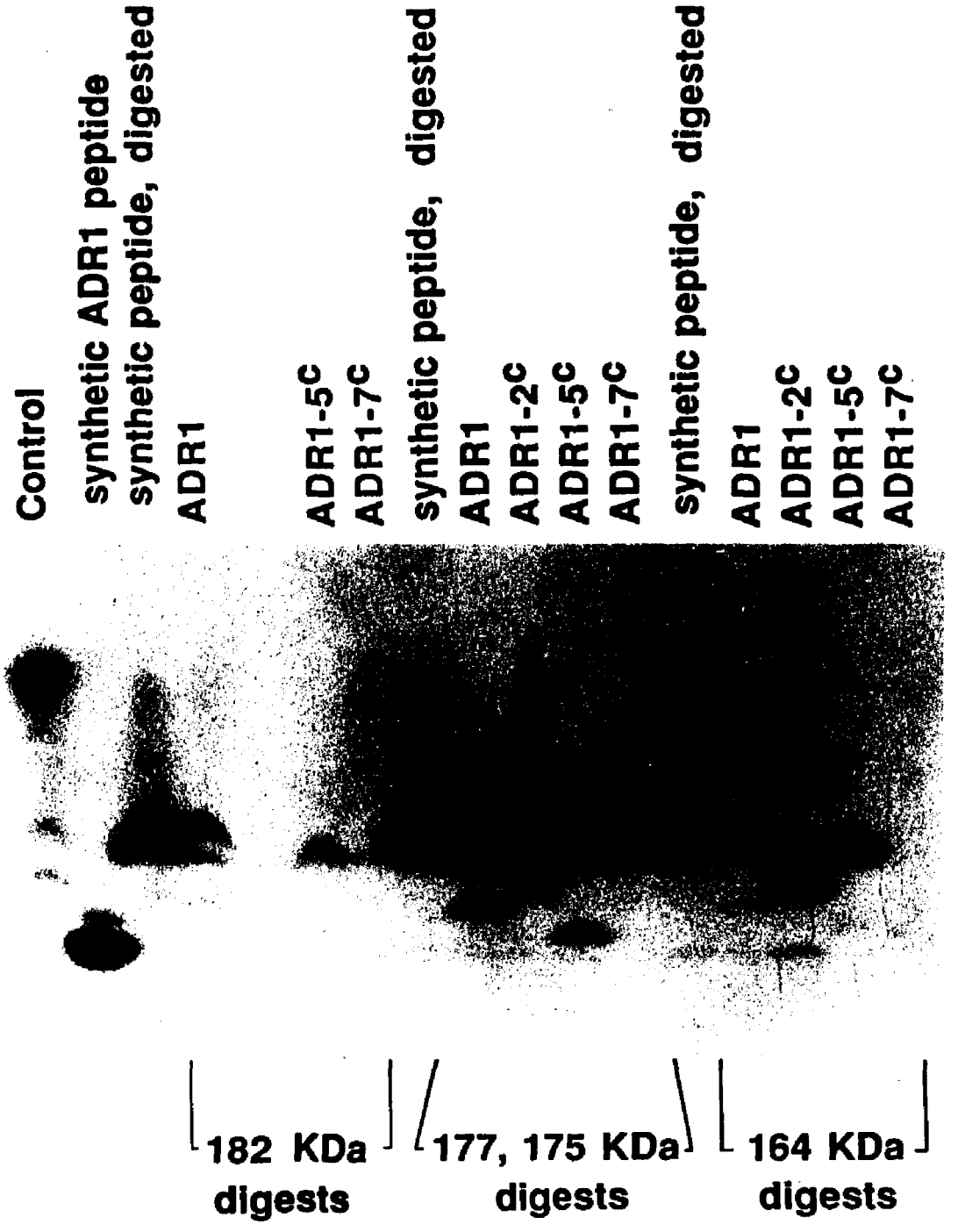

Control
synthetic ADR1 peptide
synthetic peptide, digested ADR1
ADR1-5C
ADR1-7C
synthetic peptide, digested ADR1
ADR1-2C
ADR1-5C
ADR1-7C
synthetic peptide, digested ADR1
ADR1-2C
ADR1-5C
ADR1-7C
182 KDa digests
177, 175 KDa digests
164 KDa digests

Figure 14

peptide, which was designed as a chymotryptic digest product, ran near the front on the mapping gel (Figure 14). After digestion its mobility decreased, presumably as the N-terminal four residues were removed to produce the limit digest product defined by residues 227-231.

Digestion of the ADR1/ß-galactosidase fusion proteins in the 182, 177 -175, and 164 KDa bands revealed three major phospho-labeled peptides, the slowest migrating of which co-migrated with the synthetic peptide limit digest. in both the 177,175 KDa doublet and the 164 KDa species. The peptide with the greatest mobility appears faintly in the 164 KDa digest lane (Figure 14) and is presumed to be a partial digest product which contains the primary phosphorylation site at serine-230. This conclusion is supported by the fact that both the slowest and fastest migrating peptides of the ADR1 182 and 177-175 KDa digests are not present in the digests of the ADR1-7^C 182 and 177-175 KDa fusion proteins. In contrast, the peptide having an intermediate mobility in the ADR1 182 and 177-175 KDa digest lanes is present in all the ADR1^C 182 and 177-175 KDa fusion protein digest lanes. Because the ADR1-7^C fusion protein cannot be phosphorylated at residue 230, this intermediate band must be a peptide containing a secondary phosphorylation site. The absence of this intermediate-mobility peptide in all of the 164 KDa digest lanes indicates that it is derived from sequences on the N-terminal side of residue 230. It can be

concluded that under these conditions ADR1 is selectively phosphorylated at two specific sites.

The ADR1-2^{C} mutation, which eliminates the chymotryptic cleavage site at amino acid 231, resulted in a decrease in the mobility of the partial and limit digest phosphopeptides containing residue 230 due to the decreased charge to frictional coefficient ratio caused by the extension of the peptide (see Appendix D for these calculations). The ADR1-5^{C} mutation, on the other hand, caused an increase in the mobility of these phosphopeptides relative to the ADR1 digest. Although the ADR1-5^{C} mutation does not alter any chymotrypsin sites directly, the change from an arginine to a lysine within the labeled peptides apparently causes an alteration in mobility. It should be noted that the ADR1-2^{C} partial digest peptide (having the greatest mobility) and the ADR1-5^{C} limit digest peptide (having the lowest mobility) both comigrated with the peptide containing the secondary site. These ADR1-2^{C} and ADR1-5^{C} peptides are present in the 164 KDa digest patterns further supporting that they are derived from the peptide containing the primary site of phosphorylation. The partial digest peptides containing residue 230 varied in their autoradiogram signal intensity relative to the limit digest peptide from digest to digest, supporting the conclusion that they result from partial proteolysis of the whole fusion proteins. Chymotryptic digestion and peptide analysis of the 182 KDa

ADR1/ and ADR1^{C}/ ß-galactosidase fusion proteins gave similar mapping results to the results obtained using the 177, 175 KDa protein (Figure 14). Unfortunately the ADR1-2^{C} 182 KDa digest was lost during the procedure .

Phosphorylation of ADR1/ and ADR1^{C}/ß-Galactosidase Fusion Proteins by Yeast cAMP-Dependent Protein Kinase. Purified yeast cAMP-dependent protein kinase catalytic subunit C1 encoded by the TPK1 locus was also used to phosphorylate the fusion proteins *in vitro*. The fusion proteins were phosphorylated by yeast cAPK in a manner similar to that observed with the bovine kinase. As shown in Figure 15, the ADR1, ADR1-2^{C} and ADR1-5^{C} fusion protein bands of 182, 177, 175, and 164 KDa were labeled by the C1 kinase while the ADR1-7^{C} fusion band of 164 KDa was, as seen with the bovine kinase, not phosphorylated. Phosphorylation was specifically blocked by the addition of purified yeast cAMP-dependent protein kinase regulatory subunit (R encoded by BCY1).

Although the yeast and bovine kinases were observed to phosphorylate the same fusion protein species, the two enzymes were found to differ in their apparent specificities for the ADR1-2^{C} and ADR1-5^{C} proteins. Using the bovine kinase the relative incorporation of phosphate was ADR1 > ADR1-5^{C} > ADR1-2^{C} > ADR1-7^{C}. In contrast the yeast kinase phosphorylated ADR1 > ADR1-2^{C} > ADR1-5^{C} > ADR1-7^{C} (Figure 16). On this basis it is concluded that yeast C1

kinase phosphorylates the sequence RRASSS (ADR1-2^{C}) better than the sequence RKASFS (ADR1-5^{C}), while the mammalian kinase phosphorylates the sequence RKASFS better than the sequence RRASSS. The two kinases may therefore differ in their substrate specificities.

Figure 15

Relative Phosphorylation of ADR1/, ADR1-2^{C}/, ADR1-5^{C}/, and ADR1-7^{C}/ß-Galactosidase Fusion Proteins Using Yeast cAMP-Dependent Protein Kinase C1

Autoradiogram of plasmid-bearing E. coli strain 4562 extracts phosphorylated in vitro using the purified yeast kinase C1 as described in Materials and Methods. In those lanes indicated (+) purified yeast regulatory subunit R (BCY1) was added to a concentration which was 10 fold higher than that of the catalytic subunit.

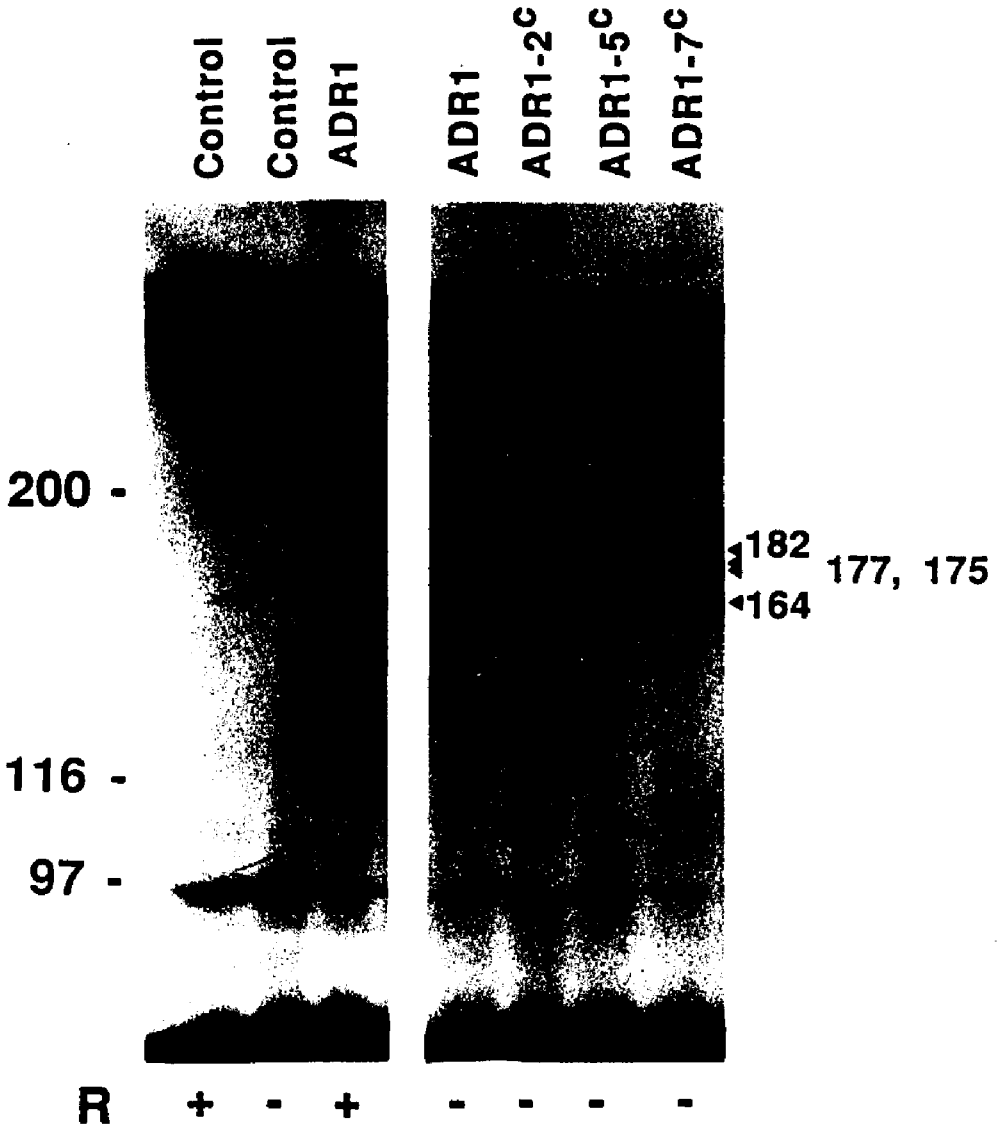

Control
Control
ADR1
ADR1
ADR1-2C
ADR1-5C
ADR1-7C
200 -
116 -
97 -
182
164
177, 175
R + - + - - - -

Figure 16. Substrate Specificities of the Yeast and Bovine Kinases

a. Graphical representation of phosphate incorporation in the 164 KDa ADR1/, ADR1-2^{C}/, and ADR1-5^{C}//ß-galactosidase fusion proteins using yeast cAMP-dependent protein kinase C1. Phosphate incorporation was measured by densitometric scanning and integration of the 164 KDa fusion protein bands appearing on the autoradiogram in Figure 15. Phosphate incorporation in each fusion protein was scaled to the amount of fusion protein present in each labeled band as determined by densitometric analysis of the silver stained polyacrylamide gel. The total amount of phosphate incorporated into the 182, 177, 175, and 164 KDa ADR1/ß-galactosidase fusion proteins was arbitrarily scaled to 1. The values for the relative phosphorylation of the ADR1, ADR1-2^{C}, and ADR1-5^{C} fusion protein species are presented as the fraction of that total.

b. Graphical representation of phosphate incorporation in the 164 KDa ADR1/, ADR1-2^{C}/, and ADR1-5^{C}//ß-galactosidase fusion proteins using bovine cAMP-dependent protein kinase. Expanded graph of phosphate incorporation into the 164 KDa fusion protein by 0.003 pmol U/µl bovine kinase as shown in Figure 11.

Figure 16a.

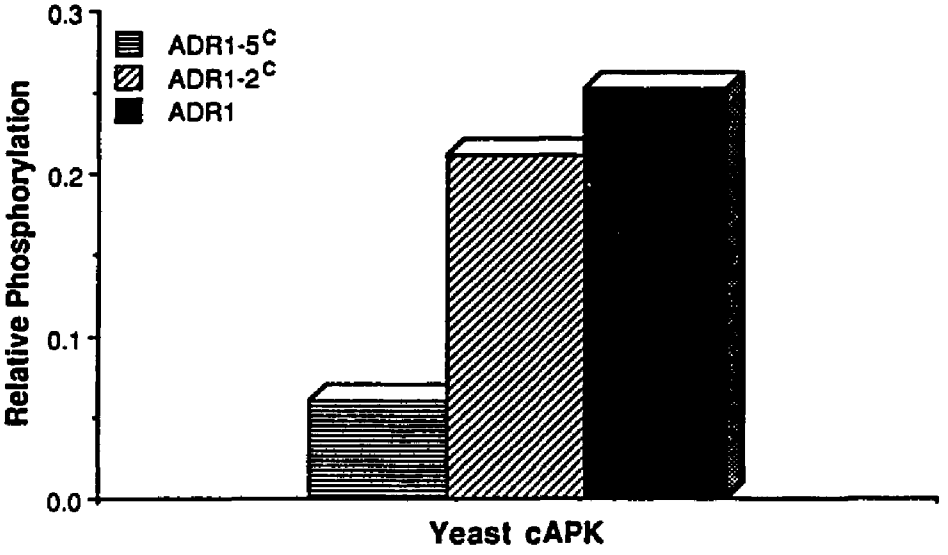
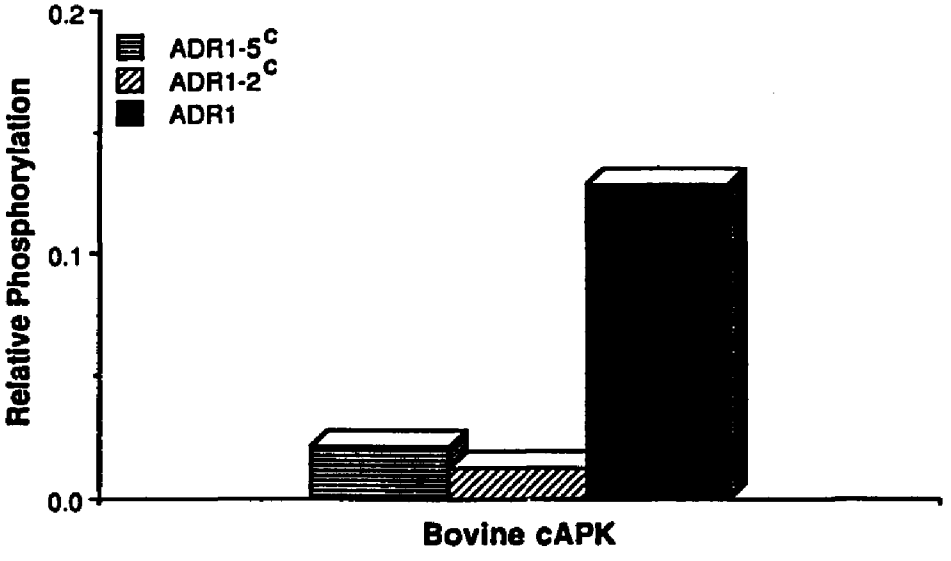


Figure 16b.



IV. Discussion

In the model proposed by Denis and Gallo[32] the glucose repression of ADH2 is mediated in part by a glucose-dependent reduction in the activity of the transcriptional activator ADR1. Based on the characterization of the ADR1-5^{C} mutation, ADR1 activity was proposed to be reduced during fermentative growth as a result of cAMP-dependent phosphorylation. The results of the work presented herein strongly support the involvement of cAMP-dependent phosphorylation in the regulation of ADR1 activity.

Using ADR1/ß-galactosidase fusion proteins expressed in *E coli* it was demonstrated that *in vitro* mammalian cAPK phosphorylates ADR1 at two distinguishable sites. A phosphorylated chymotryptic peptide was found to comigrate with a phosphorylated chymotryptic fragment derived from a synthetic peptide containing ADR1 residues 227-231. The mobility of the labeled peptide on a peptide mapping gel was altered when it contained either the ADR1-2^{C} or ADR1-5^{C} sequences. Other peptides presumed to be partial chymotrypsin digest products also displayed altered mobilities. Furthermore, when the peptide contained ADR1-7^{C} sequences (in which the phosphoacceptor serine at residue 230 is replaced by leucine), phosphorylation was eliminated (compare phosphorylation of ADR1- and ADR1-7^{C}-peptides in the 164 KDa

digests of Figure 14). These data strongly indicates that mammalian cAPK recognizes ADR1 residues 227-231, with serine-230 serving as the phosphoacceptor.

The results also indicate the existence of a second *in vitro* phosphorylation site in ADR1 which is weakly recognized by yeast and bovine cAPK relative to the primary phosphorylation site at serine-230. The 182, 177, and 175 KDa ADR1-7^C/ß-galactosidase fusion proteins were phosphorylated *in vitro* despite the fact that in these mutant proteins the phosphoacceptor serine at residue 230 is replaced by a leucine residue. Furthermore, the 164 KDa ADR1/, ADR1-2^C/, and ADR1-5^C/ß-galactosidase fusion proteins were all phosphorylated *in vitro*, while the ADR1-7^C 164 KDa fusion protein was not, indicating that the 164 KDa fusions lack the secondary phosphorylation site. Finally, peptide mapping of the 182, 177, and 175 KDa ADR1-7^C/ß-galactosidase fusion protein digests revealed a single phosphorylated peptide which is present in digests of all the comparable ADR1, ADR1-2^C, and ADR1-5^C fusion proteins. This peptide, which was not observed in the 164 KDa digests, is presumed to encompass a secondary phosphorylation site in ADR1 which is N-terminal to the primary phosphorylation site lying between residues 227-231.

ADR1 contains four regions in addition to the one between residues 227-231 which are homologous to the cAPK phosphorylation consensus sequence*

and could serve as the second phosphorylation site. Since the 164 KDa fusion proteins appeared to lack the secondary phosphorylation site, it was hypothesized that the secondary phosphorylation site occurs at a position N-terminal to the primary phosphorylation site at serine-230. Consistent with this hypothesis, the putative secondary phosphorylation sites at threonine-226, serine-211, serine-180, and serine-170 identified by homology to the cAPK phosphorylation consensus sequence are N-terminally located with respect to the primary phosphorylation site at serine-230. The putative phosphoacceptor threonine-226 in the sequence Lys-Lys-Leu-Thr-Arg lies just N-terminal to the primary phosphorylation site and occurs on the same chymotryptic peptide. Since the 164 KDa ADR1-7^{C} fusion protein digest contained no phosphorylated peptides, threonine-226 can not be the the secondary site of *in vitro* phosphorylation that was observed.

The likelihood of the other sites serving as potential phosphorylation sites was explored by examining results of previous studies on the kinetics of the *in vitro* phosphorylation of synthetic peptides. Granot and colleagues[73] found that the presence of a proline residue adjacent to the phosphoacceptor serine, as in the putative site located between residues 209-212 (Arg-Lys-Ala-Ser-Pro), decreased the k_{cat}/K_m ratio of mammalian cAPK by ~5 X 10^6 relative to an ADR1-like peptide. In other words, under identical labelling conditions the

*Arg-Arg-X-Ser-Y, where Y tends to be hydrophobic and X is any amino acid.

proline-containing peptide occurred at a rate ~10^{-6} that of the ADR1-like peptide. It thus appears unlikely that this site would be phosphorylated to any measurable extent *in vitro* in ADR1 . Of the two remaining sites, the site within residues 177-181 (Arg-Lys-Asn-Ser-Ala) is most homologous to the cAPK consensus phosphorylation sequence and is therefore more likely to serve as a substrate for cAPK than is the site between residues 167-171 (Lys-Lys-Val-Ser-Arg). Kemp and colleagues[35] have shown that the two N-terminal arginine residues within the phosphorylation consensus sequence are crucial to substrate recognition by cAPK. Substitution of the N-terminal arginine with lysine in a synthetic peptide resulted in an approximately 90-fold increase in the K_m of phosphorylation. In contrast, replacement of the C-terminal arginine with lysine resulted in a 16-fold increase in the K_m. On this basis the phosphorylation site between residues 177-181 (Arg-Lys-Asn-Ser-Ala) might be expected to serve as a better substrate for cAPK than the site between residues 167-171 (Lys-Lys-Val-Ser-Arg). The results of previous kinetic analyses using synthetic peptides as substrates for *in vitro* phosphorylation by cAPK therefore suggest that the observed secondary ADR1 phosphorylation site was serine-180. It should be emphasized that this prediction is based solely on the *in vitro* kinetic properties of the bovine cAPK and does not consider the possible effects which ADR1 tertiary structure may have on the *in*

vitro phosphorylation of serine-180. Further experiments employing two-dimensional peptide mapping techniques and/or sequencing of the relevant phosphopeptide would be required to unambiguously identify the secondary site of phosphorylation in ADR1.

The identification of a second site in ADR1 which is phosphorylated *in vitro* brings up the interesting possibility that ADR1 is phosphorylated at more than one site *in vivo.* The yeast ribosomal protein S10 has been shown to be multiply phosphorylated[74], as has the large T antigen of simian virus 40[75]. In the case of large T antigen only one of the phosphorylation sites has been demonstrated to functionally affect the protein[76], while in the S10 protein phosphorylation by cAPK appears to play no regulatory function[74]. Replacement of serine-180 with an alanine residue using site directed mutagenesis would help to clarify whether this putative secondary phosphorylation site is involved in the regulation of ADR1.

The yeast and mammalian (bovine) cAPK were observed to have slightly different substrate specificities when used in the *in vitro* phosphorylation of the fusion proteins. The yeast kinase phosphorylated the sequence Arg-Arg-Ala-Ser-Ser-Ser (ADR1-2^C) to a greater extent than the sequence Arg-Lys-Ala-Ser-Phe-Ser (ADR1-5^C) during the incubation period of the assay. In contrast, the bovine kinase had the opposite preference, phosphorylating the sequence Arg-

Lys-Ala-Ser-Phe-Ser (ADR1-5^C) to a greater extent than the sequence Arg-Arg-Ala-Ser-Ser-Ser (ADR1-2^C). These results could arise from two quite different phenomena. First, although the affinity of the enzymes for each of the two different sequences could be nearly the same, the stiochiometries of incorporation may be different. In other words, the two enzymes may share similar Michealis constants (K_m's) for the sequence Arg-Arg-Ala-Ser-Ser-Ser, but the number of serines phosphorylated by the two enzymes may differ. Alternatively, the difference observed in the levels of phosphate incorporation may simply reflect that the kinases differ in their K_m's for the two phosphorylation sequences. Differences in the substrate specificities of the yeast and mammalian kinases might result from minor differences in their respective active sites, while alterations in the stoichiometry of phosphorylation would require that more gross functional differences exist between the two enzymes. The yeast C1 catalytic subunit and mammalian catalytic subunits are only approximately 50% homologous in the conserved C-terminal 310 amino acids[49], indicating that either explanation may be responsible for the observed differences in substrate specificities. Further studies examining the stoichiometry and kinetics of phosphorylation of the sequence Arg-Arg-Ala-Ser-Ser-Ser by these two enzymes are required to clarify this issue.

The demonstration that ADR1^C/ß-galactosidase fusion proteins were less

amenable to cAMP-dependent phosphorylation than were ADR1/ß-galactosidase fusion proteins strongly supports the hypothesis that phosphorylation is physiologically significant in the regulation of ADR1 activity. Both the yeast and bovine cAPK catalytic subunits phosphorylated fusion proteins containing the ADR1^{C} amino acid substitutions poorly relative to fusion proteins containing the ADR1 sequences. *In vivo* the ADR1^{C} proteins may therefore partially bypass glucose-dependent phosphorylation, in turn allowing them to activate ADH2 transcription during glucose repression.

Krebs and colleagues[77] have suggested that in order for a physiological role for cAMP-dependent phosphorylation to be considered valid the following five criteria must be met:

1. The protein must be phosphorylated by cAPK *in vitro.*
2. *In vitro* phosphorylation must lead to a modified function.
3. A stoichiometric correlation must exist between *in vitro* phosphorylation and modified function.
4. Phosphorylation of the same protein must be demonstrated *in vivo* in response to a cAMP signal.
5. Modified function must be demonstrated in response to a cAMP signal *in vivo.*

The first of these criteria has been satisfied by the demonstration that ADR1 is phosphorylated at two distinguishable sites *in vitro* by cAPK. The second and third criteria proposed by Krebs et al., as they pertain to ADR1, concern correlating the stoichiometry of *in vitro* phosphorylation with a decreased ability

to activate transcription of ADH2. Constitutive ADR1^{C} mutants, which have an increased ability to activate ADH2 transcription during glucose repression, were less amenable to *in vitro* phosphorylation by cAPK than was the wild-type ADR1 protein, which is unable to activate transcription during glucose repression (Figures 11 and 15). It thus appears that the degree of *in vitro* phosphorylation of mutant ADR1^{C} proteins can be correlated with a known modified function *in vivo*. It is significant that strains carrying the ADR1-5^{C} mutation have slightly higher ADH II activities than strains carrying the ADR1-7^{C} mutation, although the ADR1-5^{C} protein was observed to be phosphorylated at serine-230 while the ADR1-7^{C} protein was not. These data do not necessarily preclude the possibility that a stoichiometric relationship between ADR1 phosphorylation and ADR1 activity exists. The ADR1-5^{C} and ADR1-7^{C} proteins may have tertiary or quaternary structures which are significantly different than the wild-type ADR1 protein. These structural differences may affect their ADR1 activities independently of any effect which they have on the phosphorylation state. The substitution of a leucine for the phosphoacceptor serine in the ADR1-7^{C} protein may, for instance, have two opposing effects on ADR1 activity- it may activate the protein by eliminating phosphorylation at residue 230, but it may also diminish its ability to activate transcription by altering the tertiary structure of the protein. Similarly, the substitution of a lysine for an arginine within the

phosphorylation consensus sequence in ADR1-5^{C} may not only decrease its ability to act as a cAPK substrate, but also enhance its function as a transcriptional activator by causing a beneficial alteration in the protein structure. Both of these scenarios imply that the phosphorylation sequence around serine-230 plays a role in ADR1 function even when not phosphorylated, as is indicated by the results of Bemis and Denis[23] discussed below. Without knowing the effect the ADR1^{C} mutations have on the structure of the ADR1 protein it is impossible to attribute their phenotypes solely to the effect which they have on the phosphorylation state of the protein. Determining whether there is a stoichiometric correlation between ADR1 phosphorylation and ADR1 activity will require determination of the phosphorylation state of wild-type ADR1 during repressed and derepressed growth conditions. Criteria 2 and 3 may be more directly tested if an *in vitro* assay for ADR1-mediated transcriptional activation becomes available.

Criteria 4 and 5, as they pertain to ADR1, address the question of whether a cAMP signal modifies the *in vivo* activity of the ADR1 protein. Recent genetic experiments indicate that ADR1 activity is responsive to cAMP-dependent phosphorylation *in vivo* (C. Denis, personal communication). As mentioned in the Introduction, yeast cAPK is composed of a catalytic subunit and a cAMP-responsive regulatory subunit[49,50]. Three genes encode catalytic subunits, TPK1, TPK2, and TPK3, while a regulatory subunit is encoded by a single gene

designated BCY1. The ADH II activities of strains containing only one of the catalytic subunits were compared to strains in which the gene coding for the regulatory subunit had been disrupted (bcy1). Strains with bcy1 disruptions, lacking a functional regulatory subunit, were expected to have unregulated kinase activity, while those with the BCY1 allele were expected to have had cAMP-dependent kinase activity. ADH2 derepression was shown to decrease ten to fifteen fold in two out of three strains with unregulated kinase activity (Table 5). While the three catalytic subunits have been shown to be able to functionally substitute for one another, there is some indication that they differ in their substrate specificities. For example, unregulated TPK3 kinase activity has no effect on other non-fermentative processes[50] or on ADH2 derepression, suggesting that the catalytic subunit encoded by TPK3 may be less active or not recognize ADR1 and other substrates during non-fermentative growth.

Denis next used strains containing the ADR1-5^{C} and ADR1-7^{C} in an effort to show that the effect of unregulated kinase on ADH2 expression was mediated through the ADR1 protein. He reasoned that ADR1-5^{C} would be more sensitive to unregulated kinase activity than ADR1-7^{C} based on the fact that the latter mutation encodes a protein lacking the phosphoacceptor serine. Under derepressed growth conditions unregulated TPK1 kinase activity decreased ADH II activity in the ADR1-5^{C} strain 4.4-fold, while it decreased ADR1-7^{C}

dependent activity 2.4-fold (Table 5). Although less dramatic, the glucose repressed ADH II activities show a similar trend with the ADR1-5^{C} strain decreasing 2.2-fold compared to 1.5-fold for the ADR1-7^{C} strain. Unregulated TPK3 kinase activity did not affect ADH II expression under derepressing conditions, consistent with the aforementioned suggestion that this kinase is less active or recognizes ADR1 poorly during non-fermentative growth. Under glucose-repressed growth conditions ADR1-5^{C} -dependent ADH II activity was decreased 4.7-fold by unregulated TPK3 kinase activity, while the ADR1-7^{C} -dependent activity decreased only 1.4-fold.

Taken together the *in vitro* phosphorylation studies presented in this work and the genetic analysis performed by Denis clearly demonstrate that ADR1 function is modified by cAMP-dependent phosphorylation *in vivo*.

Results previous to this work indicated that the glucose repression of ADH2 occurs at the level of transcription[16]. The isolation[24] and characterization[27,32] of dominant constitutive ADR1^{C} mutations which allow ADH2 expression to partially escape glucose repression implies that the ADR1 protein is responsive to a glucose repression signal.

There are two basic mechanisms by which a glucose repression signal could affect a regulatory protein required for transcription of ADH2. The first would be to alter the cellular concentration of the ADR1 protein by affecting its

Table 5.

Effect of BCY1 Gene Disruption on ADH2 Expression

Strains used in the preparation of this table are described in detail in the reference from which it was taken (Cherry et al.[27]). ADH II activities were determined as described[16] following growth of cultures overnight at 30° C. Culture mediums contained YEP medium (2% yeast extract, 1% Bacto-peptone, 20 mg/L adenine and uracil) supplemented with either 8% glucose or 3% ethanol. Each value represents the average of five to eleven determinations.

Table 5

relevant genotype		ADH II activity (mU/mg) Glucose	fold reduction	Ethanol	fold reduction
TPK1		41		6000	
			} 0.91		} 14
TPK1 bcy1		45		440	
TPK2		36		3800	
			} 1.3		} 15
TPK2 bcy1		28		260	
TPK3		4.2		3300	
			} 0.79		} 0.97
TPK3 bcy1		5.3		3400	
TPK1	ADR1-5^{c}	610		8700	
			} 2.2		} 4.4
TPK1 bcy1	ADR1-5^{c}	280		2000	
TPK1	ADR1-7^{c}	105		15,300	
			} 1.5		} 2.4
TPK1 bcy1	ADR1-7^{c}	69		6500	
TPK3	ADR1-5^{c}	340		7400	
			} 4.7		} 0.96
TPK3 bcy1	ADR1-5^{c}	72		7900	
TPK3	ADR1-7^{c}	90		8400	
			} 1.4		} 0.94
TPK3 bcy1	ADR1-7^{c}	66		8900	

transcription, translation, or rate of degradation. The *E. coli* lamda phage provides an interesting example of a transcriptional control mechanism by which the cellular concentration of the lambda repressor is autoregulated[78,93]. Transcription of the lytic genes and the gene encoding the lambda repressor (cI) are repressed by the lambda repressor protein when its concentration is high. When the cellular concentration of lambda repressor becomes low, repressor binding is reduced and transcription of the cI gene is stimulated. Cellular signals can also modulate the cellular concentration of regulatory proteins translationally, as has been demonstrated for the GCN4 protein in yeast[80]. GCN4 is a positive regulatory protein required for transcription of amino acid biosynthetic genes in yeast[79]. The cellular signal, amino acid starvation, removes a block on the translation of the GCN4 protein and causes an increased rate of translation[80]. The resulting increase in GCN4 protein concentration allows transcriptional activation of approximately 50 biosynthetic genes. Although there are currently no examples of a cellular signal which causes an increased rate of regulatory protein degradation, several enzymes in yeast are known to be preferentially degraded proteolytically in response to the addition of glucose to the growth medium. In the case of the gluconeogenic enzyme fructose-1,6-bisphosphatase glucose induced inactivation is at least partially mediated by a cAMP-dependent phosphorylation step[81,82]. There

appears to be a direct correlation between the degree of subunit phosphorylation, which inactivates the enzyme, and the rate of its subsequent proteolytic degradation[81,82,41]. This mechanism, termed catabolite inactivation, may also be used by the cell to selectively degrade regulatory proteins which are not required during growth on fermentable carbon sources, such as ADR1.

The second way in which a glucose repression signal could affect ADR1 activity is by inducing a transition of the protein from an active state to an inactive state. By regulating the activity of the protein rather than its synthesis or degradation, such a mechanism would offer the advantages of being faster and more energetically economical, since no *de novo* protein synthesis would be required. In *E. coli* transcription of several glucose repressed operons requires that the catabolite activator protein (CAP) be bound to operator sequences[83-86]. CAP must bind cAMP before it can bind to DNA and activate transcription. In *E. coli* intracellular cAMP levels are increased in the absence of glucose. When cells are grown in the absence of glucose, cAMP levels are elevated and CAP is activated. Thus the glucose repression of operons such as lac and ara is mediated through low cAMP levels which prevent CAP from activating transcription. The *E. coli* lac repressor provides another example in which a cellular signal modifies the activity of a regulatory protein. This negative regulatory protein binds to the lac operator and sterically blocks transcription of

the lac operon[83]. When cells are exposed to lactose an inducer is produced, allolactose, which is bound by the lac repressor causing its release from the operator.

In the examples discussed thus far the cellular messengers interact directly with regulatory proteins which bind to DNA and affect transcription. A cellular signal may also interact with a regulatory protein not directly involved with DNA-binding, but which eventually results in an effect on transcription. The galactose metabolism system in yeast, for example, is largely controlled by the positive regulatory protein GAL4 and the negative effector GAL80[87]. Transcription is dependent on binding of the GAL4 protein to upstream activating sequences (UAS's) located upstream from the site of transcription initiation[88,89]. In the absence of galactose the GAL80 protein binds to the GAL4 protein and blocks its activator function[90]. Since GAL80 does not bind to the UAS's and binding of GAL80 to GAL4 does not disrupt GAL4 binding to DNA[89], it is presumed that GAL80 masks some functional region of GAL4 which normally interacts with the transcriptional complex. In the presence of galactose, GAL80 is altered such that it no longer binds to GAL4 and transcription is activated[90]. It is unclear whether GAL80 interacts directly with galactose, but it is evident that some cellular signal indicating the availability of galactose is transmitted via protein-protein interactions to activate transcription of the genes required for galactose

metabolism.

Several studies suggest that ADR1 is not transcriptionally or translationally controlled in response to a glucose repression signal. ADR1 mRNA has been demonstrated to be constitutively synthesized independent of the carbon source in the growth medium[32]. ADR1^{c} mutations allow ADH2 transcription to partially escape glucose repression. The characterization of these mutations as single amino acid substitutions[32,27] suggests that ADR1 is translated under fermentative conditions. Therefore if the cellular concentration of ADR1 is controlled, it must occur through a post-translational mechanism.

Although it appears quite clear that the glucose repression of ADH2 is in part mediated by phosphorylation of the ADR1 protein, the mechanism by which phosphorylation reduces the ability of ADR1 to activate ADH2 transcription remains unclear. A recent report by Bemis and Denis[23] in which they studied the effects of various ADR1 gene truncations on ADH2 expression clarifies the role which the phosphorylation region plays in ADR1 function. Strains expressing only the N-terminal 220 amino acids of ADR1 (ADR1-220) did not allow ADH2 to escape glucose repression, although the truncated protein was capable of activating ADH2 under derepressing conditions. Increased expression of ADR1-220 did not allow ADH2 expression to escape glucose repression. In contrast, increased expression of any ADR1 proteins containing the phosphorylation site at serine-230 did allow ADH2 expression to bypass

glucose repression. From these data it appears that the phosphorylation site at serine-230 is required for ADR1 to bypass ADH2 glucose repression, but is not required for activation of ADH2 transcription during derepressed growth.

How can bypassing glucose repression of ADH2 require the ADR1 phosphorylation region, yet not allow ADH2 expression to escape glucose repression when removed? Bemis and Denis[23] suggest that this apparent paradox results from the decreased ability of the ADR1-220 protein to activate transcription as compared to proteins which contain the phosphorylation region. They found that the ADR1-220 protein was 2-3 fold less active than proteins containing the N-terminal 253 or 272 amino acids of ADR1 (ADR1-253 and ADR1-272) under derepressing conditions. These results indicate that the phosphorylation region, while negatively affected by phosphorylation during glucose repression, plays a positive role in ADR1 function. The ADR1-220 protein may be able to activate transcription under derepressed conditions because other proteins necessary for ADH2 transcription are present and active. Under glucose growth conditions increased expression of ADR1-220 does not allow glucose repression to be bypassed because, unlike larger ADR1 proteins, this less active truncated ADR1 protein requires other factors which are present only during derepression. Thus it appears that phosphorylation does not wholly inactivate ADR1, but merely diminishes the positive role which

the phosphorylation region plays in ADR1 function.

Of the many positive roles it could play, the phosphorylation region may facilitate the binding of the ADR1 protein to DNA. The DNA-binding zinc finger motif found between residues 99-155 appears to be necessary but not sufficient for binding of ADR1 to the ADH2 upstream sequences (E.T. Young, personal communication) indicating that other regions of the protein are necessary. Simian virus 40 large T antigen, a protein involved in viral gene regulation and replication, has been suggested to be regulated through a phosphorylation mechanism[76]. When dephosphorylated, large T antigen binds to the viral origin of replication *in vitro* with several-fold higher affinity than does the phosphorylated protein. Phosphorylation may play a similar role in the DNA-binding properties of ADR1. Since 24% of the amino acids between the C-terminal zinc finger and the primary phosphorylation site at amino acid 230 are basic residues, while only 4% are acidic (18 of 75 versus 4 of 75), it is tempting to speculate that the overall basic nature of this region aids DNA binding through electrostatic attraction. Addition of an acidic phosphate group, by decreasing the positive character of the region, may diminish such an attraction. Deletion of the basic region, as in the ADR1-220 protein discussed above, would also be expected to diminish ADR1 activity. The first step in testing this hypothesis would be to determine whether the *in vitro* phosphorylation of

ADR1 affects its ability to bind to the ADH2 UAS.

The phosphorylation region may also be involved in the transcriptional activation properties of ADR1. If ADR1 contacts a protein in the transcription complex directly through the phosphorylation region, phosphorylation may disrupt such a contact. Alternately, the phosphorylation region could contact another protein which is required for stimulation of transcription, but which is not directly involved in the transcriptional machinery. Yeast regulatory proteins GAL4 and GCN4 have been suggested to activate transcription by contacting the transcriptional machinery through acidic domains[91,92]. The basic phosphorylation region of ADR1, in order to play a role in transcription activation, may require contact with a "helper" protein which contains the necessary acidic domain. To be consistent with the results of the ADR1 truncation studies performed by Bemis and Denis discussed above, the helper protein must also either interact with ADR1 through regions other than the phosphorylation region around serine-230 or not be absolutely required for ADR1 function during derepression.

Finally, the phosphorylation region could be important to ADR1 oligomerization. ADH II activities in ADR1^{C}, adr1-x heterologous diploid strains suggest that ADR1 monomers interact with one another to activate transcription of ADH2 (C. Denis, personal communication). In addition, the palindromic

nature of the ADH2 DNA binding site suggests that a symmetrical oligomer is required for binding. Multimers of ADR1 may have better DNA binding or transcriptional activation properties than do monomer ADR1 molecules. A good example of the possible effects of phosphorylation on oligomerization is provided by glycogen phosphorylase, the enzyme which catalyses the first step in glycogen metabolism. The transition between an active dimer and a less-active monomer has been suggested to be controlled by the opposing actions of phosphorylase kinase and phosphorylase phosphatase[44].

Several lines of evidence indicate that while phosphorylation of ADR1 at serine-230 is responsible for some of the observed decrease in ADR1 activity during glucose repression, there must exist other factors which contribute to the glucose repression of ADH2. The ADH II activity in ADR1-7^{C}-containing strains was still decreased by unregulated kinase activity, despite the absence of the phosphoacceptor serine at amino acid 230. This data implies that cAMP-dependent phosphorylation occurs at another site in ADR1 or that phosphorylation of some other factor is important to glucose repression. Furthermore, ADH2 expression in strains carrying any of the ADR1^{C} mutations never completely bypasses glucose repression (Table 1). Similarly, increased dosage of the ADR1 gene allows ADH2 expression to significantly escape glucose repression, yet glucose repression of ADH2 is still evident even when

ADR1 is present at 80 copies . Factors other than ADR1 must therefore be involved in the glucose repression of ADH2.

At least two other genes with pleiotropic action are known to be required for ADH2 expression, CCR1 and CCR4[18-21]. Defects in either of these genes decrease the activities of a number of gluconeogenic enzymes which are glucose repressed. The CCR1 and CCR4 proteins may therefore be involved in the general transmission of a glucose repression signal. Genetic analysis has shown that CCR1 acts through or with ADR1 to activate ADH2 transcription[21]. CCR1 encodes a protein kinase[21], but appears not to act through the primary phosphorylation site at amino acid 230 since truncated ADR1 proteins lacking this site remain sensitive to defects in the CCR1 gene[23]. CCR1 may affect phosphorylation of ADR1 at some other site either directly or indirectly or may act on some other protein required for the derepression of glucose-repressed genes. The other positive regulatory element, CCR4, is thought to act independently of ADR1 at sequences downstream of the TATA promoter element in ADH2[94]. Because CCR4 is a pleiotropic effector it may comprise part of a general transcription complex which is sensitive to a glucose repression signal. There exists genetic evidence that CCR4 is inactivated during glucose repression by the action of two negative effectors, CRE1 and CRE2[21]. The cellular signals which CRE1 and CRE2 respond to and the mechanism of their action on CCR4 is unknown.

Much work remains if the role of phosphorylation in ADR1 regulation is to be fully understood. Among the most pressing experiments are determining the *in vivo* phosphorylation state of the ADR1 protein during repressed and derepressed growth conditions, the effect of ADR1 phosphorylation state on ADR1's DNA binding properties, and using site directed mutagenesis to determine the possible significance of the putative secondary phosphorylation site. More long-term goals should be directed toward gaining a better understanding of <u>how</u> phosphorylation molecularly affects the interaction of ADR1 with DNA or other proteins of the transcription complex.

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Appendix A

Small scale plasmid prep

(adapted from H. C. Birnboim and J. Doly (1979) Nucleic Acids Res. 7, 1513.)

1. Grow 1.5 ml cultures overnight in L broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) supplemented with appropriate antibiotic (ampicillian to 50 μg/ml).
2. Pellet cells by centrifugation for 1 minute in microcentrifuge tubes.
3. Decant L broth and air dry pellet by inversion on absorbant towels. Dried pellets should be loosened by vortexing two tubes together prior to next step.
4. Add 100 μl cold lysozyme solution (25 mM Tris-Cl, pH 8, 10 mM EDTA, 50 mM glucose, 2 mg/ml fresh lysozyme), mix thoroughly, and incubate on ice 10-30 minutes.
5. Add 200 μl NaOH-SDS (0.2 N NaOH, 1% SDS, freshly prepared by mixing 1 ml 1N NaOH, 0.5 ml 10% SDS, 3.5 ml water) and mix by vortexing. Incubate on ice 5 minutes.
6. Add 150 μl 3M sodium acetate, pH 4.8 and vortex in inverted position for 10 seconds at low speed. Vigorous mixing will result in contamination with genomic DNA. Incubate on ice 20-60 minutes.
7. Centrifuge at 4° C. for 10 minutes and remove supernatant to a fresh microtube (avoiding the pellet) using a 1 ml micropipet. This supernatant contains the plasmid DNA.
8. Add 1 ml cold ethanol to recovered supernatant, mix gently, and incubate at 4° C. for 15 minutes. This is a good stopping point, if necessary.
9. Centrifuge for 15 minutes at 4° C., decant ethanol supernatant, and allow pellets to air-dry by inversion on paper towels.
10. Resuspend dried pellets in 400 μl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
11. Extract resuspended pellet with an equal volume of TE-saturated phenol (phenol:chloroform:isoamyl alcohol; 50:1:1), centrifuge 2 minutes, and carefully remove top phase to a fresh microtube. Repeat extraction with TE-saturated chloroform until chloroform phase (bottom) is clear. One extraction is usually sufficient.
12. Add 0.1 volume 3 M sodium acetate (about 40 μl) and fill tubes with cold ethanol. Incubate at room temperature 30 minutes to overnight.
13. Centrifuge for 30 minutes to recover precipitated plasmid and dry pellet first by inversion on paper towels, then to complete dryness in Savant Speed-Vac. Wash dried pellets with 0.5 ml cold 70% ethanol, centrifuge and dry as above.
14. Resuspend dried pellet in 40-50 μl TE buffer. 5 μl of this final plasmid solution should be sufficient for restriction digestion and visualization on an agarose gel.

Appendix B

Restriction Enzyme Buffers:

LS Buffer (low salt)

0.66 ml 1M $MgCl_2$

0.66 ml 1M Tris buffer, pH 7.5

8.68 ml sterile water

MS Buffer (medium salt, $[NaCl]_{final}$= 100 mM)

0.66 ml 1M $MgCl_2$

0.66 ml 1M Tris buffer, pH 7.5

1.26 ml 5M NaCl

7.42 ml sterile water

HS Buffer (high salt, $[NaCl]_{final}$= 150 mM)

0.66 ml 1M $MgCl_2$

0.66 ml 1M Tris buffer, pH 7.5

1.89 ml 5 M NaCl

6.79 ml sterile water

KS Buffer (potassium salt, $[KCl]_{final}$= 100 mM)

0.66 ml 1M $MgCl_2$

0.66 ml 1M Tris buffer, pH 7.5

6.28 ml 1M KCl

2.40 ml sterile water

To make active buffer (2X): 20 µl sterile bovine serum albumin (1 mg/ml, $[BSA]_{final}$= 0.45 µg/ml), 10 µl 1/100 dilution ß-mercaptoethanol (14.26 M $[BME]_{final}$= 32 mM), 14 µl selected restriction buffer ($[MgCl_2]_{final}$= 21 mM, $[Tris]_{final}$= 21 mM).

Appendix C

DNA Fragment Preparation and Ligation Reactions

1. Separate restriction fragments on a 0.6% low-melting point agarose (LMP agarose, BRL or SeaPlaque agarose, FMC) run at 4° C. with TAE buffer (50X = 121 g Tris base, 11.7 g EDTA (acid form) and ~26.5 ml glacial acetic acid. Final pH should be 8.2.). Use narrow gel slots; concentrate samples by ethanol precipitation if necessary.
2. Stain gel with ethidium bromide (for a 10 X 10 cm gel use about 1 µg in 100 ml cold TAE running buffer). While gel is staining label and tare sufficient microtubes to accomodate each fragment to be excised.
3. Bands should be easily visible using a low-power UV light box. Carefully excise fragments of interest from the gel using a clean razor blade and transfer to labeled microtubes. Work quickly to minimize exposure of the DNA to damaging UV light. Photograph gels after band excision for a record.
4. Determine the weight of the agarose band and add 5 X the weight of TE (i.e. a gel slice weighing 0.1 g will get 500 µl TE). Add 1 µg yeast tRNA as a carrier (omit if the fragment is to be used as a probe).
5. Heat gel slice at 65° C. for 10 minutes to melt agarose and release the fragment.
6. Cool and extract with an equal volume STE-saturated phenol (no chloroform! STE= TE with 0.15 M NaCl). Repeat extraction with phenol:chloroform:isoamyl alcohol, then chloroform.
7. Add 0.1 volume 3 M sodium acetate and 2 volumes ethanol. Incubate overnight at 4° C. for good recovery. Centrifuge for 30 minutes at 4° C to recover fragment.
8. Resuspend fragment in 10 µl TE.
9. Run 2 µl of DNA fragments to be ligated side-by-side on a microscope slide agarose gel. Photograph and visually determine relative concentrations.
10. Mix 1 µl 10X ligation buffer (300 mM Tris, pH 7.6, 100 mM $MgCl_2$, 50 mM dithiothreitol), 1 µl 10 mM ATP, 1 µl T4 DNA ligase (approximately 100 units, New England Biolabs) and DNA fragments to be ligated in a 10 µl reaction volume. Insert to vector ratios should be between 2:1 and 5:1 for efficient ligation. Blunt end ligations should be performed at 4°-12° C., while cohesive end ligations work better at 16° C. Ligations should be allowed to react overnight, and should be diluted such that the estimated amount of ligated DNA used in transformation with BRL competent cells is ~10 ng.

Appendix D

Estimated charge/frictional coefficient ratios for possible peptides produced by chymotryptic digestion of ADR1, ADR1-2^C, ADR1-5^C and ADR1-7^C

The theoretical mobility (U) of a macromolecule during zonal electrophoresis is a function of the charge on the macromolecule (ze) and its frictional coefficient (f)[99]:

$$U = ze / f$$

Due to numerous complications introduced by interaction of macromolecules with solvent ions, endosmosis of the solvent, and distortion of the ion field around the macromolecule as it moves through the electrophoretic medium, there is no simple theoretical treatment which can accurately explain the observed mobility of proteins during electrophoresis. The calculations performed here are provided simply as a reference for comparison to observed mobilities of peptides on the 50% pH 3 gel system of West and Bonner[68].

To use the formula presented above, the charge (p) and frictional coefficients for all the ADR1 and ADR1^C chymotryptic peptides capable of being phosphorylated were estimated. For estimations of p under the gel conditions of pH 3, the following assumptions were made: N-terminal amino groups were assumed to be fully protonated and contributed +1 charge per peptide ; lysine and arginine residues were assumed to be fully protonated and contributed +1 charge per acidic residue; C-terminal carboxyl groups were assumed to contribute -0.1 charge per residue; and finally, phosphate groups were assumed to contribute -0.86 charge per phosphate incorporated. Due to the effect of electrostatic interactions may have on the ionization of charged groups (effectively lowering or raising pK_a's), charges were calculated for each peptide containing one and two phosphate groups. These two values may be used as a possible range of variability.

Frictional coefficients were estimated using two different techniques. The first simply assumed that the frictional coefficients of the various peptides were directly proportional to the length of the peptide. The second estimate assumed that the frictional coefficients of the peptides is proportional to their molecular weight raised to the 2/3 power. The latter estimate is based on empirical observations of the mobility of peptides during thin layer electrophoresis[100].

Results of these calculations are presented in Table A.

Table A

Estimated charge/frictional coefficient ratios for possible peptides produced by chymotryptic digestion of ADR1, ADR1-2^{C}, ADR1-5^{C} and ADR1-7^{C}

Column A: Sequence of predicted phosphorylated peptides produced by chymotryptic digestion of ADR1/, ADR1-2^{C}/, ADR1-5^{C}/ and ADR1-7^{C}/ß-galactosidase fusion proteins.

Column B: Calculated molecular weights of the predicted peptides.

Column C: ADR1 residues which the peptides in column A span.

Column D: Number of lysine and arginine residues within the peptide.

Column E: Calculated charge on the peptide at pH 3 with no phosphate groups incorporated, p.

Column F: Calculated charge on the peptide at pH 3 with one phosphate groups incorporated, p1.

Column G: Calculated charge on the peptide at pH 3 with two phosphate groups incorporated, p2.

Column H: Number of residues in the peptide, N.

Column I: Calculated charge on the peptide at pH 3 with one phosphate groups incorporated divided by the length of the peptide, p1/N.

Column J: Calculated charge on the peptide at pH 3 with two phosphate groups incorporated divided by the length of the peptide, p2/N.

Column K: Calculated charge on the peptide at pH 3 with one phosphate groups incorporated divided by the molecular weight of the of peptide to the two-thirds power, p1/MW^2/3.

Column L: Calculated charge on the peptide at pH 3 with two phosphate groups incorporated divided by the molecular weight of the of peptide to the two-thirds power, p2/MW^2/3.

Table A

	A	B	C	D	E	F	G	H	I	J	K	L
1	Sequence	MW	ADR1	# of Acidic	total charge, p	total charge, p1	total charge, p2	# of residues	Charge/length	Charge/length	MW^(-2/3)*p1	MW^(-2/3)*p2
2	(peptides derived from wild-type ADR1)		residues	residues	(no PO4)	(1 PO4)	(2 PO4)	N	p1/N, 1 PO4	p2/N, 2 PO4	1 PO4	2 PO4
3												
4	TRRASFSAQSASSY	1519	226-239	2	2.9	2.04	1.18	14	0.146	0.084	1.54E-02	8.93E-03
5	(at 6mb digest) TRRASF	737	226-231	2	2.9	2.04	1.18	6	0.340	0.197	2.50E-02	1.45E-02
6	LKKLTRRASFSAQSASSY	2001	222-239	4	4.9	4.04	3.18	18	0.224	0.177	2.54E-02	2.00E-02
7	KKLTRRASFSAQSASSY	1888	223-239	4	4.9	4.04	3.18	17	0.238	0.187	2.64E-02	2.08E-02
8	"Clydelde" LKKLTRRASF	1219	222-231	4	4.9	4.04	3.18	10	0.404	0.318	3.54E-02	2.79E-02
9	KKLTRRASF	1106	223-231	4	4.9	4.04	3.18	9	0.449	0.353	3.78E-02	2.97E-02
10	NRITTANTRRKASPEANVKRKYLKKLTRRASFSAQSASSY	4445	201-239	12	12.9	12.04	11.18	39	0.309	0.287	4.45E-02	4.14E-02
11	NRITTANTRRKASPEANVKRKYLKKLTRRASF	3563	201-231	11	11.9	11.04	10.18	31	0.358	0.328	4.65E-02	4.28E-02
12												
13												
14	Sequence	MW	ADR1	# of Acidic	total charge, p	total charge, p1	total charge, p2	# of residues	Charge/length	Charge/length	MW^(-2/3)*p	MW^(-2/3)*p
15	(peptides derived from ADR1-2c)		residues	residues	(no PO4)	(1 PO4)	(2 PO4)	N	p1/N, 1 PO4	p2/N, 2 PO4	1 PO4	2 PO4
16												
17	(2c 6mb digest) TRRASSSAQSASSY	1459	226-239	2	2.9	2.04	1.18	14	0.146	0.084	1.59E-02	9.17E-03
18	LKKLTRRASSSAQSASSY	1941	222-239	4	4.9	4.04	3.18	18	0.224	0.177	2.60E-02	2.04E-02
19	KKLTRRASSSAQSASSY	1828	223-239	4	4.9	4.04	3.18	17	0.238	0.187	2.70E-02	2.13E-02
20												
21												
22	Sequence	MW	ADR1	# of Acidic	total charge, p	total charge, p1	total charge, p2	# of residues	Charge/length	Charge/length	MW^(-2/3)*p	MW^(-2/3)*p
23	(peptides derived from ADR1-5c)		residues	residues	(no PO4)	(1 PO4)	(2 PO4)	N	p1/N, 1 PO4	p2/N, 2 PO4	1 PO4	2 PO4
24												
25	TRKASFSAQSASSY	1488	226-239	2	2.9	2.04	1.18	14	0.146	0.084	1.57E-02	9.06E-03
26	(5c 6mb digest) TRKASF	784	226-231	2	2.9	2.04	1.18	6	0.340	0.197	2.58E-02	1.49E-02
27	LKKLTRKASFSAQSASSY	1968	222-239	4	4.9	4.04	3.18	18	0.224	0.177	2.57E-02	2.02E-02
28	KKLTRKASFSAQSASSY	1855	223-239	4	4.9	4.04	3.18	17	0.238	0.187	2.68E-02	2.11E-02
29	"Clydelde" LKKLTRKASF	1158	222-231	4	4.9	4.04	3.18	10	0.404	0.318	3.61E-02	2.84E-02
30	KKLTRKASF	1073	223-231	4	4.9	4.04	3.18	9	0.449	0.353	3.85E-02	3.03E-02
31	NRITTANTRRKASPEANVKRKYLKKLTRKASFSAQSASSY	4412	201-239	12	12.9	12.04	11.18	40	0.301	0.280	4.48E-02	4.16E-02
32	NRITTANTRRKASPEANVKRKYLKKLTRKASF	3630	201-231	11	11.9	11.04	10.18	32	0.345	0.318	4.67E-02	4.31E-02
33												
34												
35	Sequence	MW	ADR1	# of Acidic	total charge, p	total charge, p1	total charge, p2	# of residues	Charge/length	Charge/length	MW^(-2/3)*p	MW^(-2/3)*p
36	(peptides derived from ADR1-7c (2nd site)		residues	residues	(no PO4)	(1 PO4)	(2 PO4)	N	p1/N, 1 PO4	p2/N, 2 PO4	1 PO4	2 PO4
37												
38	QETISHTKKLVSRTITKARKNSASSVKF (2nd site?)	2982	132-188	7	7.9	7.04	6.18	27	0.261	0.229	3.41E-02	3.00E-02
39												
40	LNRITTANTRRKASPEANVKRKY	2575	200-221	7	7.8	6.94	6.08	22	0.315	0.276	3.69E-02	3.24E-02
41	NRITTANTRRKASPEANVKRKY	2462	201-221	7	7.9	7.04	6.18	21	0.335	0.294	3.86E-02	3.39E-02
42	LNRITTANTRKASPE	1715	200-214	4	4.9	4.04	3.18	15	0.269	0.212	2.82E-02	2.22E-02
43	NRITTANTRRKASPE	1602	201-214	4	4.9	4.04	3.18	14	0.288	0.227	2.95E-02	2.32E-02
44												
45												
46	charge on 1 PO4, pH 3	-0.86										
47	charge on 2 PO4, pH 3	-1.72										