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Structural and functional characterization of the yeast general transcriptional activator CCR4

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Structural and functional characterization of the yeast general transcriptional activator CCR4

Malvar, Thomas M., Ph.D.

University of New Hampshire, 1990

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE YEAST GENERAL TRANSCRIPTIONAL ACTIVATOR CCR4

BY

Thomas M. Malvar

B.S. Syracuse University, 1982

DISSERTATION

Submitted to the University of New Hampshire in Partial
Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in

Genetics

December, 1990

This dissertation has been examined and approved.

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Stacia A. Sower
Associate Professor of Zoology

_____
Robert M. Zsigray
Professor of Microbiology

_____
Date

To my grandfather

ACKNOWLEDGEMENTS

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ABSTRACT

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE YEAST GENERAL TRANSCRIPTIONAL ACTIVATOR CCR4

BY

Thomas M. Malvar
University of New Hampshire, December, 1990

Transcription of the glucose-repressible ADH (ADH2 locus) in

Saccharomyces cerevisiae is controlled by two regulatory

pathways. The general transcriptional factors CCR4, CRE1, and CRE2

constitute the first pathway while the second pathway is comprised

of the trans-activators ADR1 and CCR1. Both ADR1 and CCR1 act

through upstream activation sequences (UAS) found in the

5'-regulatory region of the ADH2 structual gene. In contrast, the

action of CCR4, CRE1, and CRE2 is likely to be at sequences near the

TATAA element of ADH2.

The CCR4 locus was precisely mapped on the left arm of

chromosome I where it had previously been localized. Plasmid

constructions bearing sequences from chromosome I in the vicinity

of CCR4 were tested for their ability to complement a defective

ccr4 allele. A functional copy of CCR4 was identified and the DNA

x

sequenced to reveal a 2511-bp open reading frame that predicts a

protein with an estimated mass of 94.6-kDal. The CCR4 protein

showed similarity with a family of proteins containing a

leucine-rich tandem repeat motif. The repeats are characterized by

the 24 amino acid repeating sequence P-X-X-o-X-X-L-X-X-L-X-X-L-

X-L-X-X-N-X-o (where X = any residue; o = aliphatic residues L, I, or

V) and have been suggested to represent a domain involved in protein

binding. Deletion analysis of CCR4 indicates that these repeats are

required for its proper function. The amino terminus of CCR4 showed

similarities to a variety of transcription factors including TFIID,

the TATAA binding factor from humans and Drosophila.

Additional studies indicated that CCR4 mRNA and protein

levels were not regulated by carbon source availability or the allelic

state of the CRE genes. These results suggest that the interactions

observed between CCR4 and the CRE genes occur directly or

indirectly at a protein level.

The possible role that CCR4 plays in the transcriptional

regulation of the ADH2 locus based on 1) the sequence similarities

seen between CCR4 and other proteins and 2) the functional

characterization of deletions and disruptions created within the

coding sequences of CCR4 are discussed.

I. INTRODUCTION

The genome of *Saccharomyces cerevisiae* encodes four

NAD^+-linked alcohol dehydrogenase (ADH) isozymes. The cytoplasmic

fermentative isozyme ADH I functions in the NADH linked reduction

of acetaldehyde to ethanol. In contrast, the cytoplasmic ADH II

isozyme kinetically favors the reverse reaction in the NAD^+ linked

oxidation of ethanol to acetaldehyde. ADH II thus allows yeast to

utilize ethanol as a carbon and energy source when glucose is

limiting. The abundance of both isozymes is dependent on the

available carbon source. When yeast are switched from a

fermentable to a nonfermentable carbon source ADH 1 transcription

decreases approximately six-fold (Denis et al., 1983). Transcription

of ADH2, however, is repressed in cells grown on glucose containing

medium (Denis et al., 1981) and shows a 500-fold increase upon

depletion of glucose from the medium. The third isozyme, ADH III, is

a nuclear encoded gene which functions in the mitochondria (Young

and Pilgrim, 1987). The fourth isozyme, ADH IV (Paquin and

Williamson, 1986), has not been well characterized.

The structural gene for ADH2 has been cloned and sequenced (Williamson et al., 1981; Russell et al., 1983). The TATAA element for ADH2 resides 160-base-pairs (bp) upstream of the translation initiation site (Russel et al., 1983). Two upstream activation sequences, UAS1 and UAS2, are required for proper ADH2 regulation and are contained within a 50-bp region that is located 111-bp upstream of the TATAA element. UAS1 represents a 22-bp inverted repeat while UAS2 is contained within the 25-bp immediately upstream of UAS1. Both UAS1 and UAS2 act synergistically in response to the transcriptional activator ADR1 (alcohol dehydrogenase repressible; Yu et al., 1989).

The manner in which the glucose repression signal is manifested within the cell is not known. However, several trans-acting factors affecting the transcriptional state of ADH2 have been identified (Ciracy, 1975, 1977, and 1979; Denis, 1984). Denis (1984) proposed that alleles affecting ADH 2 transcription fall into two regulatory pathways. The first consists of the positive effectors ADR1 and CCR1 (carbon catabolite repressible), and the second consisting of the positive effector CCR4, whose action is

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controlled by the negative effectors CRE1 and CRE2 (catabolite

repressor element)

ADR1 is an essential gene for the derepression of ADH2

transcription (Denis et al. 1981). Strains harboring an adr1-1 allele,

characterized by an amber mutation at the eleventh codon, show no

ADH II activity (Bemis and Denis, 1989). The ADR1 gene has been

cloned (Denis and Young, 1983) and sequenced (Hartshorne et al.,

1986) and encodes a protein containing two DNA binding zinc fingers.

ADR1 binds to the upstream activating sequences located in the

ADH2 promoter under both fermentative and nonfermentative growth

conditions (Taylor and Young, 1990). Gel retardation studies indicate

that ADR1 binds predominantly to UAS1; however, optimum binding

is observed in the presence of both UAS1 and UAS2 (Eisen et al.,

1988). The ADR1 protein also contains a cAMP dependent protein

kinase (cAPK) recognition site thought to play a role in its ability to

activate transcription (Cherry et al.,1989). Dominant alleles of ADR1

(ADR1^c) which allow for ADH II activity under glucose repression,

have been identified as mutations which purportedly disturb the

cAPK phosphorylation of ADR1 (Cherry et al., 1989; Denis and Gallo,

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1986). The CCR1 gene identified by Ciriacy (1977) is allelic to SNF1

(Denis, 1984) and codes for a protein kinase required in the

regulation of several enzymes under glucose repression (Carlson and

Celenza, 1986). Genetic evidence indicates that CCR1 acts through or

in conjunction with ADR1 (Denis, 1984) in the regulation of ADH2

transcription. More recent findings indicate that CCR1 acts through

factors independent of ADR1 (D. Audino, pers. comm.).

The negative regulatory elements CRE1 and CRE2 were

identified as mutations which allow for the escape of ADH2 from

glucose repression as determined by the ability of cells to grow in

the presence of the respiratory inhibitor antimycin A (Denis, 1984).

The CCR4 allele was identified by mutations which render strains

containing the cre1 or cre2 alleles sensitive to antimycin A.

Mutations in these alleles were shown to effect ADH2 at the

transcriptional level (Denis,1984; Denis and Malvar, 1990). Northern

analysis identified ADH2 specific mRNA in cre1 carrying strains

under glucose repression; a condition when no ADH2 message is

normally seen. Mutations in CCR4 were found to suppress the

cre-induced ADH2 mRNA levels observed under repressed conditions

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(Denis and Malvar, 1990).

CRE2 is allelic to SPT6 and SSN20 (Denis and Malvar, 1990;

Clark- Adams and Winston, 1987; Neigeborn et al., 1987) while CRE1

was shown to be allelic to SPT10 (Denis and Malvar, 1990). The SPT

(suppressor of TY) genes were originally identified as mutations

which overcome the effects of delta insertions at the his4-912delta

locus (Clark-Adams and Winston, 1987; Winston et al., 1984). Delta

sequences represent the approximately 300-bp terminal repeat of

the yeast retrotransposon TY. Delta sequences are often left behind

following transposition of a TY element into and out of a

chromosomal location. The sites of action and allelisms of the

various trans-acting regulatory factors controlling ADH2

transcription is shown in Figure 1. The identity of several members

of the SPT gene family has been determined. The SPT11 and SPT12

genes encode histone proteins (Clark-Adams et al., 1988), SPT13 is

allelic to the transcriptional regulatory factor GAL11, required for

galactose gene expression, and SPT15 is the yeast "TATAA"- binding

factor, TFIID (Eisenman et al., 1989). The SSN20 allele was

identified by mutations which overcome a suc2 phenotype caused by

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Figure1
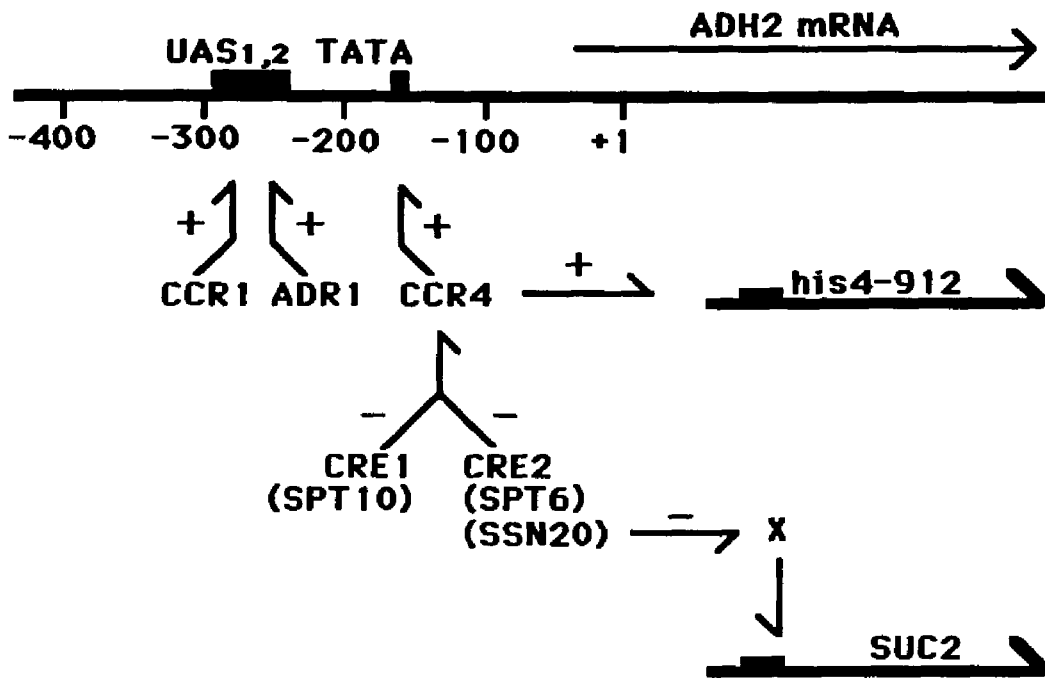
defects in the SNF2 allele, itself a general transcriptional effector
(Neigeborn et al., 1987). The SUC2 gene encodes the enzyme
invertase that acts in the metabolism of sucrose. The identification
of members of the SPT gene family as components of transcriptional
regulation and chromatin structure suggests that SPT6 (CRE2) and
SPT10 (CRE1) act in a similar capacity.

Both CCR4 and the CRE alleles are pleiotropic in their effects
on cellular processes. Mutations in CCR4 affect other
nonfermentative genes (Denis, 1984) and show a temperature
sensitive phenotype following growth on nonfermentative medium
(Denis and Malvar, 1990). Mutations in both CRE alleles affect cell
morphology resulting in an elongated cell shape (Denis, 1984). In the
case of a cre1 allele, a mutation in CCR4 was shown to suppress the
aberrent cell morphology (Denis and Malvar, 1990). Additionally,
mutations in CRE2 result in a temperature sensitive phenotype that
cannot be suppressed by a ccr4 allele (Denis and Malvar, 1990).

The effects of CCR4 and the CRE alleles occur through
sequences near the TATAA element of ADH2 (Denis and Malvar,
1990). Strains harboring deletions, disruptions, or displacements of

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sequences upstream of the ADH2 TATAA element continue to show

elevated ADH II activity under repressed conditions in a cre1 or cre2

background when compared to controls (Denis and Malvar, 1990).

Mutations in CCR4 were able to suppress the cre-induced effects in

these strains. Additionally, mutations in CCR4 suppress the effects

that cre1 (spt10) or cre2 (spt6) have in overcoming delta insertions

in the his4-912delta promoter (Denis and Malvar, 1990). The finding

that mutations in SSN20 (CRE2) are able to express SUC2 in a suc2

allele created by deletions of sequences 5' to the TATAA element

indicates that SSN20 also acts near TATAA sequences in its control

of SUC2 (Neigeborn et al., 1987).

Deletion of SPT6 (CRE2, SSN20) is lethal in haploid yeast

strains (Clark-Adams and Winston, 1987), a result consistent with

the temperature sensitive phenotype of spt6 alleles. The genes for

SPT6 and SPT10 have been cloned and sequenced (Swanson et al,

1990; G. Natsoulis, personal communication). SPT6 codes for a

nuclear protein that has an estimated mass of 170-kDal and which

contains a highly acidic amino terminus (Swanson et al., 1990). Both

increasing and decreasing the dosage of SPT6 results in the

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suppression of a his4-912delta allele and deletions in the regulatory

5' region of the SUC2 locus (Clark-Adams and Winston, 1987;

Neigeborn et al., 1987). Proteins containing a high density of acidic

residues have been implicated in chromatin binding (Earnshaw,

1987). The possibilty that SPT6 may play a role in chromatin

structure around the TATAA element is also supported by the

observation that varying the dosage of histone proteins has similar

effects on his4-912delta as that seen for SPT6 (Clark-Adams et al.,

1988).

The action of CCR4 and the CRE alleles on multiple loci

suggests they act in a general manner in the regulation of

transcription. Recent work on the initiation of transcription has

identified multiple factors that constitute components of the

transcriptional machinery (for a review see Lillie and Green, 1989).

In addition to the multi-subunit RNA polymerase II, several

heterogeneous fractions from cell extracts have been identified as

containing factors required for transcription. These include TFIIA,

TFIIB, TFIID, TFIIE, and TFIIF. Recently the gene coding for the

protein factor TFIID has been cloned and sequenced fom human,

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Drosophila, and yeast (Kao et al., 1990; Hoey et al., 1990; Cavallini

et al., 1989). The TFIID protein is required for the first step in the

initiation of transcription and acts by binding to the TATAA element

(Lillie and Green, 1989). TFIID is also thought to provide contacts

between the transcriptional machinery and transcriptional

activators which bind DNA activation sites that lie upstream of the

TATAA element (for a review see Ptashne and Gann, 1990; Lewin,

1990). Additionally, a heteromeric protein, RAP30/70, has been

identified as a component of the TFIIF fraction (Burton et al., 1988).

The protein products of CCR4 and the CRE alleles may

represent components of the transcriptional fractions discussed

above. Their action at a variety of loci support their general role in

transcriptional regulation. Additionally, their site of action appears

to be through sequences that lie near the TATAA element. The

objective of this study is to better understand the role that CCR4

plays in the regulation of transcription. As a requisite for

addressing this objective, the gene encoding CCR4 had to be cloned.

CCR4 was precisely mapped on chromosome I which allowed the use

of physically defined segments of chromosome I to be used to

identify the location of CCR4 by complementation of a ccr4 defect.

The cloning of CCR4 would allow for addressing several
questions: 1) What is the DNA sequence of the open reading frame
that represents the CCR4 gene? 2) Based on the DNA sequence what
is the predicted size and composition of the resulting polypeptide?
3) Does analysis of the protein sequence reveal any homologies to
known proteins of known function? 4) If so, does this suggest a
role(s) that CCR4 plays in the regulation of transcription?

To address these questions, plasmids that contain CCR4
sequences as determined by complementation studies were used to
determine the DNA sequence of CCR4. The predicted amino acid
sequence was searched against data bases containing the sequences
of known genes and any similarities found were analyzed for clues
as to the possible function of CRR4. Studies were also performed to
assess possible factors that regulate CCR4 at a transcriptional level
including that of carbon source and the allelic state of the CRE
genes. In order to determine regions of functional importance in the
CCR4 protein, a series of disruptions and deletions in the coding
region of CCR4 were constructed. These constructions were then

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tested for phenotypes that they impart in various genetic

backgrounds.

II. MATERIALS AND METHODS

Yeast strains and conditions of growth

Yeast strains are listed in Table 1 . Yeast cultures were grown in YEP medium (2% Bacto-Peptone, 1% yeast extract, 20mg/liter each of adenine and uracil) supplemented to a final concentration of either 8% glucose (YEP-Glc) or 3% ethanol (YEP-Et). Yd plates contained YEP medium supplemented to 2% glucose and 2.5% agar. YD8% and YET plates contained YEP-Glc or Yep-Et medium, respectively, and 2.5% agar. GLY plates contain YEP medium supplemented to 3% glycerol and 2.5% agar. Where indicated, the respiratory inhibitor antimycin A (Sigma Chemical Co.) was added to a concentration of 0.6 ug/l. Minimal plates lacking tryptophan, histidine, leucine, or uracil for selection purposes were used where indicated (Wiliamson et al., 1981).

ADH Assays

ADH II enzyme activities were assayed as previously described (Denis and Young, 1983). Activities are reported as milliUnits of ADH activity per milligram total protein.

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Table 1 : Yeast strains

Strain	Genotype
82-2b	MATo adh1-1 cre1-1 his3 leu2 trp1 ura1
85-1c	MATa adh1-1 ccr4-10:HIS3 trp1 leu2
292-6a	MATa adh1-1 ade1 his3 SPO7:LEU2 trp1
317-5b	MATo adh1-1 ccr4-10 leu2 ura3
348-13a	MATa adh1-1 ccr4-10 ura3 trp1
348-26c	MATo adh1-1 ccr4-10 his3 leu2 trp1 ura3
350-19a	MATo adh1-1 ccr4-10 trp1 ura3
350-19b	MATa adh1-1 CCR4:TRP1 his3 leu2 ura 3
350-19c	MATo adh1-1 ccr4-10 trp1 ura3
350-19d	MATa adh1-1 CCR4:TRP1 his3 lue2 ura3
500-16	MATa adh1-1 adh3 adr1-1 his4 trp1 ura1
500-16-1	MATa adh1-1 adh3 adr1-1 cre1-1 his4 trp1 ura1
500-16-14	MATa adh1-1 adh3 adr1-1 cre2-1 his4 trp1 ura3
612-1d	MATa adh1-1 his3 leu2 trp1 ura3
FW712-1d	MATo adh1-1:URA3 his4-912delta ura3-52

Bacterial and Yeast Transformation

Bacterial transformations were performed using competent DH5o or

RR1 cells (purchased from BRL, Gaithersburg, MD). Transformations

were conducted as recomended by the manufacturer.

Yeast transformations were performed using the lithium

acetate (LIAc) procedure (Ito et al., 1983).

Plasmid Constructions

DNA polymerase, DNA ligase, and restriction enzymes were

purchased from New England Biolabs (Beverly, MA). Conditions for

DNA fill in, ligation, and restriction reactions were as recomended

by the manufacturer. DNA fragments were resolved on 0.7% agarose

gels and visualized under UV light after staining with ethidium

bromide (Maniatis et al.). When required, DNA fragments were

isolated by excision from agarose gels as described in Appendix A.

Constructions lambda L4 , Lambda M7, YCp50-2c, and p89 were

gifts of D. Kaback (see Figures 2, 3a, and 3b). Lambda L4 contains

approximately 17-kilobases (kb) of chromosome I DNA in the lambda

1059 cloning vector. Lambda M7 contains approximately 18.5-kb of

chromosome I DNA in the lambda 1059 cloning vector. YCp50-2c

15
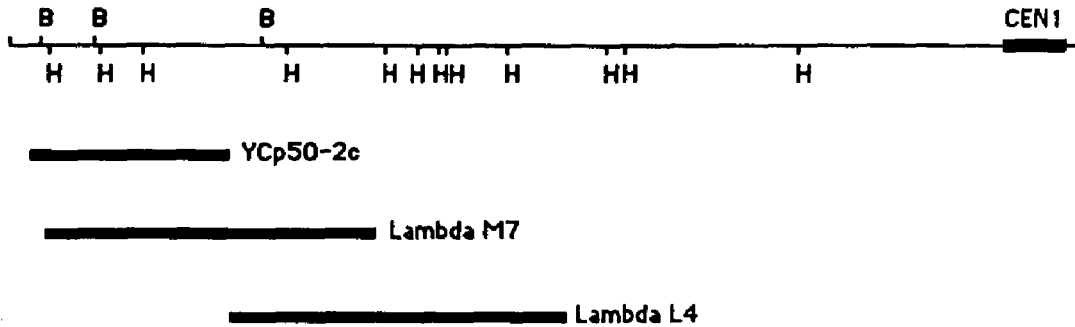
Figure 2

Figure 2 : Sequences contained on YCp50-2c, Lambda M7,
and Lambda L4.

The left arm of chromosome I is shown along with a
representation of sequences contained on YCp50-2c, Lambda
M7, and Lambda L4 (█). The restriction site designations
are B = BamHI and H = HindIII.

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Figure 3 : Sequences contained on p89, pUC18-Bm:Hd, and YRp7-3.5.

a. Detailed restriction map of chromosome I in the predicted area of CCR4 is shown. The position of a portion of Lambda L4 (■■■) is shown as a reference. Sequences contained in the constructions p89, pUC18-Bm:Hd, and YRp7-3.5 are shown as open rectangles (▭). The restriction site designations are Bm = BamHI, Bg = BglII, E = EcoRI, Hc = HincII, and Hd = HindIII

b. Scaled representation of p89, pUC18-Bm:Hd, and YRp7-3.5. The construction p89 has the 1.4-kb HincII fragment cloned into the EcoRV site of the vector pBS-KS and was a gift of D. Kaback. The vector pUC18-Bm:Hd was constructed by insertion of the 1.5-kb BamHI to HindIII fragment into the polylinker region of pUC18. YRp7-3.5 was constructed by inserting the 3.5-kb BglII fragment into the BamHI site of the yeast expression vector YRp7. Restriction sites are shown only for those sequences derived from chromosome I. Designations are : Ampicillin (AMP) = (■), Tetracycline (Tc) = (▨), TRP1 (yeast) = (▨), vector sequences = (■), and sequences from chromosome I = (▭).

Figure 3

a.
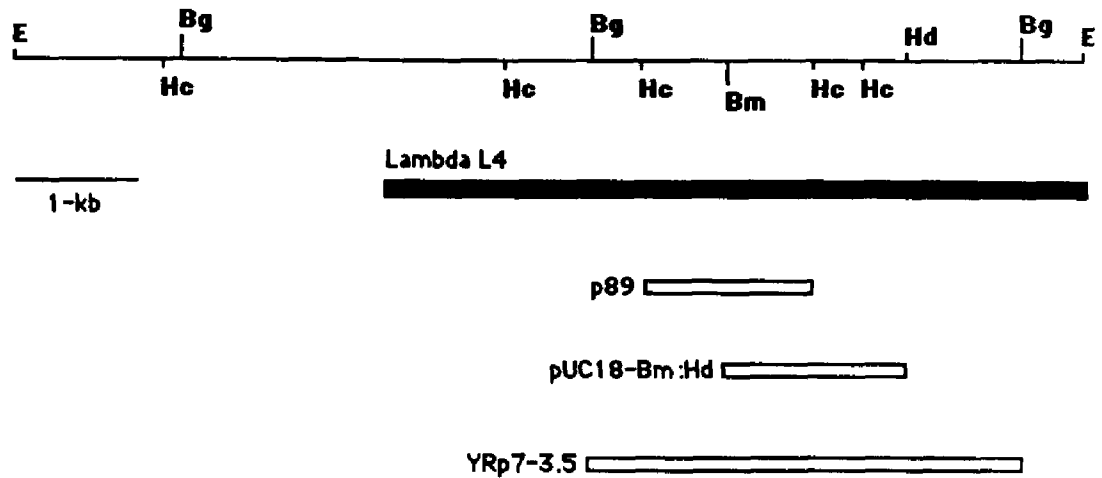
b.
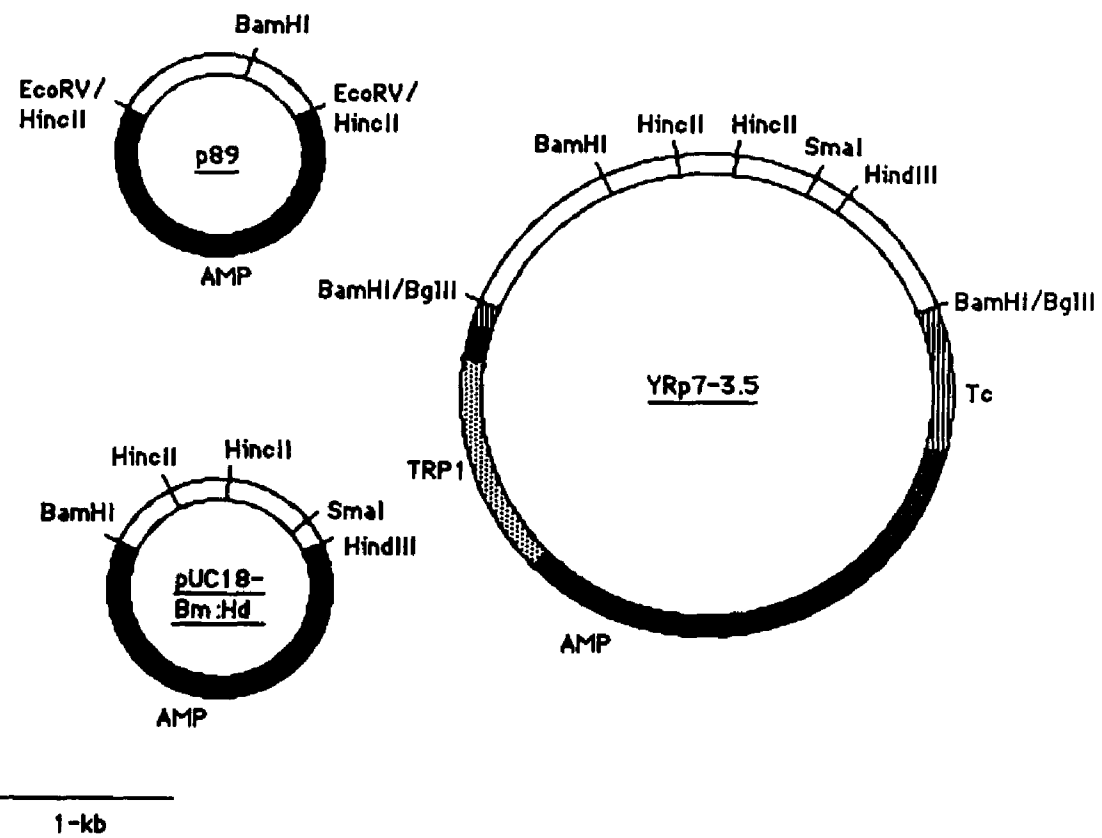
contains the yeast URA3 gene as well as approximately 12-kb of chromosome I DNA. A 1.4-kb HincII fragment cloned into the EcoRV site of pBS-KS gives the clone p89 (see Figure 3b). Plasmid pHIS3 contains a 1.7-kb BamHI fragment of the yeast HIS3 gene cloned into the polylinker region of pUC18 and was a gift from M. Wigler.

YRp7-3.5+ was contructed by subcloning the 3.5-kb BglII fragment isolated from lambda L4 into the BamHI site of the YRp7 vector (see Figures 3a and 3b). YRp7-3.5- contains the same 3.5-kb BglII fragment in the opposite orientation. The YRp7 vector also contains the yeast TRP1 gene. The clone pC1 was isolated from a yeast YEP13 genomic library (Nasmyth and Reed, 1981; see Figure 9). The library was constructed by cloning a size fractionated Sau3AI limited digestion of yeast chromosomal DNA into the BamHI site of the YEP13 vector. Clone pC1 was identified by colony hybridization (see below) using the 1.5-kb BamHI to HindIII fragment isolated from YRp7-3.5 as a probe. The pUC18-Bm:Hd clone was constructed by insertion of the 1.5-kb BamHI-HindIII fragment isolated from YRp7-3.5+ into the polylinker of pUC18 (Figures 3a and 3b). Plasmid pM-11.7 represents the 11.7-kb BamHI fragment subcloned from

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lambda M7 into the vector YRp7 (see Figure 8). Plasmid pL-6.5

represents the 6.5-kb HindIII fragment subcloned from lambda L4

into the vector YRp7 (see Figure 8)

Constructions of the disruption and deletions made in CCR4

were conducted as described below. The plasmid pC1:HIS3 was made

by digestion of pC1 at the unique BamHI site within CCR4 and

insertion of the 1.7-kb BamHI fragment of HIS3 isolated from pHIS3

(CCR4:1-668; see Figure 4). This construction represents a

disruption in the 3' end of CCR4 at base pair (bp) 2004. The plasmid

pUC18-Bm:Hd:HIS3 was constructed by replacing the 368-bp HincII

fragment in pUC18-Bm:Hd with a filled in 1.7-kb BamHI fragment of

the yeast HIS3 gene isolated from the plasmid pHIS3 (CCR4:1-392;

see Figure 5). This construction disrupts CCR4 at bp 1175 leaving

the truncated protein with two of the five leucine rich repeats

intact. An inframe deletion removing 397-bp from the middle of the

CCR4 gene was constructed by restricting pUC18-Bm:Hd with both

MluI and HincII, filling in the MluI 5' overhang, and religating the

ends (see Figure 6). The BamHI to Smal fragment containing the

deletion was then isolated from the resulting construction and used

20
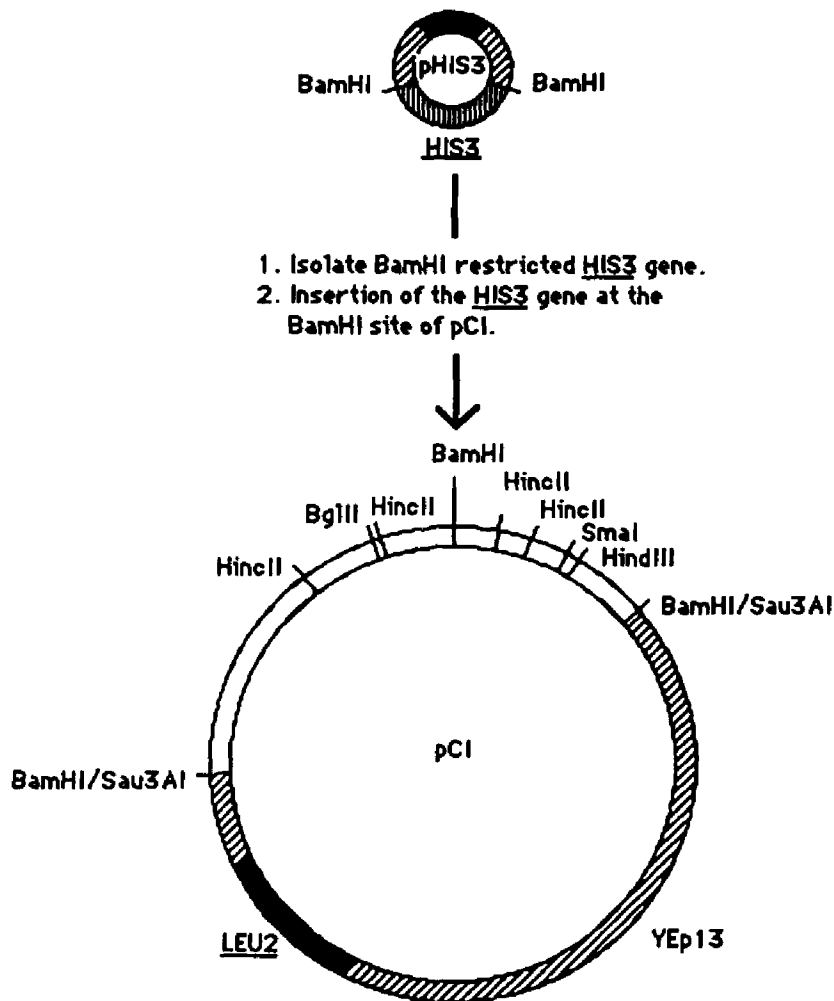
Figure 4

Figure 4 : Construction of pCI:HIS3.

CCR4 was disrupted in pCI at the internal BamHI site by insertion of the yeast HIS3 gene isolated from pHIS3. Restriction sites in the vector pCI are shown only for those sequences derived from chromosome I. Sequence designations are: ampicilin gene (■), HIS3 gene (▥), vector sequences (▨), yeast LEU2 gene (■), and sequences from chromosome I (□).

Figure 5 : Construction of pUC18-Bm:Hd:HIS3

    CCR4 was disrupted by replacment of the internal HincII
fragment (bp 1175-1543) with the yeast HIS3 gene isolated from
pHIS3. The plasmid pHIS3 was restricted with BamHI , the HIS3
gene was isolated, and the 5' overhang filled in. The plasmid
pUC18-Bm:Hd was restricted with HincII and the HIS3 gene
inserted. Restriction sites for pUC18-Bm:Hd are shown only for
those sequences derived from chromosome I. Sequence
designations are : ampicilin gene (█), HIS3 gene (▥), vector
sequences (▨), and sequences from chromosome I (☐).

Figure 5

1. Restrict with BamHI
2. Isolate HIS3 gene

1. Restrict with HincII

pHIS3
BamHI
BamHI
HIS3
AMP

HindIII
SmaI
HincII
pUC18-Bm:Hd
HincII
AMP
BamHI

HIS3
HincII/BamHI
HincII/BamHI
BamHI
pUC18-Bm:Hd:HIS3
SmaI
HindIII
AMP
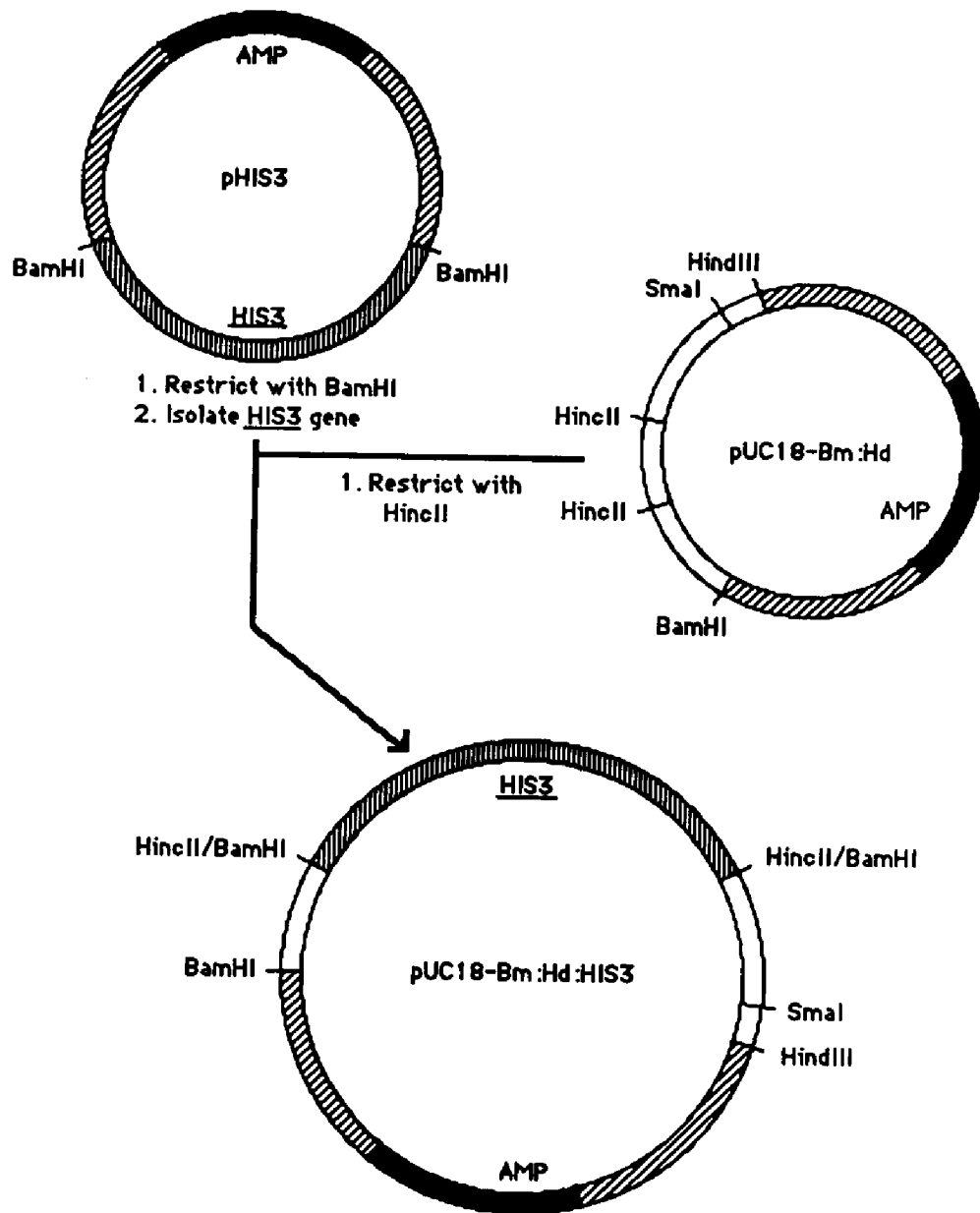
Figure 6 : Construction of YRp7-3.5:deltaMluI-HincII

The vector pUC18-Bm:Hd was restricted with both MluI and HincII, the 5' overhang of the MluI site was filled in, and the blunt ends ligated together to make pUC18-deltaMlu:Hinc. This construction gives an inframe deletion in CCR4 which removes all but one full copy of the leucine rich repeat region. In order to construct a yeast vector containing the inframe deletion, the BamHI to SmaI fragment from pUC18-deltaMlu:Hinc was isolated and used to replace the analogous BamHI to SmaI fragment from YRp7-3.5. The new construct was designated YRp7-3.5:deltaMluI-HincII. Restriction sites are shown only for those sequences derived from chromosome I.

Figure 6

1. Restrict with MluI and HincII.
2. Fill in MluI 5' overhang.
3. Ligate.

1. Isolate BamHI to SmaI frag-
   ment from pUC18-deltaMlu-
   Hinc.
2. Restrict YRp7-3.5 with BamHI
   and SmaI
3. Ligate isolated fragment with
   restricted YRp7-3.5.
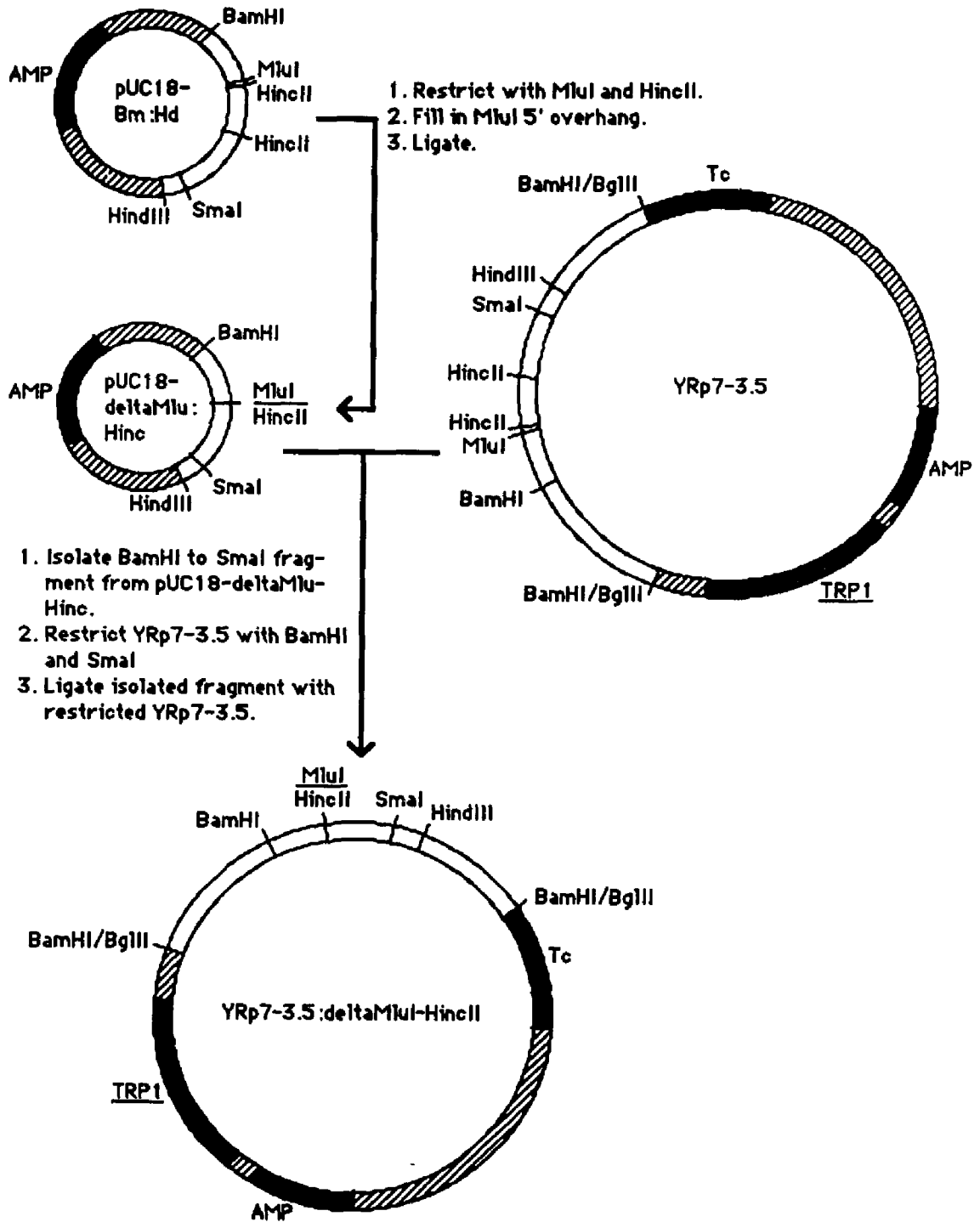
to replace the analogous1.5-kb BamHI to SmaI fragment in the vector

YRp7-3.5+. The new construction was designated YRp7-3.5:delta

MluI-HincII (CCR4:1-382/514-837; see Figure 5) and removes all but

one full repeat of the leucine rich region (residues 383 - 514).

DNA Sequencing and Computer Analysis

Double stranded template preparation and subsequent alkali

denaturation were conducted as described by Wang et al. (Appendix

B). The sequence of CCR4 was determined by using Sequenase

purchased from USB, Cleveland, OH. The sequence reactions were

conducted as described by the manufacturer. Synthetic

oligonucleotide primers were purchased from either Operon

Technologies, Alameda, CA or Promega Biotec, Madison, WI. The

^{35}S-Deoxyadenosine 5'-[0 -thio]triphosphate was obtained from New

England Nuclear, Boston, MA. The deduced amino acid sequence was

searched against the National Biomedical Research Foundation

database for homologies using the program FASTP (Lipman and

Pearson, 1985). A linear hydrophilicity plot of the CCR4 protein was

performed using the algorithm of Kyte and Doolittle in the DNA

Stryder sequence analysis program.

Colony Hybridization

Approximately 250 bacterial colonies per plate were grown overnight on AMP plates. Duplicates were made of the colonies by replica plating and one set was transferred onto Whatman 541 filter paper. The filters were then washed at room temperature in 0.5 M NaOH for ten minutes followed by three ten minute washes in 2X SCP (2 M NaCl, 0.75 M Na_2HPO_4, 10 mM EDTA). The DNA was fixed by baking the filters at 80 °C under vacuum for two hours.

The desired DNA probe was isolated from an agarose gel (Appendix A) and labeled using a random primed DNA labeling kit following the manufacturers protocol (USB, Cleveland, OH).

Prehybridization was performed for five minutes by boiling a solution of 0.6 ml 10% sarkosyl, 0.3 ml 2 mg/ml sheared salmon sperm DNA, and 3 ml H_2O for ten minutes, cooling for five minutes on ice, and addition of 1.8 ml of 20X SCP. The solution was then added to a plastic hybridization bag containing the filter. Hybridization was performed as described above with the exception of the addition of the labeled DNA probe to the prehybridization solution prior to boiling. Hybridization was carried out overnight at 65 °C. The filters

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were washed at room temperature twice in 2X SCP, 1% SDS for fifteen minutes, twice with 2X SCP for fifteen minutes, and once for fifteen minutes in 0.4% SCP. The filters were then blotted dry, wraped in plastic film, and exposed on Kodak X-OMAT diagnostic film.

Southern Analysis

Yeast chromosomal DNA was digested with restriction endonucleases, run on an agarose gel, stained in ethidium bromide, and photographed. The gel was then washed at room temperature twice for eight minutes in 0.25 M HCl, twice for fifteen minutes in 1 M NaCl, 0.5 M NaOH solution, and once for fourty-five minutes in a solution of 1 M Tris pH 7.0, 0.6 M NaCl. The DNA was transferred by blotting onto nitrocellulose (Millipore Corp., Bedford, MA) for two hours and then baked under vacuum at 80 ° C for two hours. DNA probe preparation and hybridization were conducted as described above.

Northern Analysis

The preparation of total yeast RNA has been described (Denis, Ferguson, and Young, 1983). The appropriate labeled DNA probe was

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prepared as described above. Gene Screen hybridization transfer membrane was supplied by New England Nuclear, Boston, MA. Northern analysis was performed by the glyoxal gel method as described in the manufacturer's instruction manual. Densitometric analysis of ADH2 mRNA, CCR4 mRNA, and rRNA was conducted using an E-C- 610 densitometer. The levels of ADH2 and CCR4 message were normalized to the level of rRNA loaded onto the gel as described (Denis, Ferguson, and Young, 1983).

III. RESULTS

Mapping of CCR4

Initial mapping data for CCR4 (Denis, personal communication)

showed linkage to ADE1 on chromosome I. The segregation ratios

between CCR4, ADE1, CDC24, and the centromere linked TRP1

(chromosome IV) placed CCR4 near the MAK16 locus on the left arm

of chromosome I (see Figure 7a). Several plasmid constructions

containing sequences from the predicted location of CCR4 on

chromosome I were tested for their ability to complement ccr4

allele. Strain 348-26c (ccr4) was transformed with either the

YCp50-2c or pM-11.7 plasmid and assessed for growth at 37°C on

nonfermentative media (see Figure 8). None of the plasmid

constructions were found to complement the ccr4 allele contained in

strain 348-26c.

Since the cM map distance may not always quantitatively

correlate with the physical distance between loci, a more precise

mapping of CCR4 was required in order to clone CCR4 by its

chromosomal location. Strain 317-5b (ccr4 leu2) was crossed to

Figure 7a

CDC24 .... MAK16 CCR4 .... SPO7 .... CEN1 .... ADE1

	Parental Ditype (PD)	Nonparental Ditype (NPD)	Tetratype (T)	First Division Segregation (SDS)	Second Division Segregation (SDS)
CDC24 : ADE1[a]	11	2	22	–	–
CDC24 : CCR4[a]	18	0	21	–	–
ADE1 : CCR4[a]	31	1	29	–	–
TRP1 : CCR4[a]	–	–	–	44	12

[a] Mapping data from C. Denis (personal communication).

Figure 7a : Initial mapping of CCR4.

The relative position of CCR4 with respect to other genes used for mapping purposes on chromosome I is shown above. The distance between alleles is not to scale. Genetic distances calculated between the indicated genes are : CDC24 : ADE1 = 48.6 cM, CDC24 : CCR4 = 26.9 cM, CCR4 : ADE1 = 28.7 cM, and TRP1 : CCR4 = 13.6 cM. Genetic distances were calculated by the equation:

$$cM = \frac{100}{2} \left| \frac{T + 6NPD}{PD + NPD + T} \right|$$

Centromere linkage of CCR4 using the centromere linked TRP1 locus (chromosome IV) was estimated as one half the second division segregation (SDS) frequency:

$$cM = 0.5 \left| \frac{SDS}{FDS} \right|$$

Figure 7b

Figure 7b : Diagram of the left arm of Chromosome I.

CCR4 was mapped to the left arm of chromosome I relative to SPO7 and CEN1 (see text). The direction of transcription of CCR4 is indicated by the arrow as being away from CEN1 (D. Kaback, personal communication). Distances are given in centiMorgans from CEN1 as determined from the mapping data as described in the text.

Figure 8
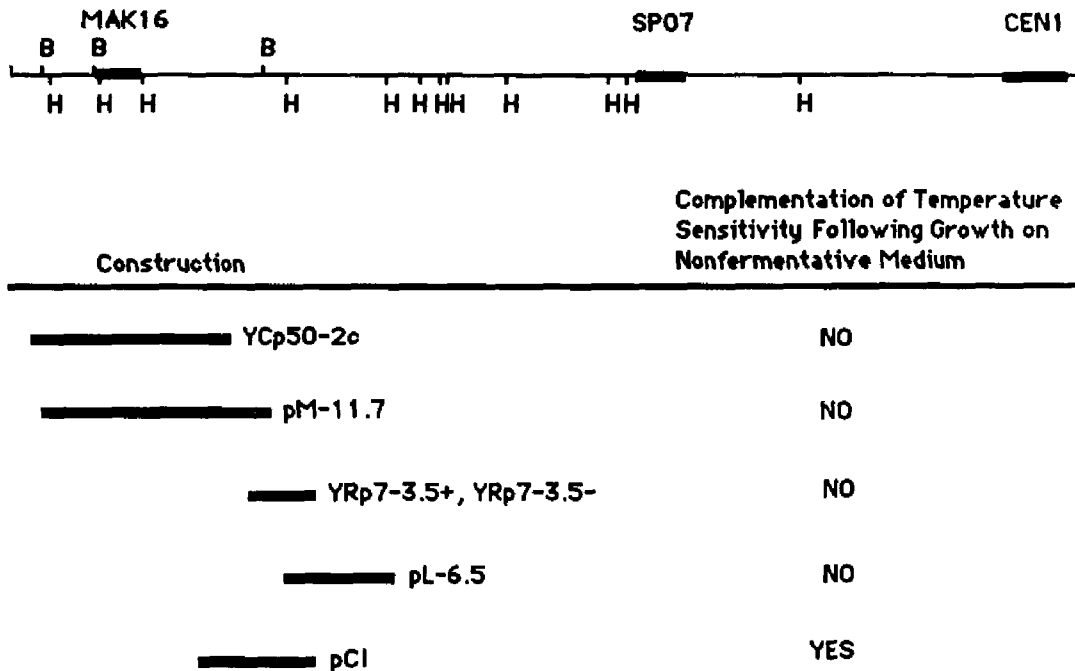
Figure 8 : Complementation of ccr4 by plasmid constructions.

Strain 348-26c (ccr4) was transformed with plasmid constructions containing sequences from chromosome I as indicated above. Complementation of the ccr4 allele was determined by the ability of the transformed strain to grow on nonfermentative medium at 37°C. Construction of the plasmids is described in the text. Restriction site designations on chromosome I are: B = BamHI and H = HindIII.

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292-6a (trp1 SPO7:LEU2) and the segregation pattern of ccr4 (low

ADH II activity, see Table 2 and temperature sensitivity following

growth on nonfermentative medium, Denis and Malvar, 1990), LEU2,

and trp1 was followed in 30 tetrads. The linkage of CCR4 to CEN1

was determined by following the segregation pattern between CCR4

and TRP1 the latter of which is tightly linked to the centromere of

chromosome IV. The segregation pattern between CCR4 and CEN1 and

between SPO7 and CEN1 gave a second degree segregation (SDS)

frequency of 0.2 (25 FDS : 5 SDS) and 0.03 (29 FDS : 1 SDS)

respectively. This places SPO7 at 1.5 cM from CEN1 and CCR4 at 10

cM from CEN1. The segregation pattern between CCR4 and SPO7

provided a ratio of 25 PD : 5 T for an estimated map distance of 8.3

cM. The resulting data placed CCR4 on the left arm of chromosome I

approximately 8.3 cM distal to SPO7 and 10 cM from CEN1 (see

Figure 7b).

Plasmid constructions pL-6.5 and YRp7-3.5 that contain

sequences in the vicinity of CCR4 estimated from the segregation

ratios provided above were tested for their ability to complement a

ccr4 allele in strain 348-26c. Like YCp50-2c and pM-11.7, both

Table 2 : Effects of ccr4/cre mutations on ADH II activity.

ADH allele	Other relevant genotype	ADH II acivity (mU/mg)	
		Glucose[a]	Ethanol[b]
ADH2[1]		8	2500
ADH2[1]	cre1	175	2200
ADH2[1]	cre1 ccr4	23	500
ADH2[1]	cre2	77	2900
ADH2[1]	cre2 ccr4	6	110
ADH2[1]	ADR1-5[c]	280	6600
ADH2[1,2]	ccr4	5	400
ADH2[1]	ccr4 ADR1-5[c]	345	335

[a] Yeast were grown on YEP medium supplemented to 8% glucose as described in Materials and Methods.

[b] Yeast were grown on YEP medium supplemented to 3% ethanol as described in Materials and Methods.

[1] ADH II values from Denis, C.L. Genetics 108:833-844. (1984).

[2] Depending on the genetic background, strains carrying the ccr4-10 allele can vary between 100 to 1000 mU/mg of ADH II activity under ethanol growth conditions.

pL-6.5 and YRp7-3.5 failed to complement the ccr4 allele (see Figure

8).

In order to provide another selectable marker for mapping, the

TRP1 gene was integrated into the left arm of chromosome I by

transformation of strain 348-26c (ccr4) with BamHI restricted

YRp7-3.5 plasmid (see Figure 3a and 3b). Analysis of the stable

TRP1 transformant 348-26cl showed elevated ADH II activity under

derepressed conditions and complementation of the temperature

sensitive phenotype following growth on nonfermentative medium

displayed by mutant CCR4 alleles (Table 3). Southern analysis of

348-26cl confirmed two copies of YRp7-3.5+ integrated at the

BamHI site on chromosome 1 (data not shown). Furthermore, analysis

of 27 tetrads resulting from a cross between 348-26cl (MATα

CCR4:TRP1) and 348-13a (MATa ccr4 trp1) showed the segregation

pattern of CCR4:TRP1, was 2 : 2 with respect to trp1, and the TRP1

phenotype segregated away from the temperature sensitive

phenotype on nonfermentative medium. One of the tetrads from the

above cross (segregants 350-19a, -19b,-19c, and -19d; Table 3)

was selected and assayed for ADH II activity under ethanol growth

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Table 3: Complementation of the CCR4 mutation.

Strain	YET growth @37°C	ADH II activity (mU/mg) Ethanol
348-26cl	+	3317
350-19a[1]	–	80
350-19b[1]	+	2373
350-19c[1]	–	8
350-19d[1]	+	2576
348-26c[a]	–	237
348-13a[a]	–	130
348-26c-Cl[2]	+	1650

[a] Segregants of 317-5b X 292-6a.

[1] Segregants of 348-26c X 348-13a.

[2] Strain 348-26c transformed with pCl. ADH II activity assayed by growth in medium lacking tryptophan and supplemented to 3% ethanol.

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conditions. Elevated ADH II activity was found to cosegregate with

CCR4:TRP1. Additionally, the presence of YRp7-3.5+ as assayed by

Southern analysis performed on the 350-19 segregants,

cosegregated with the CCR4:TRP1 phenotype and elevated ADH II

activity noted above (data not shown). These results indicate that

the YRp7-3.5 plasmid, upon integration at the BamHI site, reformed a

wild type CCR4 gene and eliminated the need for further mapping

experiments

Cloning of CCR4

While Southern analysis confirmed the integration of

YRp7-3.5+ in strain 348-26cl, it most likely did not contain a

complete functional copy of CCR4 since transformants harboring

either the YRp7-3.5+ or YRp7-3.5- intact plasmid failed to show

complementation of the mutated CCR4 allele.

In order to obtain a functional copy of CCR4, the internal

1.5-kb BamHI to HindIII fragment isolated from YRp7-3.5+ (see

Figure 3b) was used to probe a yeast genomic pool (Nasmyth and

Reed, 1982). Two thousand colonies were screened and a single clone

(designated pC1) was identified as displaying homology to the probe.

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Restriction analysis of pC1 showed that it represented an

approximately 7.0-kb insert from chromosome I including sequences

found in YRp7-3.5+ (see Figure 9). Transformation of 348-26c with

pC1 resulted in complementation of the mutant CCR4 allele as

assayed by ADH II activity and growth on nonfermentative plates at

37 ° C (see strain 348-26c-C1 in Table 3 and Figure 8). Furthermore,

the analysis of transformants selected for the loss of the pC1

plasmid following growth on nonselective medium showed that the

loss of pCl correlated with lowered ADH II activity and restoration

of the temperature sensitive phenotype on nonfermentative media.

These findings demonstrate that the pC1 construction contains a

functional copy of the CCR4 gene.

Sequencing of CCR4

The constuctions p89, YRp7-3.5+, YRp7-3.5-, pUC18-Bm-Hd,

and pC1 (Figures 3b and 9) were used to sequence both strands of

CCR4. Initial sequence data were generated from pUC18-BM-Hd using

the -20 M13 and the -24 M13 (reverse) sequencing primers.

Subsequent sequences were obtained using synthetic oligonucleotide

primers generated from previously determined sequences. The

Figure 9
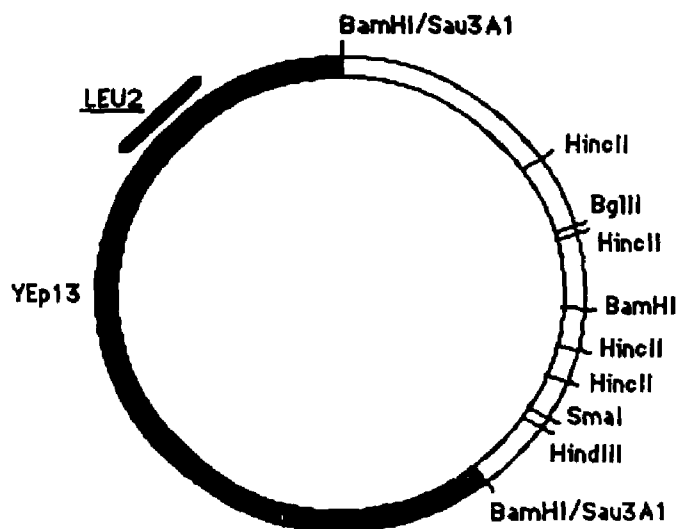
Figure 9 : Restriction map of pCI.

The construction pCI contains a functional copy of CCR4 isolated from a YEp13 yeast genomic library. Cut sites are shown only for those sequences derived from chromosome I. The YEp13 sequences are represented by darkened sections (■), while those representing sequences derived from chromosome I are open (▢).

sequencing strategy and the position and orientation of the primers

are presented in Figure 10. The sequence of CCR4 predicts an open

reading frame of 2511-bp with an estimated protein mass of

94.6-kDal. The direction of transcription is away from the

centromere (D. Kaback, personal communication). The sequence data

agrees with both the size of the CCR4 transcript as predicted by

Northern analysis (see below) and with the CCR4 protein identified

by immunoprecipitation from ^{35}S-labeled yeast cell extracts (ca.

92-kDal; R. Vallari, personal communication). Figure 11a and 11b

shows the complete sequence of CCR4 along with the corresponding

amino acid sequence. A linear plot of the hydrophobic and hydrophilic

regions the CCR4 protein is shown in Figure 12.

Protein Sequence Similarities

Analysis of the predicted amino acid sequence of CCR4 reveals

five leucine rich tandem repeats located in the middle of the protein

(residues 350 - 467, see Figure 11). This motif is charaterized by a

repeating unit with a general consensus sequence of

P-X-X-o-X-X-L-X-X-L-X-X-L-X-L-X-X-N-X-o-X-X-o (where X = any

residue and o = aliphatic amino acids, L, I, or V). A search of the

41
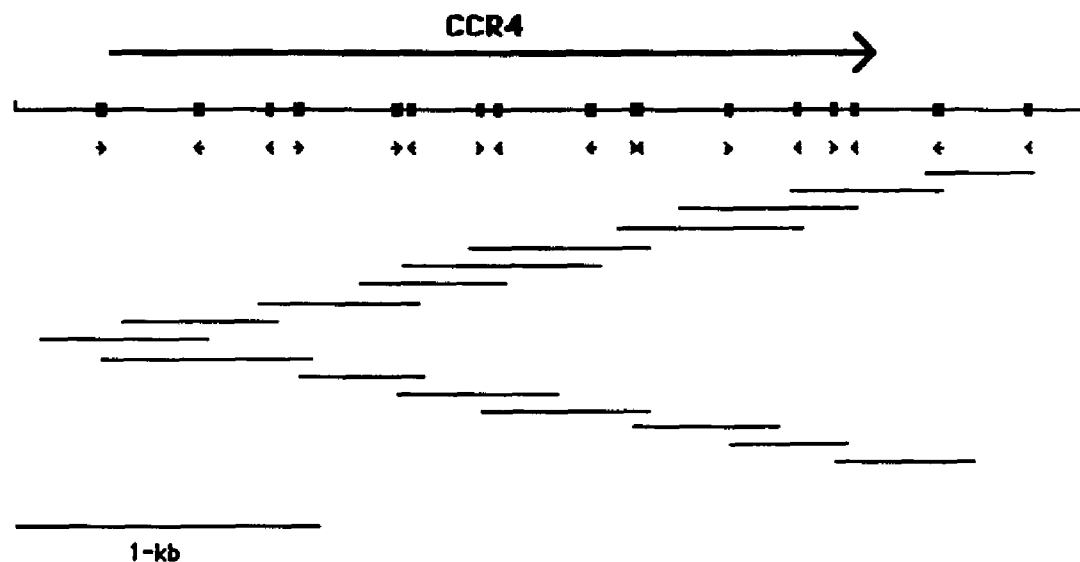
Figure 10

Figure 10 : Sequencing strategy for CCR4.

The position of synthetic primers used in sequencing CCR4 are shown above (■).The thin line (——) is representative of chromosome I and the heavy line indicates the position of CCR4 (——). The arrows indicate the orientation of the primers. Initial sequence data was generated from pUC18-Bm:Hd using the -20 and -24 (reverse) M13 sequencing primers as is described in the text. The extent of the sequence data obtained for each primer is shown.

Figure 11 : DNA and protein sequence of <u>CCR4</u>

    a. Sequence of the <u>CCR4</u> gene. The 2880-base pair sequence encompasing the 2511-base pair open reading frame is shown.

    b. Amino acid sequence of the CCR4 protein. The five leucine-rich repeat units (residues # 350-476) are underlined. Alignment of the repeats are shown in Figure 13. Also indicated are the consecutive glutamine and asparagine residues (#15-24 and #89-103, respectively) and the repeat sequence QQAQQAQQQAQQQAQQQ (#190-206).

Figure 11a

```
-153 TTTTGTTATCGCCCGTACTTGCGAAAAACGGGCCAACAACGAAAAAATTTGAAAAAGTTT
     CCACCTTAGAGAAGGTTCTCAAGCACAAGGGCACAGCATAAGGGACACCAGCAAGGGAAC
-33  TCCGACTGACGTTATCCCTGCAAACTACCGCTA
                                     ATGAACGACCCTTCTTTACTAGGCTAC
     CCTAATGTGGGCCCGCAGCAGCAACAGCAACAGCAGCAACAGCAGCATGCCGGGCTACTA
  88 GGAAAGGGTACACCAAACGCTCTACAGCAGCAGCTACACATGAACCAGCTCACCGGGATA
     CCTCCACCGGGACTCATGAACAACAGCGATGTACACACTTGCAGCAACAACAACTCGCGC
 208 CAGTTGCTCGACCAATTGGCTAATGGGAACGCGAACATGCTCAATATGAACATGGATAAC
     AACAATAACAACAATAACAACAACAACAACAACAACAACGGCGGTGGAAGCGGGGTG
 328 ATGATGAATGCCTCCACAGCCGCAGTAAATTCTATAGGGATGGTTCCTACCGTTGGTACG
     CCTGTGAACATCAATGTGAACGCCAGTAACCCTTTATTACACCCGCACTTGGATGACCCT
 448 TCTTTACTAAACAATCCGATCTGGAAGCTTCAATTGCATCTGGCTGCAGTGTCCGCACAA
     TCTCTGGGGCAACCCAACATTTATGCTAGACAAAACGCTATGAAGAAGTATTTGGCTACA
 568 CAACAGGCTCAGCAAGCTCAGCAACAAGCGCAGCAACAGGCACAGCAGCAGGTCCCGGGC
     CCATTTGGCCCCGGACCTCAGGCTGCACCACCAGCTTTGCAGCCCACCGATTTCCAGCAA
 688 TCTCACATTGCAGAAGCCTCCAAATCACTGGTAGACTGCACAAAGCAAGCCTTGATGGAA
     ATGGCCGACACTCTCACCGACAGCAAGACAGCAAAGAAACAACAACCTACGGGAGATAGC
 808 ACTCCCTCAGGCACGGCAACTAACAGTGCAGTTTCTACACCATTGACTCCCAAGATAGAG
     CTGTTTGCTAATGGCAAGGACGAAGCCAACCAGGCGCTCTTACAACACAAGAAACTGTCT
 928 CAGTACAGCATCGACGAAGATGACGACATTGAAAACAGAATGGTCATGCCCAAGGACTCG
     AAATACGACGACCAATTATGGCACGCGCTAGATTTGTCCAACTTGCAAATCTTCAATATC
1048 AGCGCCAACATCTTCAAGTACGATTTTCTAACGAGACTATATTTGAATGGCAATAGCCTC
     ACGGAACTGCCAGCGGGAGATCAAGAACCTAAGCAACCTACGCGTTTTGGACCTGTCGCAT
1168 AATAGGTTAACATCTCTACCAGCGGAACTAGGCTCATGTTTCCAATTGAAATACTTCTAC
     TTTTTTGATAACATGGTCACCACATTACCATGGGAGTTTGGGAACCTGTGTAACCTTCAG
1288 TTTCTTGGTGTAGAAGGAAACCCCTTAGAAAAACAGTTTTTAAAGATCCTTACAGAAAAA
     TCTGTCACGGGATTGATTTTCTACCTAAGGGACAACAGACCAGAGATTCCCTTACCGCAT
1408 GAACGTAGGTTCATCGAAATCAATACCGATGGGGAACCACAGAGGGAGTACGATTCTTTG
     CAGCAATCCACTGAGCATCTGGCCACCGATTTAGCCAAGAGGACGTTCACCGTCTTATCC
1528 TACAACACCTTATGTCAACACTATGCCACCCCAAAAATGTACCGTTACACACCGTCGTGG
     GCGTTAAGTTGGGATTACAGGCGCAATAAATTAAAGGAGCAGATTCTCTCGTACGACAGT
1648 GATCTGTTGTGTTTACAAGAAGTGGAGTCTAAGACTTTTGAAGAGTATTGGGTGCCCCTA
     TTGGACAAGCACGGTTATACAGGCATCTTCCATGCAAAGGCAAGAGCCAAGACCATGCAT
1768 TCCAAGGACTCCAAGAAAGTGGACGGGTGTTGCATTTTTTTCAAAAGGGACCAATTCAAG
     TTGATCACCAAAGACGCCATGGATTTCAGCGGTGCTTGGATGAAACACAAGAAGTTCCAA
1888 AGAACTGAAGATTATTTAAACCGTGCAATGAACAAAGACAACGTTGCACTGTTCTTAAAG
     CTACAACACATTCCTAGTGGCGACACCATATGGGCGGTCACCACGCATTTGCACTGGGAT
2008 CCAAAATTTAATGATGTCAAGACATTCCAAGTAGGTGTCCTGTTAGATCATCTGGAAACG
     CTGCTAAAGGAGGAGACATCGCACAATTTTAGACAGGACATTAAGAAATTTCCTGTGCTC
2128 ATTTGTGGTGACTTCAATTCATACATCAACTCCGCCGTATACGAATTGATAAATACAGGC
     CGTGTCCAAATACATCAAGAGGGAAATGGTAGAGATTTCGGTTACATGTCGGAGAAAAAT
2248 TTCTCACATAACTTGGCTCTCAAATCAAGTTATAATTGTATCGGAGAACTACCATTCACC
     AATTTCACACCGTCATTCACAGATGTTATCGACTATATATGGTTTTCTACACATGCTCTA
2368 AGGGTGCGTGGGCTATTGGGTGAAGTGGACCCTGAATACGTGAGTAAGTTTATCGGGTTC
     CCCAACGACAAATTCCCCAGTGACCATATACCATTGTTAGCAAGATTTGAATTTATGAAG
2488 ACAAACACAGGCAGTAAGAAAGTA
                             TAAACCGCACACTTTCATCCCACTCCCTCCCTCCTC
     TCTGTACACTACCTCATTATAAAATTGAAACGATCCCAATCTCACCGCACTTCCCATAAA
     GCCTTCACGTTATACTTTTTTTTCTCATCTTTTTTTTCTCTCTCTCTCTCTCTCTCTTTT
     GCGTATCTTTTTCGTCCTGTTTCTTTCTTACTTAAGTATCTTTTTATAATATTTAAGAGA
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Figure 11b

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MNDPSLLGYPNVGPQQQQQQQQQQHAGLLGKGTPNALQQQLHMNQLTGIP 50
PPGLMNNSDVHTSSNNNSRQLLDQLANGNANMLNMNMDNNNNNNNNNNNNN 100
NNNGGGSGVMMNASTAAVNSIGMVPTVGTPVNINVNASNPLLHPHLDDPS 150
LLNNPIWKLQLHLAAVSAQSLGQPNIYARQNAMKKYLATQQAQQAQQQAQ 200
QQAQQQVPGPFGPGPQAAPPALQPTDFQQSHIAEASKSLVDCTKQALMEM 250
ADTLTDSKTAKKQQPTGDSTPSGTATNSAVSTPLTPKIELFANGKDEANQ 300
ALLQHKKLSQYSIDEDDDIENRMVMPKDSKYDDQLWHALDLSNLQIFNIS 350
ANIFKYDFLTRLYLNGNSLTELPAEIKNLSNLRVLDLSHNRLTSLPAELG 400
SCFQLKYFYFFDNMVTTLPWEFGNLCNLQFLGVEGNPLEKQFLKILTEKS 450
VTGLIFYLRDNRPEIPLPHERRFIEINTDGEPQREYDSLQQSTEHLATDL 500
AKRTFTVLSYNTLCQHYATPKMYRYTPSWALSWDYRRNKLKEQILSYDSD 550
LLCLQEVESKTFEEYWVPLLDKHGYTGIFHAKARAKTMHSKDSKKVDGCC 600
IFFKRDQFKLITKDAMDFSGAWMKHKKFQRTEDYLNRAMNKDNVALFLKL 650
QHIPSGDTIWAVTTHLHWDPKFNDVKTFQVGVLLDHLETLLKEETSHNFR 700
QDIKKFPVLICGDFNSYINSAVYELINTGRVQIHQEGNGRDFGYMSEKNF 750
SHNLALKSSYNCIGELPFTNFTPSFTDVIDYIWFSTHALRVRGLLGEVDP 800
EYVSKFIGFPNDKFPSDHIPLLARFEFMKTNTGSKKV
```

Figure 12
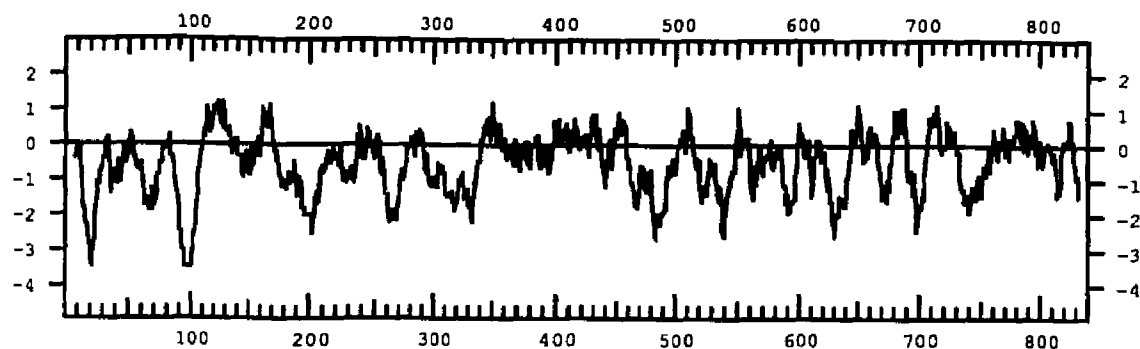
Figure 12 : Hydrophobic and hydrophilic regions of the CCR4 protein.

    A linear plot of the hydrophilic and hydrophobic regions of the CCR4 protein generated by the algorithm of Kyte and Doolittle (1982) in the DNA Stryder sequence analysis program. Residues 15 to 24 and 89 to 103 represent consecutive glutamine and asparagine residues, respectively. Residues 190 to 206 represent the repeat Q Q A Q Q A Q Q Q A Q Q Q A Q Q Q. The leucine rich tandem repeats are represented by residues 350 to 467. Negative values indicate an increasing hydrophilic nature.

National Biomedical Research Foundation database showed that CCR4

shares similarities in this region with a family of proteins

displaying a similar leucine rich repeat motif. These proteins are

found in a wide-range of species and include the yeast adenylate

cyclase (Kataoka et al., 1985), both the human placental (Lee et al.,

1988) and porcine liver (Hofsteenge et al., 1988) RNase inhibitor,

the human leucine rich α 2 glycoprotein (Takahashi et al., 1985), the

human 83-kDal subunit of carboxypeptidase N (Tan et al., 1990), the

human platelet glycoprotein lb o subunit (Lopez et al., 1987),

proteoglycan I and II from both human (Fisher et al., 1989) and

bovine (Day et al., 1987, Neame et al., 1989), the lutropin-

choriogonadotropin receptor from rat (McFarland et al., 1989), and

the Toll (Hashimoto et al., 1988) and chaoptin (Reinke et al., 1988,

Krantz and Zipursky, 1990) proteins from Drosophila. These proteins

are found to be located either extracelluar, intracellular, or

membrane bound (Kataoka et al.,1985, Lee et al.,1988, Hofsteenge et

al.,1988, Takahashi et al.,1985, Tan et al.,1990, Lopez et al.,1987,

Fisher et al.,1989, Neame et al.,1989, McFarland et al.,1989,

Hashimoto et al.,1988, and Reinke et al.,1988).

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The proteins share a common functional relationship in

mediating interactions at either a protein : protein, a protein : cell,

or a cell : cell level. Examples of which are the human placental

ribonuclease inhibitor which binds RNase at a 1 : 1 stoichiometry

(Lee et al., 1988) and the human platelet membrane glycoprotein Ib

which is involved in the interaction between the platelet and von

Willebrand factor (Titani et al., 1987). Krantz and Zipursky (1990)

have shown that chaoptin, a Drosophila photoreceptor cell specific

protein is required for proper development of and adhesion between

photoreceptor cells. The similarity shared between CCR4 and a

family of proteins thought to participate in the mediation of

interactions between various moieties suggests that CCR4 may act

in a similar capacity. CRE1, CRE2, or components of the

transcriptional machinery are possible candidates that bind CCR4

(see below). Figure 13 compares the leucine rich tandem repeats

found in CCR4 to those previously described.

The N-terminal quarter of the CCR4 protein contains several

unusual blocks of residues. This region contains 10 consecutive

glutamine residues (#15 - 24), 15 consecutive asparagine residues

Figure 13 : Homology shared between CCR4 and a family of
proteins displaying a leucine rich tandem repeat array.

a. Alignment of the 5 copies of the leucine rich repeats
found in the CCR4 protein (residues 350 - 467). Conserved
residues are indicated by being enclosed in boxes. The general
consensus sequence is shown below (x = any amino acid, * =
aliphatic amino acids leucine, isoleucine, or valine).

b. The general consensus sequence for CCR4 is compared to
those of other proteins displaying the leucine rich repeating
motif. Aliphatic amino acids leucine, isoleucine, or valine are
indicated by (*); x = any residue. The consensus sequences are
from: adenylate cyclase (Kataoka et al., 1985), Toll (Hashimoto et
al., 1988), chaoptin (Reinke et al., 1988), RNase inhibitor (human :
Lee et al., 1988; porcine : Hofsteenge et al., 1988), platelet
glycoprotein Ib o (GPIb o : Lopez et al., 1987), and the leucine
rich o $_2$ glycoprotein (GP : Takahashi et al., 1985).

Figure 13

a.

Alignment of the leucine rich repeats found in CCR4

```
S A N I F K Y D F L T R L Y L N G N S L T E L

P A E I K N L S N L R V L D L S H N R L T S L

P A E L G S C F Q L K Y F Y F F D N M V T T L

P W E F G N L C N L Q F L G V E G N P L E K Q F L K

I L T E K S V T G L - I F Y L R D N R P E I P L
```

General Consensus

```
P x x L* x x L* x x L x x L x L x x N x L* x x L*
```

* Aliphatic amino acid (leucine, isoleucine, or valine)

b.

Protein (Organism)	No. of repeats	Consensus of repeated sequence
CCR4 (Yeast)	5	P x x L*- x x L* x x L x x L x L x x N x L* x x L*
Adenylate cyclase (Yeast)	24	P x x L - x x L x x L x x L x L x x N x L x x L*
Toll (Drosophila)	15	P x x L F x H x x N L x x L x L x x N x L x x L
Chaoptin (Drosophila)	41	P x x x F x x L x x L x x L D L x x N x L x x I
RNase inhibitor (Human)	8	x x x L x x P x C x L E x L x L x x C x L T x x x C x x L
(Porcine)	7	x x x*L x x x x x L x E - L x L x x N x L G D x G*x x L
Platelet GPIbO (Human)	7	P x G L L x x L P x L x x L x L S x N x L T T L
L-rich O$_2$ GP (Human)	8	P x x L L x x x x x L x x L x L x x N x L x x L

* Aliphatic amino acids (leucine, isoleucine, or valine)

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(#89 - 103), and the repeat sequence: Q Q A Q Q A Q Q Q A Q Q Q A Q Q

Q (residues 190 - 206). Interestingly, glutamine and asparagine

residues account for 35% of the first quarter of the protein. This

region shows no significant similarity to current sequences

contained in the National Biomedical Research Foundation database.

However, several proteins from other sources have recently been

identified that display high glutamine content. Among these are the

amino-terminal region of the TATA binding factor TFIID from both

human (Kao et al., 1990) and Drosophila (Hoey et al., 1990), the

mammalian transcriptional activator SP1 (Kadonaga et al., 1987),

and the yeast transcriptional regulatory protein SPT13 (GAL11)

(Fassler and Winston, 1989).

Transcript Identification and Carbon Source Regulation

Northern analysis on total RNA harvested from strain

FW712-1d (CCR4 CRE1 CRE2) was performed using an internal 738 bp

HaeIII fragment of CCR4 isolated from pUC18-Bm:Hd. A single

transcript showing homology to the probe identified CCR4 as shown

in Figure 14, lane a. The size of the CCR4 transcript was estimated

from its mobility relative to the 25S and 18S ribosomal RNA bands

visualized by ethidium bromide in a duplicate gel (see Materials and Methods). The estimated transcript size is 2.8-kb and is consistent with an open reading frame of 2511-bp as predicted by the sequence data.

The regulation of CCR4 mRNA levels by carbon source availability was tested. Total RNA was harvested from strain FW712-1d grown in both glucose- and ethanol-containing medium and was subjected to Northern analysis as described above. Lanes b and c in Figure 14 represent equal amounts of total RNA loaded as described in Materials and Methods. Densitometric analysis of the signal from the CCR4 transcripts demonstrated that they are present in equal amounts under both fermentative and nonfermentative growth conditions. This data provided evidence that the abundance of the CCR4 transcript is not dependent on carbon source.

Effects of the CRE Alleles on CCR4 Transcription

In order to clarify how the CRE and CCR4 genes interact, the effect of a cre1 or cre2 on CCR4 mRNA levels was tested. Northern analysis on total RNA from isogenic strains 500-16 (CRE1 CRE2 CCR4), 500-16-1 (cre1 CRE2 CCR4), and 500-16-14 (CRE1 cre2

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Figure 14 : The effect of carbon source on CCR4 mRNA levels.

     CCR4 mRNA levels were measured in strain FW712-1d (CCR4 CRE1 CRE2) following growth on either glucose or ethanol containing media. CCR4 mRNA was identified as a 2.8-kb transcript under glucose growth conditions (lane a). Northern analysis of CCR4 mRNA was performed on approximately three-micrograms of total RNA from cells grown on glucose containing medium (lane b) and ethanol containing medium (lane c).

     A dulpicate gel stained with ethidium bromide for lanes b and c is displayed in the bottom panel and shows the level of the 18s rRNA. The levels of 18s rRNA and CCR4 mRNA were found to be equivalent following densitometric analysis.

Figure 14
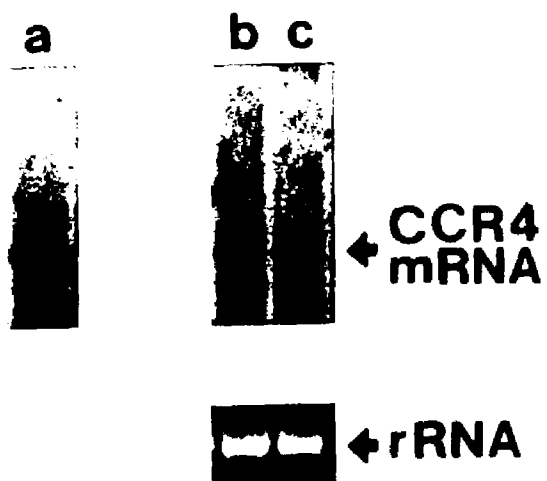
CCR4) was performed. The level of CCR4 transcript was readily

detectable in all three strains (see Figure 15). Densitometric

analysis showed an approximately 1.5 fold increase in CCR4

transcript levels in the presence of the cre alleles. Defects in the

CRE alleles have been shown to be pleiotropic in their effect on

transcription and other cellular processes (Denis, 1984, Denis and

Malvar, 1990). Therefore, the modest increase seen in CCR4

transcript level may reflect a more general increase in

transcriptional activity within the cell.

Functional Analysis of CCR4

To identify regions in the CCR4 protein of functional

importance, a series of disruptions and deletions were constructed

within the coding region of CCR4 (Figure 16). Plasmid pC1:HIS3

contains the yeast HIS3 gene inserted at bp 2004 that would result

in a truncated CCR4 protein of 668 amino acids (CCR4:1-668; see

Figure 4). The plasmid pUC18-Bm:Hd:HIS3 contains the yeast HIS3

gene placed at bp 1175 that would result in a truncated CCR4 protein

of 392 amio acids (CCR4:1-392; see Figure 5). The plasmid

YRp7-3.5:delta MluI-HincII contains an inframe deletion of the region

Figure 15 : The effect of the CRE alleles on CCR4 mRNA levels.

Northern analysis on CCR4 mRNA was performed on equal amounts of total RNA harvested from strains 500-16 (CRE1 CRE2 CCR4), 500-16-1 (cre1 CRE2 CCR4), and 500-16-14 (CRE1 cre2 CCR4) following growth on glucose containing medium. Densitometric analysis showed the level of CCR4 mRNA did not vary significantly regardless of the allelic state of the CRE genes.

Figure 15
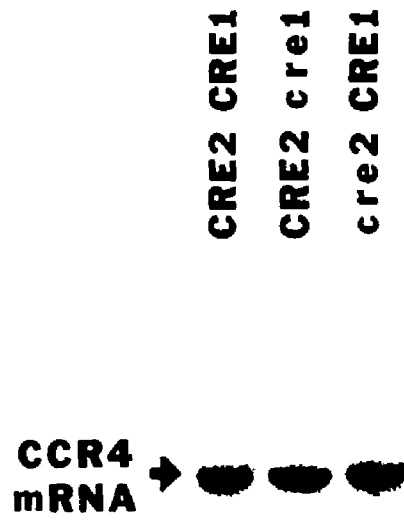
Figure 16
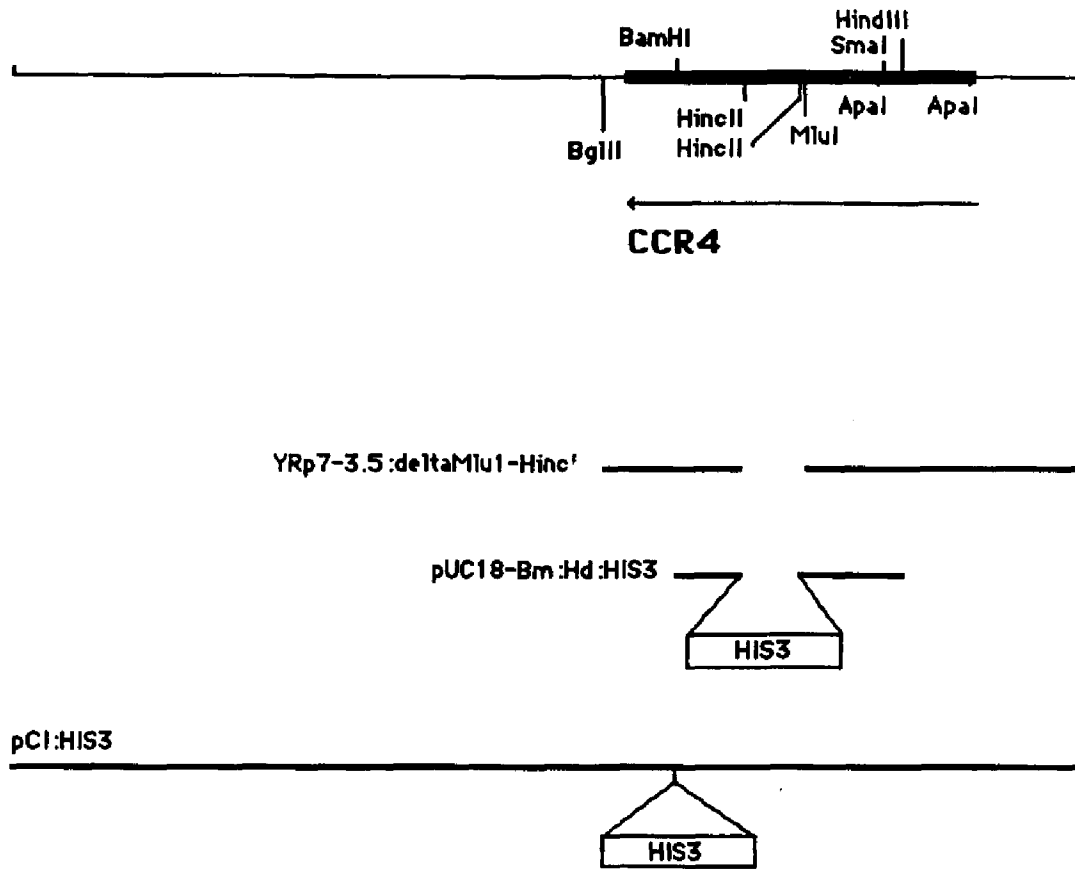
Figure16: Disruptions and deletions created in CCR4.

The thin line (—) represents sequences from the left arm of chromosome I. The position and orientation (arrow) of CCR4 is also shown. Sequences contained on plasmids used to create deletions and disruptions in CCR4 are shown below. Their construction is described in the text and Figures 5 (pCI:HIS3), 6 (pUC18-Bm:Hd:HIS3), and 7 (YRp7-3.5:deltaMlul-HincII).

from bp 1146 - 1543 in the CCR4 gene. This would result in a CCR4

protein of 704 amino acids in length (CCR4:1-382/514-837; see

Figure 6).

The effects of the constructions described above were tested

in strains 82-2b (cre1 CCR4), and 612-1d (CRE1 CCR4). Strain 82-2b

allows for the assessment of the ability of the disruptions in CCR4

to suppress cre1-induced effects. Strain 612-1d was used to

determine if the disruptions created in CCR4 would result in

increased ADH II activity under glucose repressed growth conditions.

Replacement of the endogenous CCR4 gene in strain 82-2b with

CCR4:1-668 (using plasmid pCI:HIS3) resulted in a temperature

sensitive phenotype following growth on nonfermentative medium

and sensitivity to antimycin A (Strain 82-2b-A; see Table 4 a).

Strain 82-2b-A did display, however, a slight resistance to

antimycin A after prolonged growth. This is consistent with the

slight decrease in ADH II activity measured under glucose growth

conditions that the CCR4:1-668 allele caused (see Table 4 b).

Strain 82-2b-1 contains the CCR4:1-392 allele placed at the

chromosomal locus of CCR4 using plasmid pUC18-Bm:Hd:HIS3. The

Table 4

    a. Deletions and disruption constructed in CCR4 were used
to transform yeast strains as described in the text. Plasmid
pCI:HIS3 was used to construct the carboxy-terminal disruption
of CCR4 (CCR4:1-668; strain 82-2b-A). The plasmid
pUC18-Bm:Hind:HIS3 was used to construct a disruption of the
leucine rich repeats in CCR4 (CCR4:1-392; strain 82-2b-1). The
inframe deletion generated by the construction of
YRp7-3.5:deltaMluI- HincII (CCR4:1-382/514-837) was
integrated at the TRP1 locus of 82-2b-1 to give strain
82-2b-1-1a. Strain 82-2b-1-a has a wild type copy of CCR4
contained on the plasmid YRp7-3.5+ integrated at the TRP1 locus
of 82-2b.

    Strain 612-1d was disrupted at the CCR4 locus using the
pCI:HIS3 construction (CCR4:1-668) to give strain 612-1d-2.
Strain 85-1c is a segregant of the cross 82-2b-1 X 612-1d and
carries the CCR4 disruption created using the pUC18-Bm:Hd:HIS3
(CCR4:1-392) in a wild type CRE background.

    b. ADH II activity values for strains harboring the
disruptions of CCR4 described above. Activity values were
measured following growth on glucose or ethanol containing
media as indicated (see Materials and Methods).

Table 4 : Effects of disruptions and deletions in CCR4 on strains 82-2b, 612-1d, and 85-1c.

A.

Strain	HIS3	TRP1	AT	Growth YD 30	YD 37	YET 30	YET 37	GLY 30	GLY 37
82-2b	−	−	+	+	+	+	+	+	+
82-2b-A	+	−	−	+	+	+	−	+	−
82-2b-1	+	−	−	+	+	+	−	+	−
82-2b-1-a	+	+	+	+	+	+	+	+	+
82-2b-1-1a	+	+	−	+	+	+	−	+	−
612-1d	−	−	−	+	+	+	−	+	−
612-1d-2	+	−	−	+	+	+	−	+	−
85-1c	+	−	−	+	+	+	−	+	−

B.

Strain	ADH II activity (mU/mg) Glucose	Ethanol
82-2b	105	n.d.
82-2b-A	66	n.d.
82-2b-1	32	n.d.
82-2b-1-a	235	n.d.
82-2b-1-1a	22	n.d.
612-1d	n.d.	4000
612-1d-2	n.d.	750
85-1c	n.d.	800

n.d : not determined

resultant CCR4:1-392 allele that lacks all but two copies of the

leucine rich repeats and the CCR4:1-668 allele displayed similar

phenotypes. Both disruptions in CCR4 suppressed the resistance to

antimycin A that is conferred by a cre1 allele. This result further

demonstrated the epistatic relationships previously described

between the CCR4 and CRE alleles (Denis, 1984). Strain 82-2b-1

(CCR4:1-392) shows no resistance to antimycin A, however, even

after prolonged growth. This observation is consistent with the low

ADH II activity on glucose growth conditions caused by the

CCR4:1-392 allele (see Table 4 b).

The plasmid YRp7-3.5+ was integrated into strain 82-2b-1 in

order to determine if a wild type copy of CCR4 could rescue the

phenotype caused by the CCR4:1-392 allele. Plasmid YRp7-3.5+

failed to complement a ccr4 allele in plasmid form even though

sequence data for CCR4 indicated that it contained the complete

coding region of CCR4 (discussed below). Strain 82-2b-1-a which

represents a wild type copy of CCR4 integrated at the TRP1 locus,

restored the phenoype observed in 82-2b (see Table 4 a).

Additionally, ADH II activity in strain 82-2b-1-a was increased two

fold under glucose growth conditions compared to strain 82-2b. This observation may reflect differences in the sequences flanking the CCR4 allele in terms of its overall level of expression (see below).

The construction pCl:HIS3 (CCR4:1-668) was used to create a disruption of CCR4 in strain 612-1d in order to determine its effect in a wild type CRE background. Table 4a shows that the replacement of the chromosomal CCR4 gene with the CCR4:1-668 allele (strain 612-1d-2) resulted in a temperature sensitive phenotype following growth on nonfermentative medium and a 5-fold reduction in ADH II activity under ethanol growth conditions. Attempts to create a disruption in 612-1d using the pUC18-Bm:Hd:HIS3 construction were unsuccessful. As an alternative, strain 82-2b-1 which harbors the disruption created with pUC18-Bm:Hd:HIS3 (CCR4:1-392) was crossed to 612-1d and an appropriate segregant carrying a wild type CRE1 allele and the disrupted CCR4 allele was identified (strain 85-1c). Analysis of 85-1c showed that it, like 612-1d-2, displayed a temperature sensitive phenotype following growth on nonfermentative medium (see Table 4a). Furthermore, derepressed ADH II activity values for 85-1c and 612-1d-2 were approximately

equal (see Table 4b). Analysis of the strains harboring the

disruptions described above show that they are phenotypically

similar to the mutations isolated in CCR4 as described by Denis

(1984) and Denis and Malvar (1990).

The inframe deletion of CCR4 (CCR4:1-382/514-837) was

integrated at the TRP1 locus in strain 82-2b-1 using the plasmid

YRp7-3.5:delta MluI-HincII to give strain 82-2b-1-1a. The

CCR4:1-382/514-837 allele that contains only one full copy of the

leucine rich repeats but the sequences to the C-terminal side of the

leucine rich repeats, gave a phenotype identical to those seen in the

disruptions of CCR4 described above, ie. temperature sensitivity

following growth on nonfermentative medium and anyimycin A

sensitivity. ADH II activity for strains 82-2b-1-1a and 82-2b-1

were nearly identical and represented an approximately 4-5-fold

decrease in activity when compared to strain 82-2b.

IV. DISCUSSION

Genetic studies by Denis (1984) identified components of a

second pathway involved in the transcriptional regulation of the

yeast glucose-repressible ADH II. The cre1 and cre2 alleles were

identified as mutations allowing for the expression of ADH2 under

glucose repression. Mutations which bypass cre-induced ADH2

expression under fermentative growth fall into a single

complementation group designated ccr4. Allelism has been shown

between CRE2, SPT6, and SSN20; mutations in which cause increases

in ADH2, SUC2, and his4-912delta transcription (Denis and Malvar,

1990; Neigeborn et al., 1987; Clark-Adams and Winston, 1987).

Additionally, Clark-Adams and Winston (1987) and Neigeborn et al.

(1987) have shown that SPT6 and SSN20 are essential yeast genes.

Strains carrying a cre2 allele were shown to display a temperature

sensitive phenotype which also implies that it is an essential gene

(Denis and Malvar, 1990). CRE1 is allelic to SPT10, another member

of the SPT gene family (Fassler and Winston, 1988). Like CRE2,

mutations in CRE1 allow for increased ADH2 and his4-912delta

transcription (Denis and Malvar, 1990). The epistatic relationship

described between the CRE alleles and CCR4 (Denis, 1984) is evident

by the observations that mutations in CCR4 suppress cre-induced

effects at both the ADH2 and his4-912delta loci (Denis and

Malvar,1990).

This study characterizes CCR4 and addresses the role it may

play in the regulation of ADH2 transcription. CCR4 resides on the

left arm of chromosome I (Figure 4). The genetic distance between

CCR4 and CEN1 provided by the segregation ratios from the cross

between 317-5b and 292-6a was calculated to be 10 cM. The

physical distance between CCR4 and CEN1 is approximately 37-kb.

The reported ratio of physical to genetic distance on chromosome I

is 2.6 kb/cM (Mortimer and Schild 1985) and differs from that

calculated by the mapping data for CCR4 (3.7 kb/cM). This

discrepancy may be due to a region of hypo-recombination that lies

between CEN1 and SPO7 (D. Kaback, personal communication). The

plasmid pCI (Figure 9) was shown to contain a functional copy of

CCR4 by its ability to complement the temperature sensitive

phenotype and low nonfermentative ADH II activity in strain

348-26c which harbors a ccr4-10 allele (Table 3).

The protein coded for by the 2511-bp open reading frame of CCR4 has an estimated mass of 94.6-kDal. Analysis of the DNA sequence for CCR4 shows no TACTAAC consensus sequence for intron splicing. The predicted mRNA and protein sizes agree with the Northern analysis of CCR4 mRNA which shows a single transcript with an estimated size of 2.8-kb (Figure 14, lane a) and a 92-kDal protein specifically precipitated by antibodies raised against a synthetic polypeptide to the predicted carboxy-terminal domain of CCR4 (R. Vallari, personal communication). Analysis of the CCR4 protein shows that it contains 5 tandem copies of a leucine rich repeat (residues 350 - 467). These repeats reside in the middle of the protein and are bordered by two relatively hydrophilic domains (Figure 12).

The sequence data indicate that the coding region for CCR4 is contained within the 3.5-kb BglII fragment that is present in YRp7-3.5. This is further supported by the observation that YRp7-3.5+ integrated at the TRP1 locus of strain 82-2b can complement a ccr4 allele (see strain 82-2b-1-a; Tables 4a and 4b).

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These results are in contrast to the observation that neither of the

intact YRp7-3.5 plasmids are capable of complementing a CCR4

mutation. The explanation for this discrepancy may be the fact that

increased levels of CCR4 on plasmid borne copies is detrimental to

cell growth. The YRp7 based vectors exist at a greater copy number

(50-100) within the cell than YEp13 based vectors (20-30) from

which pC1 is derived. Additionally, slow growth has been noticed in

strains harboring plasmid borne wild type copies of CCR4, including

those strains harboring pC1, when compared to strains containing

only the endogenous CCR4 allele.

The level of CCR4 transcription is not significantly altered by

either carbon source (see Figure 13, lanes b and c) or a cre1 or cre2

allele (see Figure 15). Immunoprecipitation of the CCR4 protein

showed that levels remain unchanged in cre strains when compared

to a wild type CRE background (R. Vallari, personal communication).

Additionally, CCR4 protein levels were found to be similar following

growth on fermentative and nonfermentative medium (1.7 fold

increase in nonfermentative medium over that observed in

fermentative growth conditions, R. Vallari, personal

communication).

Since the levels of CCR4 mRNA and protein remain constant

regardless of the available carbon source or state of the CRE alleles,

the interaction between CCR4 and the CRE alleles (Denis, 1984;

Denis and Malvar, 1990; this study) appears to occur directly or

indirectly at a protein level. Similarities between CCR4 and proteins

displaying the leucine rich tandem repeats support this idea. Several

findings demonstrate the role of leucine rich repeats in mediating

protein interactions. Deletions of single or multiple copies of the

repeats found in the human placental ribonuclease inhibitor disrupts

the inhibitor's ability to bind to and (or) inactivate RNase (Lee and

Vallee, 1990). Tan et al. (1990) suggest that the association of the

human carboxypeptidase N 83-kDal subunit with the 50-kDal

catalytic subunit occurs through the leucine rich repeats. The

repeats were not responsible for the interaction between the human

platelet glycoprotein Ib (GP Ib) and von Willebrand factor (Vicente et

al., 1990). However, they report that the ability of GP Ib to exist in

circulation with von Willebrand factor prior to interaction may be

due to changes in the GP Ib receptor. These changes may be in the

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form of an unidentified antagonistic substance(s). This is based on

their observation that association between GP Ib and von Willebrand

factor is seen after the platelets are exposed to high shear stress.

The binding of an antagonist to the repeats which lie adjacent to the

von Willebrand factor association site on GP Ib may prevent undue

interaction. Leucine rich repeats found in the yeast adenylate

cyclase are reported to play a role in membrane adhesion (Kataoka et

al., 1985). Their studues show that the protein sequence contains no

membrane spanning domain and that deletion of the repeats results

in cyclase activity being found in the soluble instead of the

membrane pellet of detergent free yeast cell extracts. It is not clear

if adenylate cyclase interacts directly with the membrane or is

associated with a membrane embedded protein. The identification of

the leucine rich repeat motif in a wide variety of species suggests

an important functional role for this domain in proteins involved in

association.

A series of disruption and deletions were constructed within

the coding sequences of CCR4 in order to identify regions of

importance (Table 4 a and 4b; Figure 16). Strains harboring any of

the disrupted alleles of CCR4 (82-2b-A (CCR4:1-668), 82-2b-1

(CCR4:1-392), 612-1d-2 (CCR4:1-668), and 85-1c (CCR4:1-392);

Table 4a) are phenotypically similar to previously described

mutations in CCR4; in showing temperature sensitivity following

growth on nonfermentative media (Denis, 1984; Denis and Malvar,

1990). This phenotype was also observed in strain 82-2b-1a which

contains the inframe deletion of the leucine rich repeats

(CCR4:1-382/514-837; Table 4a; Figure 8). Additionally, the effects

of CCR4 disruptions and deletions on ADH II activity are also seen

(Table 4b). Strain 82-2b displays the typical elevated repressed

activity seen in a cre1 background. Strain carrying mutations which

disrupt CCR4 (82-2b-A, 82-2b-1, and 82-2b-1-1a) act to lower the

repressed ADH II activities by varying amounts. The glucose activity

values for strains 82-2b-1 and 82-2b-1-1a are approximately 4-5

fold lower than that seen in strain 82-2b and is evident by their

sensitivity to antimycin A (see Table 4a and 4b). Strain 82-2b-A

has an ADH II activity that is 2-fold higher on glucose containing

media when compared to strains 82-2b-1 and 82-2b-1-1a (see Table

4b). As a result, strain 82-2b-A displays a slight resistance to

antimycin A after prolonged growth. This suggests that the protein

resulting from the CCR4:1-668 allele retains partial function. The

wild type 612-1d displays characteristic derepressed levels of ADH

II activation which are lowered in strains carrying the disruptions

in CCR4 (612-1d-2 and 85-1c). These results imply that CCR4 is not

an essential yeast gene. However, as the persistent temperature

sensitive phenotype and lowered ADH II activity (Table 2) indicate,

it plays a required role in derepression at the ADH2 locus.

Additionally, the temperature sensitive phenotype observed under

nonfermentative growth conditions of strain 82-2b-1-1a implies

that the leucine rich repeats are required for proper function (see

Table 4a and 4b; Figure 16). However, providing a functional protein

is made, a model where the leucine rich repeats are solely

responsible for interaction with the CRE proteins is unlikely since

strain 82-2b-1-1a is antimycin A sensitive. It should be noted that

the inframe deletion of the leucine rich repeats described in Figure

6 was confirmed by sequencing (data not shown).

ADH II activity under glucose growth conditions increases

approximately 2-fold when the YRp7-3.5+ vector is inserted at the

TRP1 locus of strain 82-2b-1 (see strain 82-2b-1-a; Table 4b). This

may reflect an overstimulation of transcription from the integrated

CCR4 allele due to adjacent pBR322 sequences contained on the YRp7

vector (Beier and Young, 1982). This is in contrast to the observation

that there is no increase in ADH II activity under glucose growth

conditions when CCR4 dosage is increased by transforming with the

plasmid pCI. Strain 612-1d transformed with plasmid pCI remains

sensitive to antimycin A and shows a repressed ADH II activity equal

to that seen for 612-1d without pCI (data not shown). Strain

348-26c-CI which harbors the pCI plasmid complements the

temperature sensitive phenotype on YET medium and increases ADH

II activity 7-fold on ethanol containing media when compared to

348-26c (See Table 3) yet remains sensitive to antimycin A (data

not shown). These results may be reconciled by considering the

relative level of overexpression of CCR4 in each case. Large

increases in CCR4 dosage, as would be expected in transformations

with pCI, appear to be detrimental to cell growth as noted above.

CCR4 transcription induced by adjacent pBR322 sequences when

YRp7-3.5 is integrated at the TRP1 locus may not achieve levels

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that would be detrimental to cell growth. An alternative explanation

may lie in the ability to detect a two-fold increase in ADH II

activity. A two-fold increase in the ADH II activity values under

glucose growth conditions in a cre1 background (ie. from 105 to 235

mU; see strains 82-2b-1 and 82-2b-1-a; Table 4b) is more readily

detectable than a similar increase from wild type levels of ADH II

activity (ie. 8 mU on glucose growth medium; see Table 2).

The amino-terminal domain of CCR4 contains several unusual

blocks of amino acids. This region shows similarities to the amino-

terminal domain of the TATA box binding factor TFIID from both

human and Drosophila (Peterson et al., 1990; Hoey et al., 1990; Kao

et al., 1990). As described above, CCR4 contains a block of 10

consecutive glutamine and 15 consecutive asparagine residues along

with the repeat Q Q A Q Q A Q Q Q A Q Q Q A Q Q Q; all residing within

the first 206 residues (see Figure 11). Human TFIID contains 38

consecutive glutamine residues while TFIID from Drosophila

displays two blocks of 6 and 8 glutamines each within their

respective amino termini (Peterson et al., 1990). Additionally, the

amino terminus of CCR4, like both human and Drosophila TFIID, is

rich in proline, methionine, and serine or threonine. In comparison,

the yeast TFIID (Eisenmann *et al.*, 1989; Horikoshi *et al.*, 1989;

Schmidt *et al.*, 1989) shares similarity with the DNA binding

carboxy-terminal domain of human and Drosophila TFIID but lacks

the amino-terminal domain altogether (Figure 17). The mammalian

transcriptional activator SP1 also contains a glutamine rich domain

that appears to play a role in its ability to activate transcription

(Mitchell and Tjian, 1989). However, SP1 does not contain the

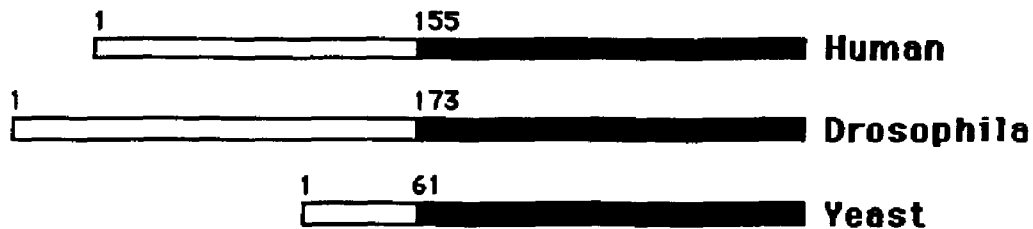extensive blocks of glutamine residues found in CCR4 and TFIID.

Figure 17 : Comparison of the TFIID sequences between species. The filled in segments represent the conserved DNA binding domains.

What role may CCR4 play in transcriptional regulation? Two

recent review articles (Ptashne and Gann, 1990; Lewin, 1990)

summarize the action of transcriptional activators and their

relationship to TFIID. Ptashne and Gann (1990) propose two classes

of transcriptional activators. First, `universal' activators consist of

proteins containing two distinct domains; one enables binding to

DNA and the other, characterized by an `acidic blob', serves to

activate transcription. The second class represents activators that

contain only one of the required domains and therefore require a

second protein to provide the missing domain. Studies indicate that

TFIID is the target seen directly or indirectly by both classes of

transcriptional activators (Sringer et al., 1990; Kelleher et al.,

1990; Berger et al., 1990; Pugh and Tjian, 1990; Peterson et al.,

1990; review: Ptashne and Gann, 1990; Lewin, 1990). The ability of

activators to contact TFIID is apparently dependent on the amino

terminus of TFIID and the presence of additional factor(s) or

`co-activators' (Kelleher et al., 1990; Berger et al., 1990; Stringer

et al., 1990; Pugh and Tjian, 1990; Peterson et al., 1990). Although

co-activators have yet to be isolated, their requirement has been

demonstrated. First, cloned mammalian TFIID which supports basal

level transcription, is incapable of supporting activation in response

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to SP1 or a GAL4-VP16 fusion protein in cell extracts depleted of

the TFIID fraction (review Ptashne and Gann, 1990). This is thought

to be due to the lack of a component(s) in preparations of cloned

TFIID that are present in the heterogeneous TFIID fraction. Second,

two members of the `dependent' class of transcriptional activators,

SP1 and E1a (an adenovirus activator bearing no acidic domain) are

capable of activation in mammalian cells but not in yeast (Ptashne

and Gann, 1990). This may reflect differences seen in the amino-

terminus of TFIID. This is supported by the finding that an excess of

either yeast TFIID or a truncated Drosophila TFIID lacking the

N-terminus are incapable of supporting activation in response to

SP1 or a GAL4-VP16 heterologous protein; even though basal level

transcription remains unaltered. However, excess Drosophila TFIID

has no effect on the ability of SP1 or GAL4-VP16 to promote basal

level or activated transcription (Ptashne and Gann, 1990; Pugh and

Tjian, 1990). Presumably, the lack of N-terminal sequences in yeast

TFIID and the truncated Drosophila TFIID render them incapable of

responding to the transcriptional activator and any co-activator(s)

(Ptashne and Gann, 1990; Lewin, 1990).

Analysis of the structural and functional domains in yeast

TFIID (Horikoshi et al.1990) show that unlike TFIID from human and

Drosophila, there is no distinction between the regions responsible

for DNA binding and basal level transcription. If, as proposed in

mammalian systems, the N-terminal domain of TFIID is the target

seen by transcriptional activators, then interactions between yeast

TFIID and transcriptional activators may occur two ways. First,

interactions may occur within the 60 amino-terminal residues as

proposed by Horikoshi et al. (1990). Secondly interactions are

mediated by co-activators which are capable in part of

compensating for the amino terminus of mammalian TFIID; which is

absent in yeast.

The role of CCR4 in the transcriptional regulation of ADH2 may

be in a capacity similar to that of a co-activator. This is supported

by the finding that CCR4 acts at or near the TATA box of ADH2 (Denis

and Malvar, 1990). When regions containing all known responsive

elements which lie upstream of the ADH2 TATA sequence are deleted

or displaced by insertions, ADH II activity remains sensitive to

mutations in CCR4 and the CRE alleles. Additionally, of potential

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interest is the similarities seen between the arrangement of the

amino termini of TFIID from human and Drosophila and that of CCR4.

The hydrophilic domains of CCR4 (see Figure 12) would presumably

be exposed and therefore have the capacity for interactions. The

action of CCR4 at the promoter to elicit full derepression of ADH2

may be to provide proper interaction between the DNA binding

transcriptional activator ADR1 (Eisen et al., 1988) and the

transcriptional machinery. Note that as the temperature sensitive

phenotype displayed by CCR4 disruptions indicate, it appears only to

be required for derepression. This may be explained in that while

contacts made by CCR4 are not required for low derepressed activity

which is characteristic of a ccr4 allele (Table 2), its presence at

elevated temperatures adds to the stability of the transcriptional

apparatus. This may explain why disruptions of and mutations

isolated in CCR4 display the same phenotype. Additional support for

the requirement of CCR4 in promoting full derepression is seen in

analysis of the constitutive mutation ADR1-5^c. Strains carrying

ADR1-5^c show elevated ADH II activity on fermentative and

nonfermentative media (Table 2). Mutations in CCR4 lower

derepressed ADH II activity in an ADR1-5^c background 20 fold (Denis, 1984) yet do not effect fermentative values.

The action imparted by CCR4 can be viewed as an auxiliary rather than an essential component of transcription. This is analogous to the argument made by Martin et al. (1990) on the role of adaptors in E1a transcriptional regulation. E1a, unlike acidic activators, is dependent on additional uncharacterized factor(s) or adaptors. Their argument is based on experiments using heterologous constructs bearing the GAL4 DNA binding region and various acidic activating domains. The ability of these constuctions to promote activation is sensitive to excess levels of VP16, a strong acidic activator, but not to excess E1a. In contrast, a LexA-E1a fusion protein was found to be sensitive to an excess of either VP16 or E1a. Martin et al. (1990) reason that the same target (presumably TFIID) is contacted in either case but that in the case of E1a an adaptor is required for proper contact. The adaptor, therefore, cannot be an essential component of the transcriptional machinery.

What role do the CRE alleles play in the epistasis seen between them and CCR4? As described above, the amount of CCR4 protein

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remains unchanged when cells are grown fermentatively; a condition

not requiring ADH2 activation. The presence of CCR4 protein is not

surprising since both the CRE alleles and CCR4 have been shown to

effect multiple loci (Denis, 1984; Denis and Malvar, 1990).

Additionally, Taylor and Young (1990) have shown that ADR1 is bound

to the UAS1 of ADH2 irrespective of carbon source. Therefore a

mechanism must exist which inhibits the action of CCR4 at the ADH2

locus under conditions such as fermentative growth. The CRE

proteins may act to inhibit the action of CCR4 by binding directly to

CCR4 or act indirectly by blocking contacts made by CCR4.

More recent findings on the action of SPT6 (CRE2) by Swanson

et al. (1990) suggest additional means by which CCR4 may regulate

transcription. The pleiotropic effects described for CCR4 and the

CRE alleles (Denis, 1984; Denis and Malvar, 1990) may be a result of

the general effect they have on chromatin structure. This view is

also supported by the proposed site of action of CCR4 and CRE

alleles noted above in which they may exert their effect on

chromatin structure near the TATA box. Swanson et al (1990) find

that SPT6 codes for a nuclear localized 170-kDal protein that is

extremely acidic in its amino terminus (residues 1 - 484 have a net

charge of -81). Proteins containing highly charged regions are

implicated in chromatin binding (Earnshaw, 1987). Additionally,

increased or decreased dosage of SPT6 suppress his4-912delta

mutations (Clark-Adams and Winston, 1987) and deletions in the

regulatory region of the SUC2 locus (Neigeborn et al., 1987. Similar

effects are seen at the his4-912delta locus in histone gene dosage

experiments (Clark-Adams et al., 1988). As noted above for strains

carrying multi-copy plasmids bearing the CCR4 gene, growth was

noticably slower. The effects of changes in the dosage of these

alleles suggests that any complexes formed between their products

is dependent on their stoichiometry. The possibility that CCR4 acts

through protein interactions implicated by the leucine rich repeats

is supported by the report that SPT6 contains sequences similar to

the leucine zipper motif shown to mediate protein : protein

interactions (Swanson et al. 1990).

The lethality caused by a null allele of SPT6 would suggest its

essential role in chromatin structure. This is in contrast to the

observation that all mutations and disruptions in CCR4 are

temperature sensitive only on nonfermentative media. If CCR4 is

involved in chromatin stucture then these observations would again

imply an auxiliary rather that an essential role as discussed above.

Transcription at the ADH2 locus under derepressed conditions

described by Denis (1984) is dependent on 1) the release from

glucose repression and 2) the state of the two regulatory circuits in

which ADR1 and CCR1 comprise one circuit and CCR4 and the CRE

alleles consitute the second. The manner in which the glucose signal

is transmitted within the cell is not known. The findings presented

in this study show no direct evidence of protein interactions

involving CCR4 and the transcriptional control of the ADH2 locus.

The ideas presented are intended to propose interactions between

the circuits based on predictions inferred from the mutational

studies and sequence data generated for CCR4. The two views

presented on the proposed action of CCR4 and the CRE alleles are not

necessarily mutually exclusive. While studies indicate that these

alleles exert their effects near the TATA box, they may act through

different sequences. The ability of CCR4 to function in an activating

capacity may be dependent on the chromatin structure as influenced

by the CRE proteins. Much work remains in order to acertain the role

that CCR4 plays in transcription. Answers to questions concerning

protein interactions mediated by CCR4 most likely will follow

purification of the protein. Additional mutational work on CCR4 is

needed to further characterize functional domains within the

protein. Likewise, mutational studies in the promoter region of ADH2

are needed to further define sequences required for the action of

CCR4 and the CRE alleles.

Appendix A

DNA fragment excision from agarose gels

1. DNA was visualized under UV light after staining with ethidium bromide (Maniatis et.al.)

2. The appropriate band was excised from the gel and placed into a microfuge tube containing 200 microliters of water.

3. The gel fragment was mashed in the tube using either a pestal or a thin pointed spatula.

4. An equal volume of phenol (equilibrated with TE pH 7.5) was added.

5. The tube was vortexed at high speed for 3 minutes and rapidly placed in a dry ice/ethanol bath for 2 minutes.

6. The phases were separated by a 20 minute high speed spin in a microfuge and the upper phase collected to a new tube.

7. 1/50 volume of a 5M NaCl solution and 2 volumes of cold ethanol was added and the DNA precipitated at -20 $^\circ$ C for 2 hours or longer.

8. The DNA was collected by centrifugation, drying under a vacuum, and resuspension in sterile water.

Appendix B

Double stranded template preparation and alkali denaturation

1. Grow a 2 ml culture of the bacterial clone overnight.

2. Pellet 1.5 ml of the bacteria in a microfuge for 1 minute and remove media by aspiration.

3. Resuspend pellet in 0.21 ml of STET (8% glucose, 0.5% Triton X-100, 50 mM EDTA, 10 mM Tris-Cl pH 8.0).

4. Add 15 microliters of fresh lysozyme (10 mg/ml in TE 8.0) and vortex.

5. Place tube in boiling water bath for 50 seconds and immediately centrifuge at RT for 6 minutes.

6. Transfer supernatant to a new tube and add 1/10 volume of 3 M NaOAc and 1 volume of isopropanol.

7. Place on ice for 10 minutes, centrifuge, wash with 70% ethanol, dry, and resuspend in 30 microliters of TE.

8. To denature the DNA prior to sequencing; 8 microliters of the DNA, 6 microliters of 1 N NaOH, and water to 24 microliters was added to a microfuge tube.

9. The tube was allowed to stand at RT for 5 minutes and then 3 microliters of cold ammonium acetate (2 M, pH 4.5) and 70 microliters of cold ethanol was added.

10. The DNA was precipitated as above, resuspended in 7 microliters of water and 2 microliters of Sequenase reaction buffer. 1 microliter of the appropriate primer was added and sequenced as described by the manufacturer.

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