Winter 1990

Structural and functional characterization of the yeast general transcriptional activator CCR4

Thomas M. Malvar
University of New Hampshire, Durham

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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

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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Oct 15, 1990
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 structural 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
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 ADH2 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
controlled by the negative effectors \textit{C}RE\textsubscript{1} and \textit{C}RE\textsubscript{2} (catabolite repressor element)

\textit{ADR1} is an essential gene for the derepression of \textit{ADH2} transcription (Denis et al. 1981). Strains harboring an \textit{adr1-1} allele, characterized by an amber mutation at the eleventh codon, show no \textit{ADH II} activity (Bemis and Denis, 1989). The \textit{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. \textit{ADR1} binds to the upstream activating sequences located in the \textit{ADH2} promoter under both fermentative and nonfermentative growth conditions (Taylor and Young, 1990). Gel retardation studies indicate that \textit{ADR1} binds predominantly to UAS1; however, optimum binding is observed in the presence of both UAS1 and UAS2 (Eisen et al., 1988). The \textit{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 \textit{ADR1} (\textit{ADR1}\textsuperscript{C}) which allow for \textit{ADH II} activity under glucose repression, have been identified as mutations which purportedly disturb the cAPK phosphorylation of \textit{ADR1} (Cherry et al., 1989; Denis and Gallo, 1989).
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.
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
Figure 1

ADH2 mRNA

UAS1,2 TATA

-400 -300 -200 -100 +1

CCR1 ADR1 CCR4

his4-912

CRE1 CRE2
(SPT10) (SPT6)
(SSN20)

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

Both \textit{CCR4} and the \textit{CRE} alleles are pleiotropic in their effects on cellular processes. Mutations in \textit{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 \textit{CRE} alleles affect cell morphology resulting in an elongated cell shape (Denis, 1984). In the case of a \textit{cre1} allele, a mutation in \textit{CCR4} was shown to suppress the aberrant cell morphology (Denis and Malvar, 1990). Additionally, mutations in \textit{CRE2} result in a temperature sensitive phenotype that cannot be suppressed by a \textit{ccr4} allele (Denis and Malvar, 1990).

The effects of \textit{CCR4} and the \textit{CRE} alleles occur through sequences near the TATAA element of \textit{ADH2} (Denis and Malvar, 1990). Strains harboring deletions, disruptions, or displacements of
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 SSN2 (CRE2) are able to express SUC2 in a suc2 allele created by deletions of sequences 5′ to the TATAA element indicates that SSN2 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-kDa and which contains a highly acidic amino terminus (Swanson et al., 1990). Both increasing and decreasing the dosage of SPT6 results in the
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 possibility 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 from human,
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
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 (Williamson 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.
**Table 1: Yeast strains**

<table>
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<tr>
<th>Strain</th>
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<tr>
<td>82-2b</td>
<td>MATo adh1-1 cre1-1 his3 leu2 trp1 ura1</td>
</tr>
<tr>
<td>85-1c</td>
<td>MATa adh1-1 ccr4-10:HIS3 trp1 leu2</td>
</tr>
<tr>
<td>292-6a</td>
<td>MATa adh1-1 ade1 his3 SP07:LEU2 trp1</td>
</tr>
<tr>
<td>317-5b</td>
<td>MATo adh1-1 ccr4-10 leu2 ura3</td>
</tr>
<tr>
<td>348-13a</td>
<td>MATa adh1-1 ccr4-10 ura3 trp1</td>
</tr>
<tr>
<td>348-26c</td>
<td>MATo adh1-1 ccr4-10 his3 leu2 trp1 ura3</td>
</tr>
<tr>
<td>350-19a</td>
<td>MATa adh1-1 ccr4-10 trp1 ura3</td>
</tr>
<tr>
<td>350-19b</td>
<td>MATa adh1-1 CCR4:TRP1 his3 leu2 ura3</td>
</tr>
<tr>
<td>350-19c</td>
<td>MATo adh1-1 ccr4-10 trp1 ura3</td>
</tr>
<tr>
<td>350-19d</td>
<td>MATa adh1-1 CCR4:TRP1 his3 leu2 ura3</td>
</tr>
<tr>
<td>500-16</td>
<td>MATa adh1-1 adh3 adrl-1 his4 trp1 ura1</td>
</tr>
<tr>
<td>500-16-1</td>
<td>MATa adh1-1 adh3 adrl-1 cre1-1 his4 trp1 ura1</td>
</tr>
<tr>
<td>500-16-14</td>
<td>MATa adh1-1 adh3 adrl-1 cre2-1 his4 trp1 ura3</td>
</tr>
<tr>
<td>612-1d</td>
<td>MATa adh1-1 his3 leu2 trp1 ura3</td>
</tr>
<tr>
<td>FW712-1d</td>
<td>MATo adh1-1:URA3 his4-912delta ura3-52</td>
</tr>
</tbody>
</table>
Bacterial and Yeast Transformation

Bacterial transformations were performed using competent DH5α or RR1 cells (purchased from BRL, Gaithersburg, MD). Transformations were conducted as recommended 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 recommended 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
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 $B = \text{BamHI}$ and $H = \text{HindIII}$. 
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 (l2l). 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 HindIII 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) = (□□□), TRPI (yeast) = (□□□), vector sequences = (□□□), and sequences from chromosome I = (□□□).
Figure 3

a.

![Diagram of restriction enzyme digests of Lambda L4](image)

- Lambda L4
- p89
- pUC18-Bm: Hd
- YRp7-3.5

1-kb scale

b.

![Diagram of restriction enzyme digests of p89 and pUC18-Bm: Hd](image)

- p89
- pUC18-Bm: Hd
- YRp7-3.5

1-kb scale
contains the yeast URA3 gene as well as approximately 12-kb of chromosome I DNA. A 1.4-kb HindII 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
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 HindIII 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 HindIII, 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
Figure 4

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 ( ).
CCR4 was disrupted by replacement of the internal HindIII 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 HindIII and the HIS3 gene inserted. Restriction sites for pUC18-Bm:Hd are shown only for those sequences derived from chromosome I. Sequence designations are: ampicillin gene (■), HIS3 gene (■■■), vector sequences (■■), and sequences from chromosome I (□).
1. Restrict with BamHI
2. Isolate HIS3 gene
Figure 6: Construction of YRp7-3.5:deltaMlu:Hi11

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 Smal fragment from pUC18-deltaMlu:Hinc was isolated and used to replace the analogous BamHI to Smal fragment from YRp7-3.5. The new construct was designated YRp7-3.5:deltaMlu:Hi11. Restriction sites are shown only for those sequences derived from chromosome I.
Figure 6

1. Isolate BamHI to Smal fragment from pUC18-deltaMlu-Hinc.
2. Restrict YRp7-3.5 with BamHI and Smal
3. Ligate isolated fragment with restricted YRp7-3.5.

1. Restrict with MluI and HindIII.
2. Fill in MluI 5' overhang.
3. Ligate.
to replace the analogous 1.5-kb BamHI to Smal fragment in the vector YRp7-3.5+. The new construction was designated YRp7-3.5:delta Miul-Hincll (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 35S-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 Na₂HPO₄, 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 H₂O 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
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, wrapped 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 forty-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
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

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:

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

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

\[
\text{cM} = 0.5 \left| \frac{\text{SDS}}{\text{FDS}} \right|
\]

<table>
<thead>
<tr>
<th></th>
<th>Parental Ditype (PD)</th>
<th>Nonparental Ditype (NPD)</th>
<th>Tetraype (T)</th>
<th>First Division Segregation (SDS)</th>
<th>Second Division Segregation (SDS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDC24 : ADE1</td>
<td>11</td>
<td>2</td>
<td>22</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CDC24 : CCR4</td>
<td>18</td>
<td>0</td>
<td>21</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ADE1 : CCR4</td>
<td>31</td>
<td>1</td>
<td>29</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TRP1 : CCR4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>44</td>
<td>12</td>
</tr>
</tbody>
</table>

* Mapping data from C. Denis (personal communication).
Figure 7b: Diagram of the left arm of Chromosome I.

CCR4 was mapped to the left arm of chromosome I relative to SP07 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: 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 = HindII.
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.

<table>
<thead>
<tr>
<th>ADH allele</th>
<th>Other relevant genotype</th>
<th>ADH II activity (mU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>ADH2&lt;sup&gt;1&lt;/sup&gt;</td>
<td>cre1</td>
<td>8</td>
</tr>
<tr>
<td>ADH2&lt;sup&gt;1&lt;/sup&gt;</td>
<td>cre1 ccr4</td>
<td>175</td>
</tr>
<tr>
<td>ADH2&lt;sup&gt;1&lt;/sup&gt;</td>
<td>cre2</td>
<td>23</td>
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<tr>
<td>ADH2&lt;sup&gt;1&lt;/sup&gt;</td>
<td>cre2 ccr4</td>
<td>77</td>
</tr>
<tr>
<td>ADH2&lt;sup&gt;1&lt;/sup&gt;</td>
<td>ADR1-5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6</td>
</tr>
<tr>
<td>ADH2&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td>ccr4</td>
<td>280</td>
</tr>
<tr>
<td>ADH2&lt;sup&gt;1&lt;/sup&gt;</td>
<td>ccr4 ADR1-5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Yeast were grown on YEP medium supplemented to 8% glucose as described in Materials and Methods.

<sup>b</sup> Yeast were grown on YEP medium supplemented to 3% ethanol as described in Materials and Methods.


<sup>2</sup> 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 (MATo 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.
Table 3: Complementation of the CCR4 mutation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>YET growth @37°C</th>
<th>ADH II activity (mU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>348-26c1</td>
<td>+</td>
<td>3317</td>
</tr>
<tr>
<td>350-19a¹</td>
<td>-</td>
<td>80</td>
</tr>
<tr>
<td>350-19b¹</td>
<td>+</td>
<td>2373</td>
</tr>
<tr>
<td>350-19c¹</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>350-19d¹</td>
<td>+</td>
<td>2576</td>
</tr>
<tr>
<td>348-26c²</td>
<td>-</td>
<td>237</td>
</tr>
<tr>
<td>348-13a²</td>
<td>-</td>
<td>130</td>
</tr>
<tr>
<td>348-26c-Cl²</td>
<td>+</td>
<td>1650</td>
</tr>
</tbody>
</table>

² 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.
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.
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 pC1 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 constructions 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

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 35S-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 characterized by a repeating unit with a general consensus sequence of \( \text{P-X-X-o-X-X-L-X-X-L-X-X-L-N-X-o-X-o} \) (where \( X = \) any residue and \( o = \) aliphatic amino acids, L, I, or V). A search of the
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 1 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 CCR4

a. Sequence of the CCR4 gene. The 2880-base pair sequence encompassing 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 QQAQQQAQQQAQQQQAQQQ (#190-206).
Figure 11a

-153  TTTGGTTATCCGGCGGTTACCTGGAGAAAAACGGCCACAAACGAAAAATTTGGAAGTTT

CCAGCTTATAGAAGGTTCTCATTGACAAAGCAGGATAATGGGAGTCTCAGCTGGAACAGGAA

-33  TCCGACTGACCTTACTCCTGGACACCCCTACTG

ATGAACGACCCTTCTTTACTAGGCTAC

CCTAATGTGGGCCCGCAGCAGCAACAGCAACAGCAGCAACAGCAGCATGCCGGGCTACTA

88  GGAAAGGCTGACACCCCTACTTGACTGCGTTATCGGTAGGTTACGCGTAACACCCCT

CCCTGGGACTGACGTTATCCCTGCAAACTACCGCTA

ATGAACGACCCTTCTTTACTAGGCTAC

CCTAATGTGGGCCCGCAGCAGCAACAGCAACAGCAGCAACAGCAGCATGCCGGGCTACTA

121  GGAAAGGCTGACACCCCTACTTGACTGCGTTATCGGTAGGTTACGCGTAACACCCCT

CCCTGGGACTGACGTTATCCCTGCAAACTACCGCTA

ATGAACGACCCTTCTTTACTAGGCTAC

CCTAATGTGGGCCCGCAGCAGCAACAGCAACAGCAGCAACAGCAGCATGCCGGGCTACTA

156  TTTGGTTATCCGGCGGTTACCTGGAGAAAAACGGCCACAAACGAAAAATTTGGAAGTTT

CCAGCTTATAGAAGGTTCTCATTGACAAAGCAGGATAATGGGAGTCTCAGCTGGAACAGGAA

115  TTTGGTTATCCGGCGGTTACCTGGAGAAAAACGGCCACAAACGAAAAATTTGGAAGTTT

CCAGCTTATAGAAGGTTCTCATTGACAAAGCAGGATAATGGGAGTCTCAGCTGGAACAGGAA

144  TTTGGTTATCCGGCGGTTACCTGGAGAAAAACGGCCACAAACGAAAAATTTGGAAGTTT

CCAGCTTATAGAAGGTTCTCATTGACAAAGCAGGATAATGGGAGTCTCAGCTGGAACAGGAA

173  TTTGGTTATCCGGCGGTTACCTGGAGAAAAACGGCCACAAACGAAAAATTTGGAAGTTT

CCAGCTTATAGAAGGTTCTCATTGACAAAGCAGGATAATGGGAGTCTCAGCTGGAACAGGAA

202  CAGTGGCAGCACGTGACGTTATCCCTGCAAACTACCGCTA

ATGAACGACCCTTCTTTACTAGGCTAC

CCTAATGTGGGCCCGCAGCAGCAACAGCAACAGCAGCAACAGCAGCATGCCGGGCTACTA

241  TTTGGTTATCCGGCGGTTACCTGGAGAAAAACGGCCACAAACGAAAAATTTGGAAGTTT

CCAGCTTATAGAAGGTTCTCATTGACAAAGCAGGATAATGGGAGTCTCAGCTGGAACAGGAA

270  TTTGGTTATCCGGCGGTTACCTGGAGAAAAACGGCCACAAACGAAAAATTTGGAAGTTT

CCAGCTTATAGAAGGTTCTCATTGACAAAGCAGGATAATGGGAGTCTCAGCTGGAACAGGAA

309  TTTGGTTATCCGGCGGTTACCTGGAGAAAAACGGCCACAAACGAAAAATTTGGAAGTTT

CCAGCTTATAGAAGGTTCTCATTGACAAAGCAGGATAATGGGAGTCTCAGCTGGAACAGGAA

348  TTTGGTTATCCGGCGGTTACCTGGAGAAAAACGGCCACAAACGAAAAATTTGGAAGTTT

CCAGCTTATAGAAGGTTCTCATTGACAAAGCAGGATAATGGGAGTCTCAGCTGGAACAGGAA

443  TTTGGTTATCCGGCGGTTACCTGGAGAAAAACGGCCACAAACGAAAAATTTGGAAGTTT

CCAGCTTATAGAAGGTTCTCATTGACAAAGCAGGATAATGGGAGTCTCAGCTGGAACAGGAA
MNDPSLLGYPNVGPQQQQQQQQQQHACLLGKGTNPALQQQLHMNQLTGI 50
PPGLMNNSDVHTSSNNNSRQLLDQLANGNANMLNMDNNNNNNNNNNNN 100
NNNGGGSGVMNASTAAAVNSIGMVTGTPVPNINVNASNPLLHPHLDPS 150
LLNRPWKLQLHALAVSAQSLQPNIYARQNMKLLATQQAQAAQQAQAO 200
QQAAQQVPFGFGGQAPPAQQPDFQOSH1AEASKSLVDCTKQALMEM 250
ADTLTSTAKKQQPTGDSGTATNSAVSTPLTPKLTFANGKDEANQ 300
ALLQHKKLSQYSIDEDEDDINRMVMPDKSRYDDQLWHALDLNLQIINFIS 350
ANIFKYDFLTRYLLNSLTELPAEIKNLSNLRLSDLNRLTLSLPAELG 400
SCFQLKYFYFFDNNVTTLPWFQGNLCNLQFLGVEGNPLPKQFLKILTEKS 450
VTGLFYLRDNRPEIPLPHERRFIEINTDGPQREYDSLQQSTEHLATDL 500
AKRTFTVLSYNTLQCQHYATPKMYRTPSWALSWDYRRNKLEQILSYSD 550
LLCLQEVESTFEEYWVPLLDDKHYTGIFHAKARAKTMHDSKVDGCC 600
IFFKRDQFKLITKADMDFSAWKKHKFQRTEDYLNRMKDNVALFLKL 650
QHIPSGDTIWAVTTLHWDKPNIVKTFQGVVLDHLETALLKEETSHNFR 700
QDIKFPVLICGDFNSYINSAVLYELINTGRVQIHQEGNGRDFGYMEKSF 750
SHNLALKSSYCIGELPFTNFTPSFTDVYDFIWFSTHALRVRLGGEVDP 800
EYVSFIGFNDKFPDSHPLARFEFMKNTGSKKV

Figure 11b
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 A Q Q A 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-kDa subunit of carboxypeptidase N (Tan et al., 1990), the human platelet glycoprotein Ib α 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 extracellular, 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).
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 Iib 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 Iib 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

<table>
<thead>
<tr>
<th>Protein (Organism)</th>
<th>No. of repeats</th>
<th>Consensus of repeated sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR4 (Yeast)</td>
<td>5</td>
<td>P xxL<em>xxL</em>xxLxxLxLxxNxxLxxL<em>xxL</em></td>
</tr>
<tr>
<td>Adenylate cyclase (Yeast)</td>
<td>24</td>
<td>P xxL-xxLxxLxxLxxLxxNxxLxxL*</td>
</tr>
<tr>
<td>Toll (Drosophila)</td>
<td>15</td>
<td>P xxLFxxHxxNLxxLxxLxxNxxLxxL</td>
</tr>
<tr>
<td>Chaoptin (Drosophila)</td>
<td>41</td>
<td>P xxxFxxLxxLxxLDLxxNxxLxxL</td>
</tr>
<tr>
<td>RNase inhibitor (Human) (Porcine)</td>
<td>8</td>
<td>xxxLxxPxCxLxE-LxxLxxCxxCxxL</td>
</tr>
<tr>
<td>Platelet GPIbO (Human)</td>
<td>7</td>
<td>P xGLLxxLPxxLxxLSxNxxLTTL</td>
</tr>
<tr>
<td>L-rich O2 GP (Human)</td>
<td>8</td>
<td>P xxLLxxxxLxxLxxLxxNxxLxxL</td>
</tr>
</tbody>
</table>

* Aliphatic amino acid (leucine, isoleucine, or valine)
(#89 - 103), and the repeat sequence: Q Q A Q Q A Q Q A 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 HaellII 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
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 duplicate 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

- a
- b c
- CCR4 mRNA
- rRNA
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 amino acids (CCR4:1-392; see Figure 5). The plasmid
YRp7-3.5:delta Mlu-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

CCR4 mRNA -> CRE2, CRE1, cre1, cre2, CRE1
Figure 16: Disruptions and deletions created in CCR4.

The thin line (—) represents sequences from the left arm of chromosome 1. 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 (pCl:HIS3), 6 (pUC18-Bm:Hd:HIS3), and 7 (YRp7-3.5:deltaMlu1-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 4a). 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 4b).

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-1). 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>HIS3</th>
<th>TRP1</th>
<th>AT</th>
<th>YD 30</th>
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<tr>
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<td>+</td>
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<tr>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>612-1d</td>
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<td>+</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>85-1c</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>Strain</th>
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<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>82-2b</td>
<td>105</td>
<td>n.d.</td>
</tr>
<tr>
<td>82-2b-A</td>
<td>66</td>
<td>n.d.</td>
</tr>
<tr>
<td>82-2b-1</td>
<td>32</td>
<td>n.d.</td>
</tr>
<tr>
<td>82-2b-1-a</td>
<td>235</td>
<td>n.d.</td>
</tr>
<tr>
<td>82-2b-1-1a</td>
<td>22</td>
<td>n.d.</td>
</tr>
<tr>
<td>612-1d</td>
<td>n.d.</td>
<td>4000</td>
</tr>
<tr>
<td>612-1d-2</td>
<td>n.d.</td>
<td>750</td>
</tr>
<tr>
<td>85-1c</td>
<td>n.d.</td>
<td>800</td>
</tr>
</tbody>
</table>

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 phenotype 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 pCI: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, i.e. 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.
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 BgIII 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).
These results are in contrast to the observation that neither of the intact YRp7-3.5 plasmids are capable of complementing a \textsc{CCR4} mutation. The explanation for this discrepancy may be the fact that increased levels of \textsc{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 \textsc{CCR4}, including those strains harboring pC1, when compared to strains containing only the endogenous \textsc{CCR4} allele.

The level of \textsc{CCR4} transcription is not significantly altered by either carbon source (see Figure 13, lanes b and c) or a \textsc{cre1} or \textsc{cre2} allele (see Figure 15). Immunoprecipitation of the CCR4 protein showed that levels remain unchanged in \textsc{cre} strains when compared to a wild type \textsc{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...
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
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 studies 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 4a and 4b; Figure 16). Strains harboring any of
the disrupted alleles of $\text{CCR4}$ (82-2b-A ($\text{CCR4:1-668}$), 82-2b-1 ($\text{CCR4:1-392}$), 612-1d-2 ($\text{CCR4:1-668}$), and 85-1c ($\text{CCR4:1-392}$); Table 4a) are phenotypically similar to previously described mutations in $\text{CCR4}$; 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 ($\text{CCR4:1-382/514-837}$; Table 4a; Figure 8). Additionally, the effects of $\text{CCR4}$ disruptions and deletions on $\text{ADH II}$ activity are also seen (Table 4b). Strain 82-2b displays the typical elevated repressed activity seen in a $\text{cre1}$ background. Strain carrying mutations which disrupt $\text{CCR4}$ (82-2b-A, 82-2b-1, and 82-2b-1-1a) act to lower the repressed $\text{ADH II}$ 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 $\text{ADH II}$ 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
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 A Q Q A Q A Q A 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.](image)

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
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
interest is the similarities seen between the arrangement of the amino termini of TFIIID 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 ADR1-5c. Strains carrying ADR1-5c show elevated ADH II activity on fermentative and nonfermentative media (Table 2). Mutations in CCR4 lower
derepressed ADH II activity in an *ADR1-5c* 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 constructions 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
remains unchanged when cells are grown fermentatively; a condition not requiring \textit{ADH2} activation. The presence of CCR4 protein is not surprising since both the \textbf{CRE} alleles and \textbf{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 \textit{ADH2} irrespective of carbon source. Therefore a mechanism must exist which inhibits the action of CCR4 at the \textit{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 \textit{SPT6} (CRE2) by Swanson et al. (1990) suggest additional means by which \textbf{CCR4} may regulate transcription. The pleiotropic effects described for \textbf{CCR4} and the \textbf{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 \textbf{CCR4} and \textbf{CRE} alleles noted above in which they may exert their effect on chromatin structure near the TATA box. Swanson et al (1990) find that \textit{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 noticeably 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 structure then these observations would again imply an auxiliary rather than 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 constitute 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 ascertain 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.
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 °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.
REFERENCES


