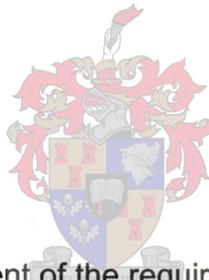

**Deletion analysis of the Ure2p in *Saccharomyces cerevisiae* and
effect of NCR on the production of ethyl carbamate during wine
fermentations**

by

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Science at the University of Stellenbosch

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Declaration

I the undersigned hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

D. J. Erasmus

Summary

The wine yeast *Saccharomyces cerevisiae* has the ability to utilize several different nitrogenous compounds to fulfill its metabolic requirements. Based upon different growth rates of the yeast in a particular nitrogen source, nitrogen compounds have been classified as either good or poor nitrogen sources. In an environment which contains different quality nitrogen sources, such as grape must, the yeast first utilizes good and then the poor nitrogen sources. This discrimination between good and poor nitrogen sources is referred to as nitrogen catabolite repression (NCR). Examples of good nitrogen sources are ammonia, glutamine and asparagine. Nitrogen sources such as allantoin, γ -aminobutyrate (GABA), arginine and proline are poor quality nitrogen sources.

Several regulatory proteins, Ure2p, Gln3p, Dal80p, Gat1p and Deh1p, mediate NCR in *S. cerevisiae*. These *trans*-acting factors regulate transcription of NCR sensitive genes. All these proteins, except Ure2p, bind *cis*-acting elements in the promoters of genes that are responsible for degradation of poor nitrogen sources. Gln3p is an activator of NCR sensitive genes in the absence of good nitrogen sources. The predominant mechanism by which NCR functions is by using Ure2p to inactivate the activator Gln3p in the presence of a good nitrogen source.

Several research groups have studied the Ure2p, mainly due to its prion-like characteristics. The Ure2p has two domains: a prion inducing domain located in the N-terminal region and a NCR regulatory domain located in the C-terminal domain. The aims of this study were (i) to determine the part of the C-terminal domain which is responsible for NCR, (ii) to establish if *ure2* deletion mutants produce less ethyl carbamate during wine fermentations and (iii) if NCR functions in industrial yeast strains. Nested deletions of the *URE2* gene revealed that the NCR regulatory domain resides in the last ten amino acids of the Ure2p. This was established by Northern blot analysis on the NCR sensitive genes *DAL5*, *CAN1*, and *GAP1* genes.

Ethyl carbamate in wine is produced by spontaneous chemical reaction between urea and ethanol in wine. Urea is produced by *S. cerevisiae* during the metabolism

of arginine. Arginine is degraded to ornithine and urea by arginase, the product of the *CAR1* gene. Degradation of urea by *S. cerevisiae* is accomplished by urea amidolyase, a bi-functional enzyme and product of the *DUR1,2* gene which is subject to NCR. This study investigated if a *ure2* mutant strain produced less ethyl carbamate during wine fermentations.

Wine fermentations were conducted with diploid laboratory strains: a *ure2* mutant strain and its isogenic wild type strain. GC/MS analysis of the wine revealed that the *ure2* mutant produced less ethyl carbamate but more ethanol than the wild type strain when arginine, di-ammoniumphosphate, asparagine or glutamine were added as nitrogen sources, in combinations and separately. There was no significant difference between the wild type fermentation and the *ure2* mutant fermentation when no nitrogen was added. It was found that a combination between the deletion of *URE2* and the addition of a good nitrogen source resulted in lower levels of ethyl carbamate.

High density micro array analysis done on an industrial strain wine yeast in Chardonnay grape must revealed that the *GAP1*, *CAN1*, *CAR1* and *DUR1,2* genes, responsible for transport and metabolism of arginine and degradation of urea, are NCR sensitive. These data strongly suggest that NCR functions in industrial yeast strains.

Opsomming

Die wyngis *Saccharomyces cerevisiae* kan verskillende stikstofbronne gebruik om in sy stikstofbehoefte te voldoen. Stikstofbronne word as goeie of swak stikstofbronne geklassifiseer op grond van die groeitempo van die gis op die betrokke stikstofbron. 'n Goeie stikstofbron laat die gis vinniger groei as wat dit op 'n swak stikstofbron sou groei. In omgewings soos druiwemos waar daar 'n verskeidenheid van stikstofbronne teenwoordig is, sal die gis eers die goeie bronne en daarna die swak bronne benut. Stikstofbronne soos ammonium, asparagien en glutamien word geklassifiseer as goeie bronne. Allantoïen, γ -amino-butaraat (GABA), prolien en arginien word as swak stikstofbronne geklassifiseer. Die meganisme waarmee *S. cerevisiae* tussen die stikstofbronne onderskei, staan as stikstof kataboliet onderdrukking (NCR) bekend.

Die proteïene wat vir verantwoordelik is NCR naamlik Ure2p, Gln3p, Gat1p, Dal80p en Deh1p, bind met die uitsondering van Ure2p, almal aan *cis*-werkende elemente in die promoters van NCR-sensitiewe gene. Die *trans*-werkende faktore reguleer die transkripsie van NCR-sensitiewe gene. NCR werk hoofsaaklik deur die inhibering van Gln3p deur Ure2p in die teenwoordigheid van 'n goeie stikstofbron. Die oorgrote meerderheid NCR-sensitiewe gene word deur Gln3p in die afwesigheid van 'n goeie stikstofbron geaktiveer.

Heelwat navorsing is op die prionvormings vermoë van Ure2p gedoen. Ure2p het twee domeine: 'n N-terminale domein wat vir prionvorming verantwoordelik is en die C-terminale domein waar die NCR funksie van Ure2p gesetel is. Die doel van die studie was (i) om te bepaal waar in die C-terminale domein van Ure2p die NCR regulering geleë is, (ii) of *ure2* delesie mutante minder etielkarbamaat tydens wynfermentasies produseer en (iii) of NCR in industriële gisrasse funksioneel is. Delesie analyses van *URE2* het getoon dat die NCR regulerings domein in die laaste tien aminosure gesetel is. Dit is vas gestel m.b.v. noordlike klad tegniek analyses op die *DAL5*, *CAN1* en *GAP1* gene.

Etielkarbamaat in wyn word deur die spontane chemiese reaksie tussen ureum en alkohol geproduseer. Ureum word gedurende die metabolisme van arginien in *S. cerevisiae* geproduseer. Arginien word deur arginase, produk van die *CAR1* geen, na ornitien en ureum afgebreek. Die bi-funksionele ureum amidoliase, gekodeer deur die *DUR1,2* geen, breek ureum na CO_2 en NH_4^+ af. As gevolg van die NCR-sensitiwiteit van dié gene is ondersoek ingestel na 'n *ure2* mutant se vermoë om minder etielkarbamaat tydens wynfermentasies te produseer. Chardonnay druiwemos is met 'n diploiede laboratorium ras en die isogeniese *ure2* mutant gefermenteer. GC/MS analise op die wyn het getoon dat die *ure2* mutant minder etielkarbamaat, maar meer alkohol in vergelyking met die wilde tipe gis produseer, as arginien, di-ammoniumfosfaat, asparagien en glutamien, afsonderlik of gesamentlik byvoeg is. Daar was egter nie 'n merkwaardige verskil tussen die fermentasies waar geen stikstof bygevoeg is nie. Dit dui daarop dat 'n kombinasie van 'n *URE2* delesie en die byvoeging van stikstof etielkarbamaat vlakke verlaag.

Mikro-skyfie analise van 'n industriële gis in Chardonnay mos het getoon dat die *GAP1*, *CAN1*, *CAR1* en *DUR1,2* gene wat verantwoordelik is vir die transport en metabolisme van arginien en degradasie van ureum, wel NCR-sensitief is. Dit dui daarop dat NCR wel in industriële gisrasse funksioneel is.

Biographical Sketch

Daniel J. Erasmus was born in Middelburg, Mpumalanga, South Africa, on December 20, 1975. He matriculated from Hottentots Holland High School in Somerset West, Western Province, 1993. After receiving his B.Sc. in Microbiology and Biochemistry in 1996 and his Hons. B.Sc. in Microbiology in 1997 from the University of Stellenbosch, he enrolled for his M.Sc. in Microbiology at the University of Stellenbosch.

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INTRODUCTION

Nitrogen plays an important role in the physiology and metabolism of all living organisms as nitrogen is an essential part of nucleic and amino acids, as well as other important metabolic molecules.

The yeast *Saccharomyces cerevisiae* assimilates nitrogen found in ammonia, asparagine, proline, allantoin and several other compounds. In an environment where there are multiple forms of assimilable nitrogen available, the yeast metabolizes nitrogen discriminately. This discrimination is based on how readily the yeast grows on the different nitrogen sources. Nitrogen sources such as ammonia, glutamine and asparagine allow the yeast to grow faster than allantoin, proline and gamma-amino butyric acid (GABA). Nitrogenous compounds that allow faster growth are considered to be good nitrogen sources and those that result in comparatively slow growth are poor nitrogen sources. The yeast will therefore utilize the best nitrogen source first followed by the poor nitrogen sources when both are available. This phenomenon is known as nitrogen catabolite repression (NCR) (Cooper and Sumrada, 1983).

NCR requires an intricate system to regulate the expression of genes involved in the assimilation and utilization of various nitrogenous compounds. This regulation occurs through the modulation of steady state mRNA levels (Rai *et al.*, 1987) and is upheld by a number of regulatory proteins that repress or activate the expression of genes involved in the transport and assimilation of nitrogen (Ahmad and Bussey, 1986). This regulatory system consists of a number of global regulators namely Gln3p, Dal80p, Deh1p, Gat1p, and Ure2p (Coffman *et al.*, 1997).

Of these global regulators, Ure2p is the only one that functions in the cytoplasm. Gln3p and the other proteins function as DNA-binding proteins in the nucleus (Cunningham *et al.*, 1996; Stanbrough and Magasanik, 1996; Rowen *et al.*, 1997; Edkes *et al.*, 1999). Neither the mechanism by which Ure2p functions nor the part of Ure2p responsible for NCR activity has been identified. However, it is known that the function of Ure2p lies within the COOH-terminal domain of the protein. Numerous publications indicate that the N-terminal domain of Ure2p has the ability to induce the

formation of prions (Wickner, 1994). Prions (**proteinaceous infectious particles**) are proteins, encoded by normal genomic genes, that undergo secondary structural change that result in the formation of new protein structures that cause diseases in mammals (Prusiner, 1982). Prion-like proteins have been found in yeast (Wickner, 1994), as well as other fungi (Wickner, 1997).

The metabolism of arginine and allantoin in grape must leads to the production of urea. Urea can react spontaneously with ethanol to form ethyl carbamate. The mutagenicity of ethyl carbamate has been studied as early as the 1940's. Data obtained confirmed that ethyl carbamate is a carcinogen in a variety of experimental animals (Zimmerli and Schlatter, 1991). The metabolic pathways that lead to the formation and breakdown of urea are NCR-sensitive (Genbauffe and Cooper, 1986). The *URE2* gene might therefore play a substantial regulatory role in the formation of urea.

The aims of this study were:

1. To establish the NCR regulatory domain in the Ure2p by deletion and mutation analysis of the *URE2* gene;
2. To determine the regulatory effect of *URE2* on the production of ethyl carbamate during wine fermentations;
3. To investigate if NCR functions in industrial wine yeast strains during vinification.

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NITROGEN CATABOLITE REPRESSION IN *Saccharomyces cerevisiae*

The yeast *S. cerevisiae* has the ability to utilize several nitrogenous compounds as sole sources of nitrogen. These nitrogenous compounds include some of the naturally occurring L-amino acids, polyamines, ammonia and some purine degradative products. The nitrogen generated from these nitrogen sources are either converted to glutamate or deaminated to produce ammonia (Figure 1). Ammonia is incorporated into either α -ketoglutarate or glutamate to produce glutamate or glutamine, respectively. The incorporation of nitrogen occurs solely through this pathway in *S. cerevisiae* (Magasanik, 1992).

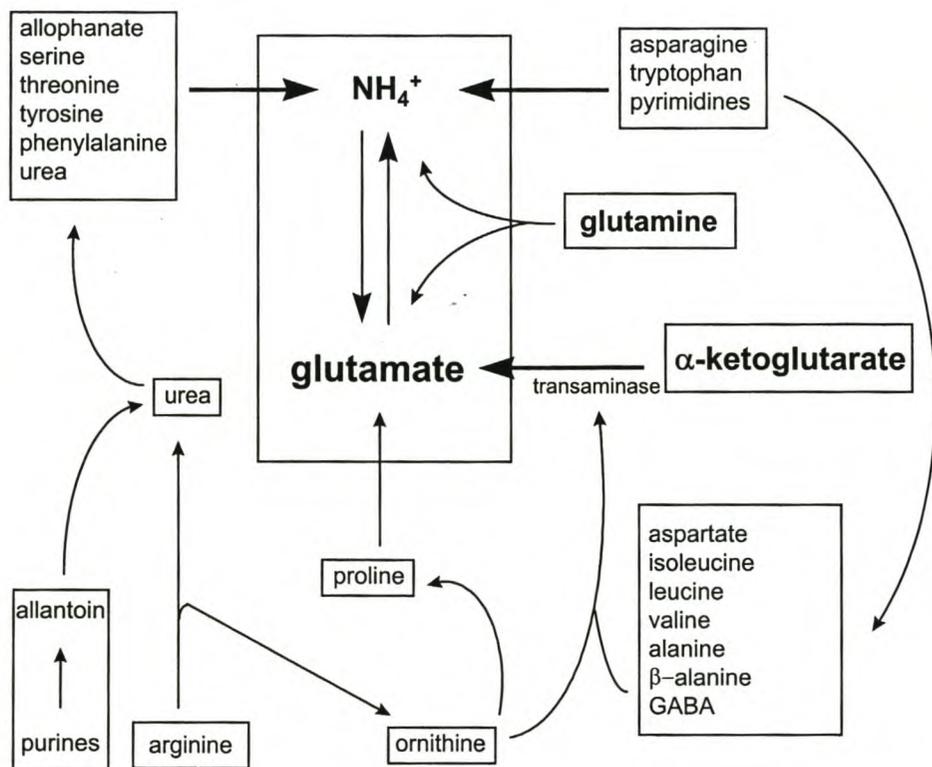


Figure 1. General metabolism of nitrogen in *S. cerevisiae* (adapted from Boulton *et al.*, 1996).

In an environment where there are multiple forms of assimilable nitrogen available, the yeast metabolizes nitrogen discriminately. This discrimination is based on how

readily the yeast grows on the different nitrogen sources. Nitrogen sources such as ammonia, glutamine and asparagine allow the yeast to grow faster than allantoin, proline and GABA. Nitrogenous compounds that allow faster growth are considered to be good nitrogen sources and those that result in comparatively slow growth are poor nitrogen sources. The yeast will utilize the good nitrogen source first and then the poor nitrogen sources when both are available. This phenomenon is known as nitrogen catabolite repression (NCR) (Cooper and Sumrada, 1983).

NCR requires an intricate system to regulate the expression of genes involved in the assimilation and utilization of various nitrogenous compounds. This regulation occurs through the modulation of steady state mRNA levels (Rai *et al.*, 1987) and is upheld by a number of regulatory proteins that repress or activate the expression of genes involved in the transport and assimilation of nitrogen (Ahmad and Bussey, 1986). This regulatory system consists of a number of global regulators, including Gln3p, Dal80p, Deh1p, Gat1p and Ure2p (Coffman *et al.*, 1997).

In addition to these global regulators, the yeast has to sense what type of nitrogen source is available for its metabolism. The mechanism of sensing environmental nitrogen has not been fully elucidated. What is known, is that some of the NCR-sensitive permeases are able to detect ammonia levels in the yeast's surroundings. The Mep2 protein is able to sense ammonia in its external environment. New studies have indicated that Mep2p might be the first protein in the signal transduction pathway for sensing nitrogen (Lorenz and Heitman, 1998a). Regulation of this protein is highly dependent on the activity of Ure2p. There has also been a report that suggests glutamine tRNA is responsible for sensing nitrogen (Murray *et al.*, 1998), however, recent data contradicts this evidence (Beeser and Cooper, 1999). Iraqui *et al.* (1999) showed that an amino acid permease homologue, Ssy1p, acts as a sensor for external amino acids. They showed data implicating Ssy1p in the induction of several amino acid permeases, thereby coupling the availability of amino acids to transcriptional regulation. Several permeases that transport nitrogenous compounds are not sensitive to NCR. The branched chain amino acid permease (*BAP2*) is one such example. One explanation for Bap2p NCR insensitivity is that it transports amino acids for incorporation into proteins. Therefore, it is to the yeast's

advantage that permeases like Bap2p are not regulated by NCR (Didion *et al.*, 1996).

Utilization of poor nitrogen sources

S. cerevisiae contain specific metabolic pathways to metabolize poor nitrogen sources. It is often the case that some of these compounds, such as allantoin and arginine, share intermediates and enzymes in their metabolism. This sharing makes the utilization of poor nitrogen sources more efficient. The regulation of catabolic NCR sensitive genes is controlled, by the global regulators of NCR, at transcriptional level. Numerous examples of genes regulated by Ure2p exist: *CAR1*, *DAL1*, *DAL2*, *PUT1*, *PUT2*, *UGA1* etc (Coffman *et al.*, 1994). These genes encode for catabolic enzymes of the arginine, allantoin, GABA and proline systems. All these catabolic pathways degrade their particular nitrogen source to generate ammonia ions (except proline and GABA) that are then incorporated into α -ketoglutarate or glutamate for further metabolism (Figure 2). Proline and GABA are converted to glutamate. Therefore, in the presence of a good nitrogen source NCR sensitive pathways are down-regulated by Ure2p.

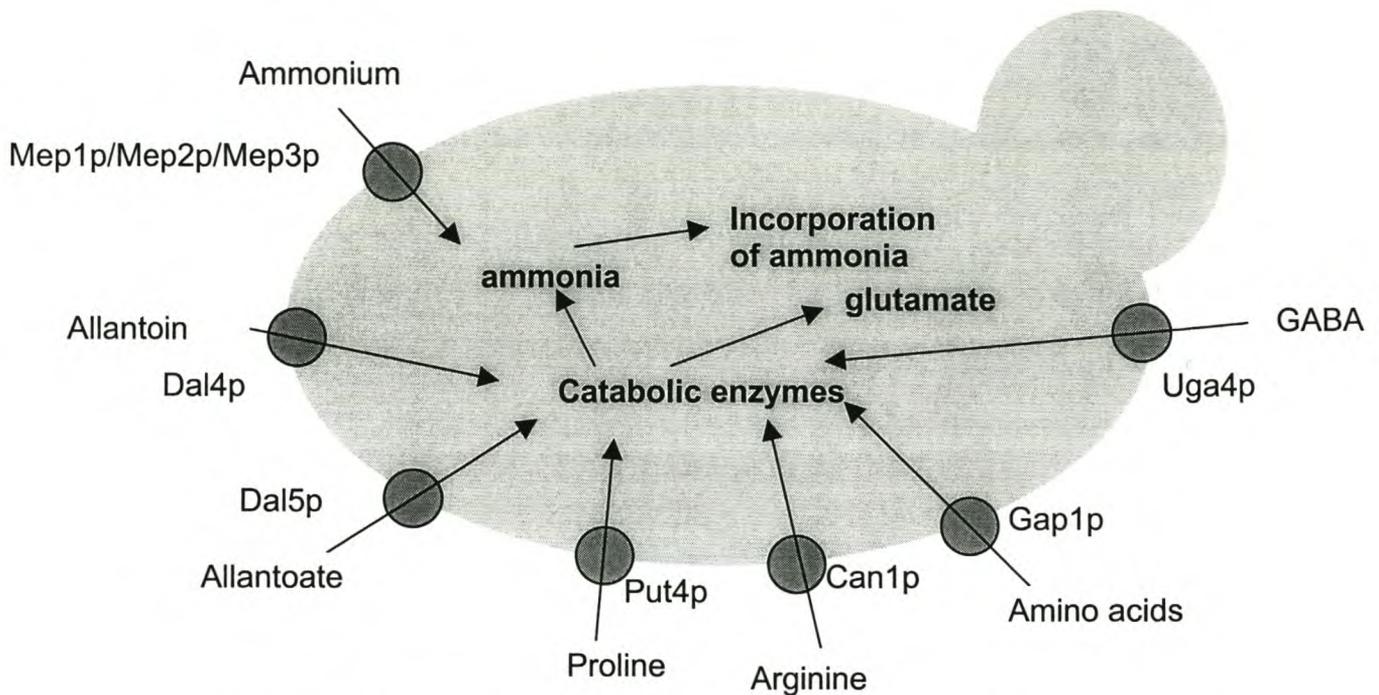


Figure 2. The utilization of poor nitrogen sources in *S. cerevisiae* and subsequent incorporation of ammonia into metabolites.

In addition to sharing metabolites, some of the NCR genes are grouped together in clusters on the yeast's chromosomes. One example of such clustering is the *DAL* cluster situated on the right arm of chromosome IV (Chisholm and Cooper, 1982). Clustering of genes is also found in other fungi such as *Aspergillus nidulans* (Marzluf, 1997). Genes closer to the telomeres of a chromosome are more accessible to the transcription machinery than those genes closer to the centromere. Packing genes away in clusters close to the centromere is an advantage if the genes are only to be utilized in circumstances that do not arise often. Furthermore, the sharing of enzymes and metabolites conserve energy even more so because these pathways are probably only active in near starvation environments and the yeast needs to conserve as much energy as possible.

Metabolism of the purine degradative product allantoin

Allantoin is an intermediate product of purine catabolism. This compound can be used by *S. cerevisiae* as a sole nitrogen source when there are no other nitrogen sources available to the yeast. For this reason *S. cerevisiae* has a subset of genes

that are responsible for allantoin degradation which generates the necessary ammonia to fulfill in the nitrogen requirements of the yeast. Allantoin is degraded to four molecules of ammonia, four molecules of carbon dioxide and one glyoxylate molecule. This metabolic pathway (Figure 3) has two permeases encoded by *DAL4* and *DAL5*, and five of catabolic enzymes encoded by *DAL1*, *DUR1,2*, *DAL2*, *DAL3*, and (Yoo *et al.*, 1985).

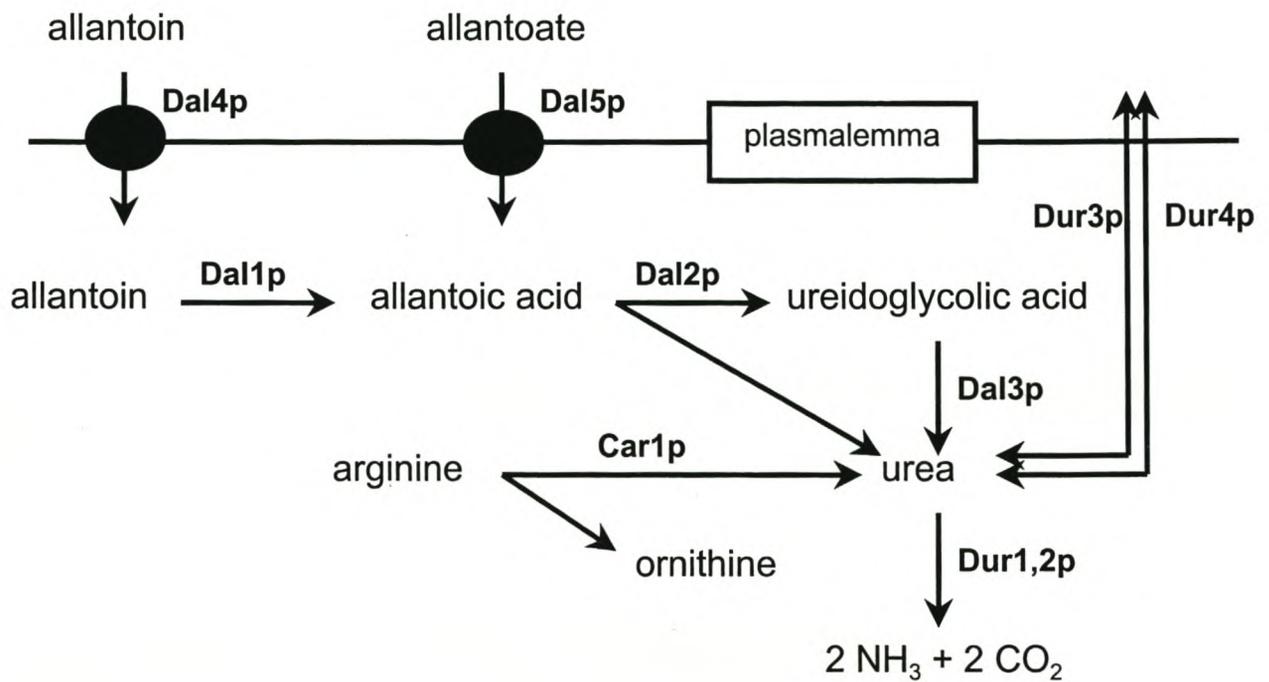


Figure 3. The allantoin pathway in *S. cerevisiae*. Abbreviations used: Dal1p, allantoinase; Dal2p, allantoicase; Dal3p, ureidoglycollate hydrolase; Dal4p, allantoin permease; Dal5p, allantoate permease; Dur1,2p, urea amidolyase; Dur3p, urea (low K_m) permease; Dur4p, urea (high K_m) permease; and Car1p, arginase.

The metabolism of allantoin is heavily regulated by NCR with *URE2*, *GLN3* and *DAL80* being the major role players. *DAL81* (Turoscy and Cooper, 1982; Turoscy *et al.*, 1984) and *DAL82* (Olive *et al.*, 1991) act as inducer proteins that are responsible for the induction of genes such as *DAL7* (Van Vuuren *et al.*, 1991) and *DAL4* in the presence of allophanate (Cooper *et al.*, 1987). The *DAL5* gene has been used as an indicator for the loss of NCR by several research groups (Masison *et al.*, 1997).

Transcriptional regulation of the allantoin permease gene (*DAL5*). The Dal5p is responsible for the uptake of allantoin, the first degradative product of allantoin catabolism. This compound is actively transported into the cell with a K_m of approximately 50 μ M (Turoscy Chisholm *et al.*, 1987). Sequence analysis of *DAL5* revealed that it encodes a highly hydrophobic protein containing alternating beta-pleated sheets and alpha-helices, suggesting strongly that Dal5p is an integral membrane protein. The protein has no signaling sequences similar to those found in secreted proteins (Rai *et al.*, 1988).

The transport of allantoin is sensitive to feedback inhibition by intracellular allantoin and trans-inhibition by D- and L- amino acids (Turoscy Chisholm *et al.*, 1987). *DAL5* expression is highly suppressed in the presence of a good nitrogen source (Rai *et al.*, 1987). Allantoin uptake is therefore regulated at two levels: transcription by NCR and at protein level by feedback inhibition.

In contrast with *DAL4*, *DAL5*'s expression is inducer independent (Sumrada *et al.*, 1978; Turoscy Chisholm *et al.*, 1987). The *DAL5* and the catabolic *DAL3* genes are the only two genes in the allantoin pathway that are constitutively expressed once NCR is uplified. The promoter of *DAL5* contains eight putative UAS_{NTR} elements. Transcriptional analysis revealed the elements between -414 to -335 are responsible for 98% of *DAL5* transcription. The remaining UAS_{NTR} elements supports only 1% to 2% of the total expression (Rai *et al.*, 1989).

The expression of *DAL5* is highly dependent on Gln3p, which is responsible for transcriptional activation. During nitrogen repressive conditions, Ure2p is responsible for inactivation of Gln3p resulting in a shutdown of *DAL5* expression. Transcription of the *DAL5* gene is NCR insensitive in an *ure2* mutant (Coffman *et al.*, 1994). This makes *DAL5* an ideal gene to use as an indicator gene when studying the nitrogen related functionality of *URE2*. Other scientists have also used *DAL5* as an indicator for studying loss of Ure2p function (Masison *et al.*, 1997).

Metabolism of arginine

Amino acids, once in the yeast cell, have one of two metabolic fates: they are either incorporated into proteins or catabolized to fulfill in the yeast's nitrogen and carbon requirements (Olivera *et al.*, 1993). Arginine can be used as a nitrogen source by the yeast. The pathway that is responsible for the utilization of arginine consists of a permease (*CAN1*) and several catabolic enzymes, i. e. arginase (*CAR1*), ornithine amino transferase (*CAR2*) and urea amidolyase (*DUR1,2*) that degrade arginine and its intermediates (Figure 3) to ammonia and carbon dioxide.

The *CAN1* gene was identified in a mutant of *S. cerevisiae* that was resistant to canavanine, a toxic analog of arginine. This mutant could not transport canavanine into the cell for degradation, thereby enabling the mutant to grow on plates containing canavanine (Ahmad and Bussey, 1986). The gene was named *CAN1* due to the fact that this particular mutation in the gene resulted in CANavanine resistance. This mutant, *arg-p1*, was identified to be responsible for the transport of basic amino acids such as L-arginine, D-arginine, L-lysine, L-histidine, L-ornithine and L-canavanine. Can1p is responsible for 96% of all arginine that enters the cell.

Transcriptional regulation of the arginine permease gene (*CAN1*). Daugherty *et al.* (1993) reported that *CAN1* expression is very much NCR-sensitive and relies a great deal on Gln3p for its expression. The regulation of *CAN1* by *URE2* seems to follow the same pattern as that of *DAL5* (Coffman *et al.*, 1994). Northern blot analysis revealed that *CAN1* is transcribed when *S. cerevisiae* is cultivated in a good nitrogen source, such as asparagine, when *URE2* is deleted. The deletion of *URE2* allows Gln3p to activate *CAN1* transcription in the presence of a good nitrogen

source. In the wild type cells, *CAN1* expression is repressed due to a functional Ure2p.

The promoter of *CAN1* contains both UAS_{NTR} as well as URS_{GATAA} elements to which Gln3p and Dal80p bind, respectively. Assaying for *CAN1* expression by northern blot analysis in a *gln3* background strain allowed researchers to implicate Gln3p in *CAN1* transcriptional regulation. The absence of *GLN3* resulted in failure of transcription of *CAN1*. *CAN1* expression is remarkably elevated in a *dal80* mutant background, thereby implicating Dal80p in *CAN1* expression as well (Daugherty *et al.*, 1993).

However, it was later shown, by Coffman *et al.* (1995) that *CAN1* can be expressed independently of Gln3p and Dal80p. This evidence uncovered a third DNA-binding protein, Gat1p, that plays a role in the transcription of *CAN1*.

Metabolism of 4-amino butyric acid (GABA)

4-Aminobutyric acid (GABA) transport is facilitated by a number of proteins: the general amino acid permease (Gap1p), the proline permease (Put4p) and the 4-aminobutyrate permease (Uga4p) (Marini *et al.*, 1997). The uptake of GABA by these three transporters is tightly regulated and highly NCR-sensitive (Andre *et al.*, 1995). The regulation of *GAP1* by Ure2p will be discussed later in this chapter.

Transcriptional regulation of the *UGA4* gene. The regulation of *UGA4* is facilitated by negative and positive regulatory systems (Vissers *et al.*, 1989). The negative regulatory systems consist of Ure2p (which functions in the presence of a good nitrogen source) and Uga43p/Dal80p (which down-regulates transcription of *UGA4* when NCR is uplifted) (Figure 4). Ure2p functions by inactivating Gln3p, which is then unable to activate transcription of *UGA4*.

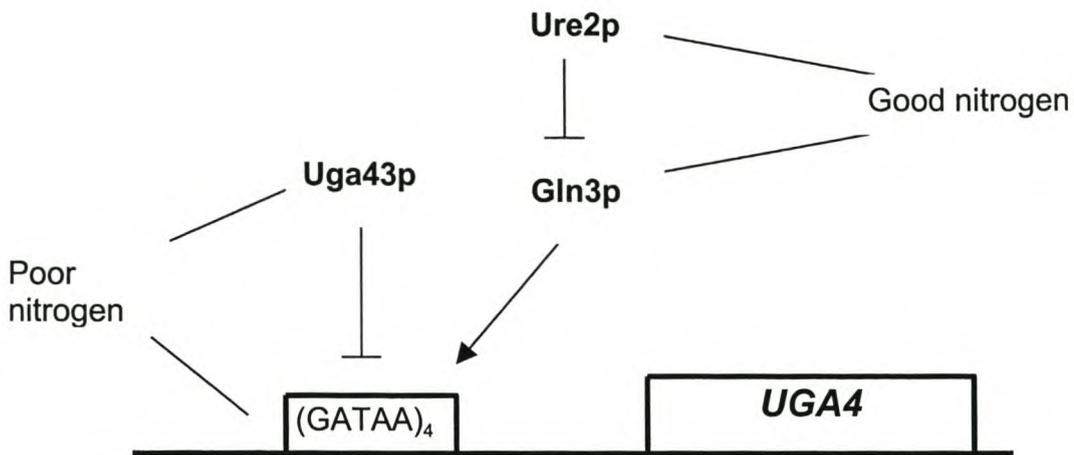


Figure 4. The *UGA4* gene is down-regulated at two levels: in the presence of a good nitrogen source via Ure2p and in the presence of a poor nitrogen source, Uga43p and Gln3p compete for the same binding site in the promoter of *UGA4*.

The promoter of *UGA4* contains a UAS_{NTR} that consists of four direct repeats, 5'-CGAT(A/T)AG-3'. Both Gln3p and Uga43p bind to this element. As soon as there is no good nitrogen source available, Gln3p binds the UAS_{NTR} , activate transcription of *UGA4* with eventual transport of GABA (Andre *et al.*, 1995; Cunningham *et al.*, 1994).

Induction of *UGA4* depends on the binding of two proteins to the UAS_{GABA} site of the *UGA4* promoter. These two positive regulatory proteins, the inducer-specific Uga3p (Andre, 1990) and the universal inducer protein *DAL81/UGA35/DURL*, bind DNA with Cys_6-Zn_2 finger motifs (Vissers *et al.*, 1990; Coornaert *et al.* 1991; Andre *et al.*, 1993). The Uga35p can bind to the UAS_{GABA} when GABA or allantoin is supplied as a nitrogen source. On the other hand, Uga3p only binds to UAS_{GABA} if the inducer molecule, GABA, is supplied as the nitrogen source (Figure 5). The UAS_{GABA} is 19bp long consisting of a 5'-CCG-3' triplet at each end, and a GC-rich core containing a perfect palindromic sequence 5'-CCGCCGGCGG-3' for *UGA4*. In the case of the *UGA1* gene an imperfect palindrome 5'-CCGCGGGCGG-3' is present in the promoter (Talibi *et al.*, 1995).

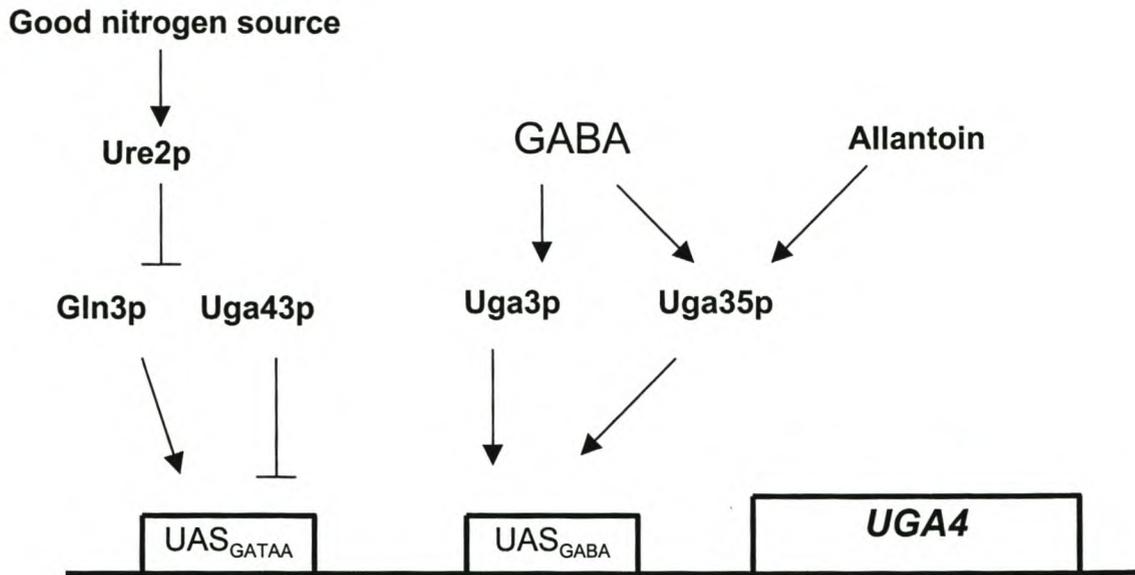


Figure 5. In addition to the regulation of *UGA4* by NCR (involving Ure2p, Gln3p and Uga43p), induction of *UGA4* transcription occurs (in the absence of a good nitrogen source) in the presence of GABA and allantoin via Uga3p and Uga35p.

Proline metabolism

The amino acid proline can be used as the sole nitrogen source by yeast. The yeast, however, grows remarkably slower on proline in comparison to other amino acids such as glutamine and asparagine. A possible reason is that proline has to be converted to glutamate first before it can be used as a nitrogen source (Figure 6). This requires additional catabolic enzymes whose function and synthesis put an extra demand for energy on the cell (Brandriss and Magasanik, 1979).

The proline degradation pathway shares intermediates with both the proline biosynthetic pathway and the arginine-ornithine catabolic pathway. The *PUT1* and *PUT2* genes are required by the yeast if proline is to be used as the sole nitrogen source (Brandriss and Magasanik, 1979). The transport of proline is facilitated by Put4p.

Xu *et al.* (1993) reported that *PUT1* expression is regulated by *URE2*, but independent from *GLN3*. Coffman *et al.* (1995) explained this observation by stating that the Put3p is responsible for the induction of *PUT1* in the presence of proline and that this might seem as if *PUT1* transcription is Gln3p-independent. The *PUT3* gene encodes for an activator protein that binds to a 21bp partial palindromic sequence that functions as an UAS in the transcription of both *PUT1* and *PUT2*. Activation by Put3p only occurs in the presence of proline, although it is bound to the promoter regardless the presence of proline (Siddiqui and Brandriss, 1989). *PUT1* is highly inducible and levels of mRNA increases up to 50-fold upon full induction in the presence of proline (Wang and Brandriss, 1986). There are two binding sites (UAS elements) for Put3p in the promoter of *PUT1* (Siddiqui and Brandriss, 1989). These two UAS elements function in combination with UAS_{NTR} to achieve transcriptional activation. The UAS_{NTR} and the UAS elements bound by Put3p will give an altered activation profile, compared to a promoter containing just UAS_{NTR} elements when the cells are grown on poor nitrogen sources (Rai *et al.*, 1995).

Coffman and co-workers (1995) contradicted Xu *et al.*'s (1993) findings that *PUT1* expression is Gln3p independent. They showed that Gln3p, Ure2p, as well as Gat1p (all of which are global regulators of NCR), influence the expression of *PUT1*. Northern blots done by this group on a strain with a *ure2GAT1gln3* background, showed *PUT1* was not transcribed when these cells were grown in glutamine. A *ure2GLN3gat1* background, however, permitted expression of the *PUT1* gene when the cells were grown on glutamine (Coffman *et al.*, 1996). Two-hybrid analysis indicated that Gat1p functions in the presence of a poor nitrogen source such as proline. Therefore, when cells are grown in proline, it can be expected that *GAT1* might function under these conditions as a transcriptional activator of *PUT1* (Coffman *et al.*, 1996).

The general amino acid permease

The *GAP1* gene encodes for the general amino acid permease. The Gap1p is an integral cell membrane protein that has 33 -40% homology with other amino acid permeases such as Can1p, Hip1p and Put4p (Jauniaux and Grenson, 1990). This permease is able to actively transport most naturally occurring neutral and basic L-

amino acids, except proline, and some of their analogs. The Gap1p functions at optimal ability under severe nitrogen limiting conditions. Gap1p activity decreases up to 400-fold when grown in good nitrogen sources. As in the case of the permeases discussed earlier, *GAP1* is also NCR-sensitive (Olivera *et al.*, 1993). In addition to its regulation at transcriptional level by NCR, Gap1p is also regulated post-translationally (Grenson, 1983a).

Regulation of Gap1p activity. Grenson and co-workers (1970) isolated three mutations that had an effect on the activity of Gap1p. The first mutant, *pgr*, affected only *GAP1*, whereas the other two mutations, *mut2* and *mut4*, liberated other ammonia-sensitive permeases from their regulation as well (Grenson and Acheroy, 1982). The *pgr* mutation was identified as a *cis*-acting element in the *GAP1* gene promoter with *MUT2* and *MUT4* exerting a negative effect, *trans*-acting on *GAP1* expression by binding to the PGR site. The *mut2* and *mut4* mutants were later identified as the *NPI1* and *NPI2* genes (Jauniaux and Grenson, 1990). At the time of identification of *pgr*, *mut2* and *mut4*, the *NPR1* (nitrogen permease reactivator) gene were also implicated in *GAP1* regulation. The *NPR1* gene seems to be a positive regulator of *GAP1* activity (Vandenbol *et al.*, 1990). This was determined by the observation that Gap1p was inactivated when *npr^{ts}* cells were transferred from a permissive temperature to a non-permissive temperature. The Npr1p does not affect *GAP1* transcription, but seems to be involved in the prevention or inactivation of degradation of Gap1p. Npr1p has a high degree of homology with protein kinases. It was therefore assumed that Npr1p activates Gap1p by phosphorylation, or it can have an indirect effect on Gap1p by countering Npi1p and Npi2p activity (Stanbrough and Magasanik, 1995). This is supported by the fact that Npr1p is not required for *GAP1* expression when *MUT2* and *MUT4* are impaired (Grenson, 1983b).

Gap1p contains a di-leucine motif in its C-terminal domain. Post-translation degradation or inactivation of Gap1p is induced by ammonia. This inactivation is achieved by ubiquitination of Gap1p at the di-leucine motif and requires the Npi1p and Rsp5p genes (Hein and Andre, 1997; Springael and Andre, 1998). Ubiquitination of proteins in yeast targets them for degradation.

Sophianopoulou and Diallynas (1993) implicated the *AUA1* gene (**A**mino acid **U**ptake **A**ctivation) in Gap1p activity. The mechanism by which *AUA1* works, has not yet been elucidated: Aua1p blocks the effect of ammonia on Gap1p, Aua1p interacts with Npr1p or Aua1p binds to an element in the promoter of *GAP1*. A *aua1* null mutant results in only 50% activity in comparison with the wild type.

Transcriptional regulation of *GAP1*. The transcriptional regulation of *GAP1* is NCR-sensitive. Jauniaux and Grenson (1990) were the first researchers to implicate Ure2p in *GAP1* regulation by northern blot analysis of *GAP1* in an *ure2* mutant strain. The repression of Ure2p on *GAP1* is achieved by the inhibition of Gln3p by Ure2p (Grenson, 1983a; Coffman *et al.*, 1994). Gln3p and Gat1p bind to five 5'-GATAA-3' sequences in the promoter of *GAP1*. Gln3p seemed to be the main activator when cells were grown in glutamate, whereas Gat1p functioned in combination with Gln3p as activators in urea- or proline-grown cells. Although mRNA levels were the same in cells grown in either glutamate or urea as sole nitrogen sources, there was a 15-fold higher activity in urea-grown cells (Stanbrough and Magasanik, 1995; 1996).

DEH1 (negative global regulator of NCR) also has an effect on *GAP1* transcription. The elimination of *DEH1* resulted in expression of *GAP1* by means of transcriptional activation by *GAT1* (positive global regulator of NCR). This observation was made in glutamine-grown cells with either a *NIL1gln3* or *nil1GLN3* genotype (Rowen *et al.*, 1997) (Rowen *et al.* referred to *GAT1* as *NIL1*).

Incorporation of ammonia ions into metabolites

The yeast has three sources of ammonia. Firstly, ammonia generated from the catabolism of poor nitrogen sources. Secondly, ammonia ions present in the environment and thirdly, ammonia generated from the utilization of good nitrogen sources that either release ammonia ions or takes part in a transamination reaction of α -ketoglutarate to form glutamate. The incorporation of ammonia, no matter what the source, occurs exclusively by its incorporation into α -ketoglutarate or glutamate

(Figure 7). The role of ammonia is further complicated by the fact that *S. cerevisiae* uses ammonia for inter colony communications (Palkova *et al.*, 1997).

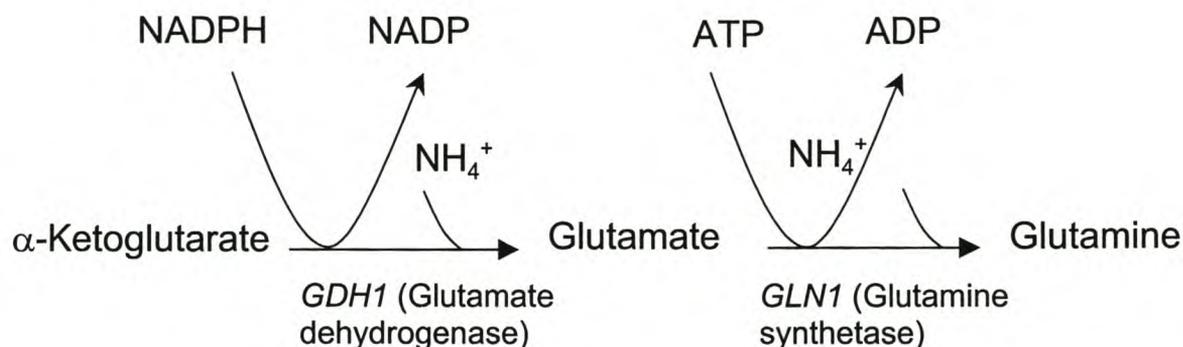


Figure 7. The incorporation of ammonia into α -ketoglutarate and glutamate to form glutamate and glutamine, via NADP-glutamate dehydrogenase and glutamine synthetase, respectively.

The NADP-dependent glutamate dehydrogenase, encoded by *GDH1*, is responsible for the reaction between α -ketoglutarate and ammonia to form glutamate (Figure 7). It was shown that *GDH1* contains two upstream CCAAT boxes at -333 and -438 that are essential for full activation of *GDH1*. It was proposed that these CCAAT boxes are part of the regulation of *GDH1* by carbon metabolism (Dang *et al.*, 1996). The influence of carbon metabolism on *GDH1* expression or activity is illustrated by the fact that Gdh1p activity is reduced by 80% if cells are starved for glucose (Mazon, 1978). Transcription of *GDH1* is also NCR-sensitive (Drillen *et al.*, 1973; Mitchell and Magasanik, 1984a; Courchesne and Magasanik, 1988). Dang and co-workers (1996) suggested that *GDH1* regulation is the point where carbon and nitrogen regulation are combined and co-ordinated.

GLN1, encoding for glutamine synthetase, is responsible for the incorporation of ammonia into glutamate to form glutamine (Figure 7). Transcriptional activation of *GLN1* depends on three regulatory systems (Figure 8). *GLN1* contains 5'-GATAA-3' sequences in its promoter to which Gln3p binds. *GLN1* is activated in the presence of ammonia and glutamate, but growth on glutamine results in repression of *GLN1*

transcription. *GLN1* mRNA levels decrease by 75% within 5 minutes after glutamine is added, indicating that *GLN1* transcripts are NCR-sensitive and relatively unstable with a half life of approximately 3 minutes (Benjamin *et al.*, 1989).

Glutamine, product of the reaction catalized by Gln1p, is a precursor for the synthesis of purines and many amino acids. Therefore, it is only logical that transcription of *GLN1* be regulated by purines and the availability of amino acids. When cells are starved for purines, there is an increase in activity of Gln1p and neither *GLN3* nor *GCN4* are required. Even when glutamine is present, a 10-fold increase in activity of Gln1p was seen in response to purine starvation (Mitchell and Magasanik, 1984b). The Gcn4p protein is an activator protein responsible for activation of some 30 genes in the biosynthesis of eleven amino acids in response to amino acid starvation (Albrecht *et al.*, 1998). Glutamine is a precursor of several of these amino acids, therefore, *GLN1* is activated when cells are starved for amino acids. Gln1p is synthesized to incorporate ammonia to form glutamine (Figure 8).

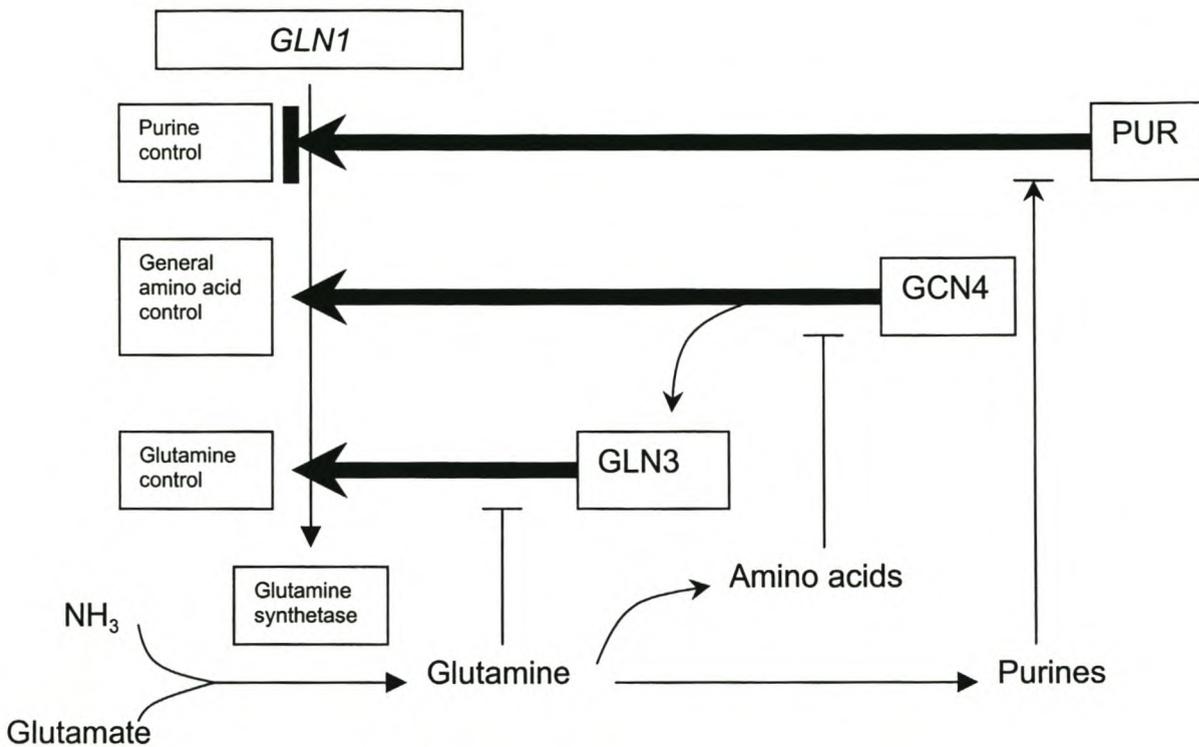


Figure 8. Three regulatory circuits controlling transcription of *GLN1* (↑) positive regulation, (⊥) negative regulation, (↑⊥) positive or negative regulation (Adapted from Mitchell and Magasanik, 1984a)

Degradation of poor nitrogen sources to generate ammonia has been discussed in relative detail. In the following section, uptake of ammonia ions from the environment by ammonia permeases will be discussed since these permeases are also NCR-sensitive.

Uptake of ammonia. When ammonia is present at a relatively high concentration in the environment, ammonia diffuses across the cell membrane. However, when extracellular ammonia reaches relatively low concentrations, it is transported actively by three proteins: Mep1p, Mep2p and Mep3p. Strains lacking these three proteins are not able to grow on media containing less than 5mM ammonium as the sole

nitrogen source. The individual proteins can facilitate transport of ammonia by themselves and therefore allow growth at low ammonium concentrations. These proteins are, however, not essential for growth when the ammonium concentrations exceed 20 mM. The main difference between these three proteins is probably their affinity towards ammonium. Mep2p displays the highest affinity (K_m of 1 to 2 μ M), followed by Mep1p (K_m of 5 to 10 μ M) with Mep3p displaying quite a low affinity of 1.4 to 2.1 mM (Marini *et al.*, 1997).

The presence of 5'-GATAA-3' sequences in upstream regions of these three transporter genes indicates that NCR might play a role in the regulation of their expression. Expression of *MEP2* requires the activator proteins Gln3p and Gat1p, depending on the nitrogen source available. In proline grown cultures, Gln3p and Gat1p activate transcription with both being able to compensate for the other activator's absence. However, in glutamate-grown cells, *MEP2* expression is entirely dependent on Gln3p. The transcription of *MEP3* and *MEP1* is dependent on Gln3p for activation, regardless of whether the nitrogen source is proline or glutamate. Marini *et al.* (1997) observed that Gat1p actually inhibited the expression of *MEP1* and *MEP3* during growth on poor nitrogen sources. This is probably due to the fact that Gat1p binds to the 5'-GATAA-3' site, hindering Gln3p from binding and resulting in failure to transcribe *MEP1* and *MEP2*.

The *mep2* mutant cannot form pseudohyphae. The Mep2p protein is essential for pseudohyphal differentiation in response to an ammonium shortage. It was proposed by Lorenz and Heitmann (1998a) that Mep2p functions as an ammonium sensor, which is responsible for generating a signal in an ammonium, deprived environment, resulting in pseudohyphal growth to fulfill the yeast's nitrogen requirements.

Strains lacking *MEP2* are defective in pseudohyphal growth when ammonia acts as the source of nitrogen. Homozygous diploid mutants of *GLN3*, *URE2* and *NPR1*, are also defective for pseudohyphal growth in ammonia, glutamine and proline respectively. This indicates that Mep2p is not the only protein responsible for sensing nitrogen. Confirmation of this was made when *MEP2* was re-introduced

under the control of the inducible *GAL1* promoter in cells in which *GLN3*, *URE2* and *NPR1* were deleted. These transformed mutants could still not form pseudohyphae (Lorenz and Heitman, 1998a; 1998b).

Proteins responsible for Nitrogen Catabolite Repression

The genes that encode the enzymes responsible for assimilation of poor nitrogen sources are tightly regulated. Proteins that enforce NCR facilitate this regulation (Coffman *et al.*, 1994). An environment containing only poor nitrogen sources will induce the transcriptional activation of the catabolic enzyme genes that are NCR-sensitive. In the presence of a good nitrogen source, these NCR-sensitive genes are down-regulated, resulting in low levels of expression (Daugherty *et al.*, 1993).

There are a number of ways by which *S. cerevisiae* manifests NCR. Firstly, NCR facilitated by the Ure2p inactivates Gln3p and an unidentified protein or proteins, resulting in the repression of genes responsible for assimilation of poor nitrogen sources. In some instances Ure2p enforces NCR without the involvement of Gln3p (Coffman *et al.*, 1995). Secondly, proteins with homology to Gln3p also act as regulatory proteins; these include Gat1p, Deh1p and Dal80p (Figure 9). As in the case of Gln3p, these proteins contain zinc fingers that bind specifically to elements containing 5'-GATAA-3' sequences in the promoters of genes responsible for assimilation of poor nitrogen sources. The presence of 5'-GATAA-3' sequences in promoters indicate that a particular gene might be NCR sensitive (Coffman *et al.*, 1994). Thirdly, there are unidentified regulatory proteins that are also involved in NCR (Coffman and Cooper, 1997).

The mechanism of NCR is depicted in Figure 9. Data generated by several research groups have identified five proteins that are involved in the global regulation of NCR. Gln3p and Gat1p are activator proteins, Dal80p, Deh1p and Ure2p are repressors. Gln3p, Gat1p, Dal80p and Deh1p all bind DNA whereas Ure2p does not.

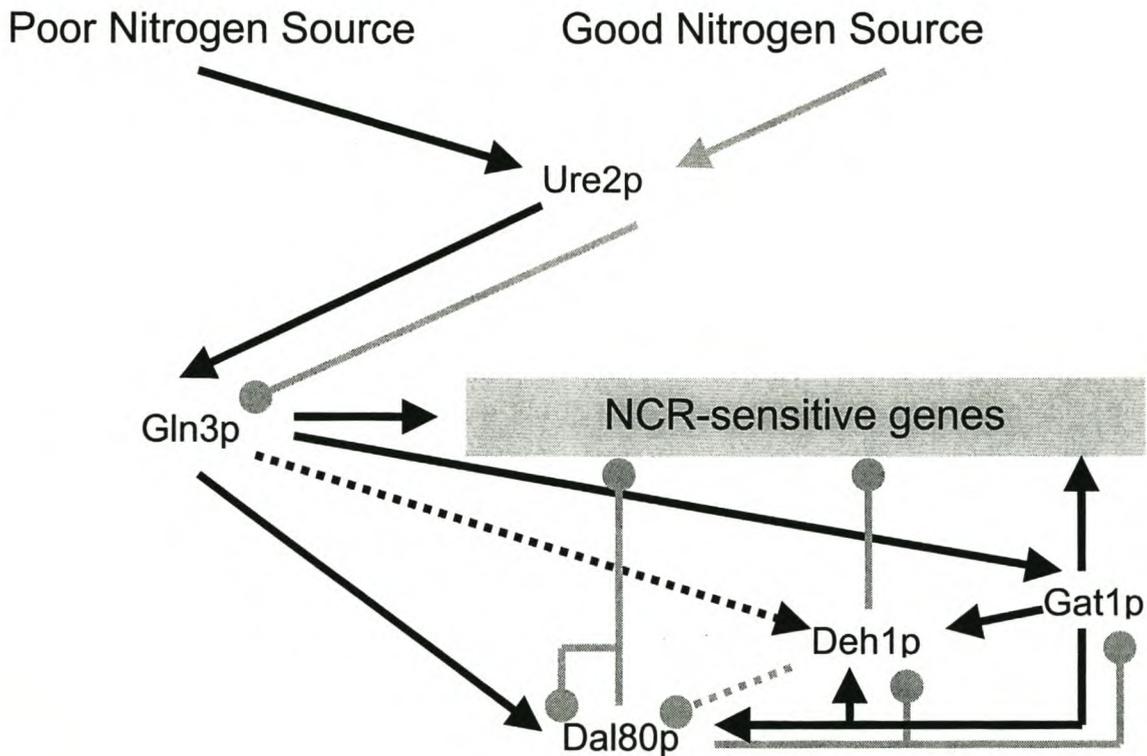


Figure 9. Integrated schematic of the proteins responsible for nitrogen catabolite repression. Arrows indicate positive regulation and bars ending in a circle indicate negative regulation.

The *URE2* protein

The *URE2* gene was first isolated as two mutants that altered the expression of the genes encoding for the two glutamate dehydrogenases of *S. cerevisiae* (Drillien *et al.*, 1973). Since one of these mutants was later identified as a prion-like protein a discussion of the prion-like characteristics of Ure2p is necessary to get a global picture of Ure2p's function in *S. cerevisiae* (Wickner, 1994).

Protein structure of Ure2p. The Ure2p is a 40.224 kDa protein of 354 amino acids that can be divided into two domains; a N-terminal domain and a C-terminal domain (Wickner, 1994). The N-terminal domain consists of 40% asparagine and 20% serine and threonine residues (Wickner, 1994). This domain is responsible for

conversion of Ure2p to its prion-like isoform, [URE3]. A prion is a protein that misfolds. This misfolding results in a change from a predominantly alpha-helix structure to a beta-sheet structured protein. The C-terminal domain is responsible for its cellular function as regulatory protein of NCR (Masison and Wickner, 1995; Wickner 1994).

Prion formation by the N-terminal domain. The prion-like isoform of Ure2p is known as [URE3]. The initial observation was that [URE3] and *URE2* are independent from each other (Drillien and Lacroute, 1972). Indications that [URE3] and Ure2p are linked were attributed to the fact that both have similar phenotypes, although the *ure2* is inherited in a Mendelian way and [URE3] non-Mendelianly (Aigle and Lacroute, 1975). Both *ure2* and [URE3] mutants are able to use ureidosuccinate in the presence of ammonium. Wickner (1994) presented evidence that [URE3] is an altered protein form of Ure2p and that [URE3] is a prion-like protein in yeast. The N-terminal, in particular, was identified as essential in prion formation and therefore referred to as the “prion-inducing” domain. There are a number of reasons why [URE3] is considered to be a prion-like protein and will be discussed in more detail:

- ◆ Overproduction of Ure2p results in elevated levels of [URE3].
- ◆ The propagation of [URE3] depends on the chromosomal *URE2* gene.
- ◆ Reversible curability of [URE3].

In addition to these reasons, there are two more observation that strengthen the argument that [URE3] is a prion-like protein:

- ◆ [URE3] has a fibrillar structure.
- ◆ Translation imbalance can result in [URE3] formation.

Overproduction of Ure2p results in elevated levels of [URE3]. Prion studies done in mice revealed that prion formation depends to a great extent on the PrP (prion protein) gene dosage (Carlson *et al.*, 1994). The overproduction of Ure2p on a multi-copy plasmid results in the formation of [URE3] at higher frequencies. This can be as high as 200-fold more than in strains with the normal *URE2* dosage (Masison and Wickner, 1995) (Figure 10). This was determined by expressing *URE2* under the

GAL1 promoter in two separate nitrogen conditions: proline (a poor nitrogen source) and ammonia (a good nitrogen source). Both these nitrogen sources were tested in combination with two sugars, dextrose or galactose. The *URE2* gene is under the control of the *GAL1* promoter and therefore its transcription will be repressed in media containing glucose and induced in media containing galactose. It was found that [URE3] was only formed in significant numbers when galactose was used as a carbon source instead of dextrose and that the quality of nitrogen source had no influence on post-translational modification of Ure2p that might result in [URE3]. This is a clear indication that overproduction and not the nitrogen source had an effect on the formation of [URE3] (Masison *et al.*, 1997).

Propagation of [URE3] depends on the URE2 gene. In the early 1980's, Chesebro and co-workers (Chesebro *et al.*, 1985) cloned a human genomic gene of which the protein can misfold to a prion in humans. In addition to misfolding of into a prion, the gene encoding for this protein is expressed at the same levels in infected and uninfected organisms (Chesebro *et al.*, 1985; Oesch *et al.*, 1985). Wickner (1994) showed [URE3] to be dependent on the *URE2* gene for its propagation (Figure 10).

Reversible curability of [URE3]. After a prion phenotype has been cured, it should have the ability to revert spontaneously since a prion is encoded by a native gene in the yeast, unless the agent responsible for the prion phenotype is a foreign piece of DNA or RNA. [URE3] cells cured with 5 mM guanidine reverted back to a [URE3] phenotype spontaneously and investigations confirmed that [URE3] was reformed. This is unlike nucleic acid replicons which, once cured, do not return unless re-introduced from other cells (Wickner, 1994; Wickner *et al.*, 1999) (Figure 10). Further evidence indicating that Ure2p is a prion, is the fact that *URE2* mRNA could not induce prion formation. This was established by making frame shift deletions in the *URE2* gene. The mutated gene's protein was able to complement the mutant, but could not significantly induce [URE3] formation in comparison to the wild type. This indicates that protein, and not nucleic acid, is responsible for [URE3] formation. If foreign nucleic acid was responsible for the formation of [URE3], it needs to be re-introduced to develop the [URE3] phenotype (Wickner, 1994).

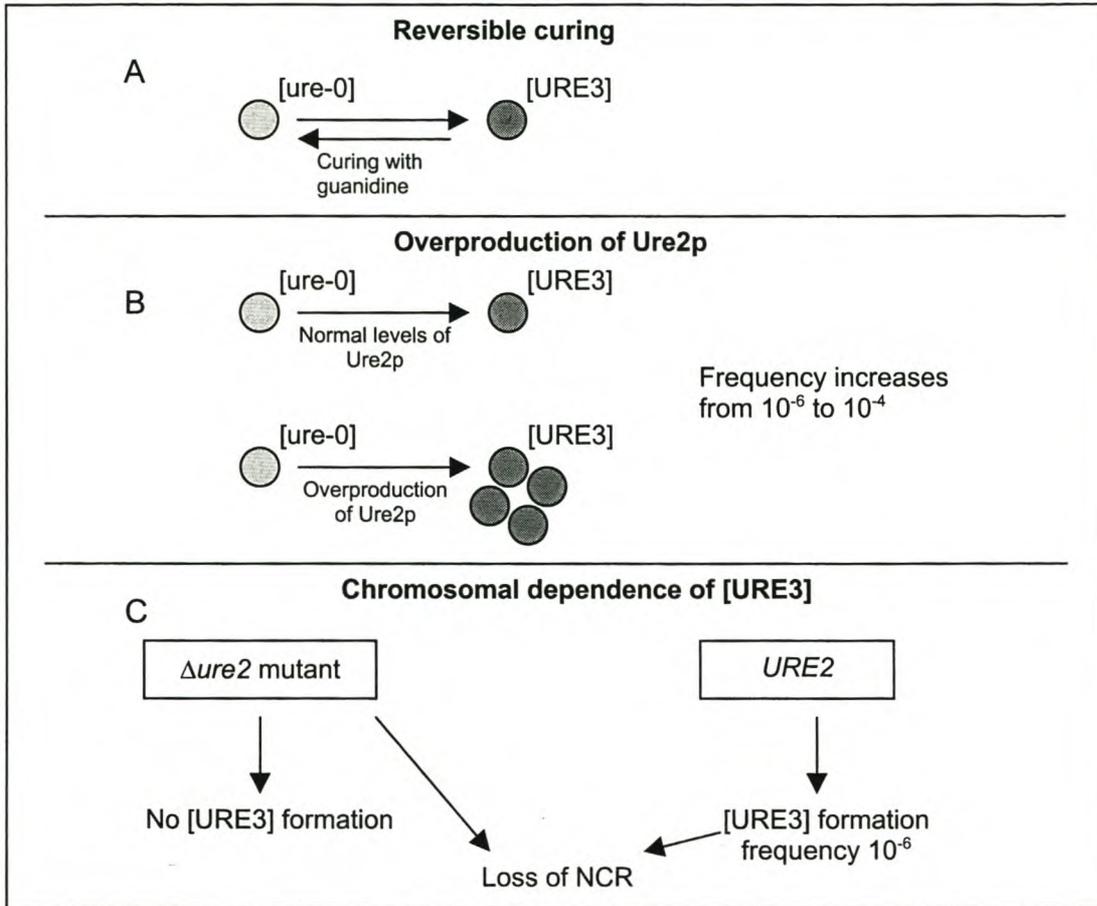


Figure 10. Genetic evidence that [URE3] is a prion-like protein. (A) Reversible curability of [URE3]. (B) The overproduction of Ure2p results in formation of [URE3] at higher frequencies. (C) *ure2* and [URE3] have the same phenotypes because both lack the normal Ure2p. [URE3] is also dependent on the *URE2* gene.

[URE3] has a fibrillar structure. Prions in mammals have been detected as abnormal fibrils. Electron microscopy studies revealed that aggregated [URE3] has a fibrillar structure similar to that of prion proteins in mammals (Forloni *et al.*, 1993; Thual *et al.*, 1999). The fibrillar structure of mammalian prions is highly resistant to proteinase K. This was also found for [URE3] (Masison and Wickner, 1995). In-depth studies revealed that the N-terminal domain has a higher sensitivity to proteinase K treatment than the C-terminal domain of Ure2p (Thual *et al.*, 1999). Treatment with proteases revealed that different sized peptides of the N-terminal

were not stable for more than one minute, whereas some of the C-terminal domain polypeptides were stable for more than an hour (Edkes *et al.*, 1999).

It was shown *in vitro* that synthetic ure2p1-65 induced full-length Ure2p to co-polymerize. This polymerization takes on the form of an amyloid structure which is defined as a filamentous protein structure that stains with Congo red (CR) to produce green birefringence under polarized light. This amyloid structure is usually protease resistant and has an anti-parallel β -sheet structure. Additional studies done on ure2p1-65 to see if it could induce an amyloid structure in other proteins, revealed that none of these proteins could enter the peptide precipitate with ure2p1-65. The proteins used were BSA, γ -globin, myoglobin, ovalbumin, thyroglobin, ribonuclease A and aldolase. Introduction of Alzheimers β -peptide, a peptide with an amyloid structure, could not induce the full length of Ure2p to enter with the β -peptide into a precipitate. This strongly suggests that ure2p1-65 has specificity to full length Ure2p only and *vice versa* (Taylor *et al.*, 1999). Ure2p has been isolated as a 130 kDa fraction from a gel filtration chromatography column, although Ure2p has a molecular weight of 42 kDa. Sedimentation studies revealed that Ure2p is a heterogenous mixture of monomeric, dimeric, tetrameric as well as higher oligomers, when Ure2p was expressed in *E. coli* (Thual *et al.*, 1999).

URE2 has been expressed in *E. coli* to generate sufficient quantities to study the formation of [URE3]. In the expression of the heterologous proteins codon usage comes into play. It is known that the AGA codon for arginine is extremely slowly expressed in *E. coli*. Attempts to enhance the expression of Ure2p in *E. coli* revealed that if the AGA codons at positions 253 and 254 could be changed to CGT, it would enhance expression up to 100-fold in *E. coli*. This is a great innovative tool to generate enough protein for structural studies (Komar *et al.*, 1998).

Translation imbalance results in [URE3] formation. The role of chaperone molecules in protein folding has been established and is well known for the other prion-like protein, [PSI], in *S. cerevisiae* (Newman *et al.*, 1999). Experiments were done where Ure2p was translated *in vitro* in cell free wheat germ extracts (WGE) and rabbit reticulate lysate (RRL). *URE2* translated in WGE was significantly more resistant to

proteinase K than when it was synthesized in RRL (Komar *et al.*, 1997). The prolonged proteinase K resistance of Ure2p in WGE is characteristic of [URE3]. An imbalance of the cellular translation system, or the role of chaperone molecules, might play a far more important role in Ure2p folding than we think.

Analysis of [URE3] by deletion studies. Deletion studies revealed that removal of the amino-terminal domain, amino acids 2 to 65, eliminated the ability of the Ure2p to induce [URE3] when overexpressed. The remaining C-terminal on a single-copy plasmid under the control of the *URE2* promoter had a reduced ability to complement a chromosomal *ure2* deletion and induce [URE3] formation (Masison and Wickner, 1995).

The C-terminal domain of Ure2p is only inactivated if covalently linked to an N-terminal-domain. This was shown by the fact that if Ure2p C-terminal and N-terminal fragments were expressed separately in *ure2* deletion strains, they were still NCR functional. This was also found when [URE3] was introduced in a strain where functional fragments of the C-terminal domain were expressed. Expression of both these fragments in wild type cells resulted in a loss of NCR. This data strongly suggest that the interaction of the N-terminal domains are responsible for the conversion to [URE3] when [URE3] is introduced into Ure2p cells (Masison *et al.*, 1997).

The N-terminal and C-terminal are not two domains acting on their own. They have specific interactions that are suggested by the following: the N-terminal improves the C-terminal's ability to maintain NCR when required; the C-terminal seems to stabilize the N-terminal and therefore reducing the chance of forming [URE3]; and the C-terminal is not altered unless it is attached to the N-terminal (Masison and Wickner, 1995).

Cellular function lies within the C-terminal domain. The deletion of *URE2* not only uplifts the down-regulation of NCR sensitive genes (Coffman *et al.*, 1994), but also grows remarkably slower than the isogenic wild type strain (Coschigano and Magasanik, 1991). A *ure2* mutant constructed by Marczak and Brandriss (1989)

showed a temperature sensitivity phenotype. Three hours of heat shock at 45°C resulted in a reduced ability to recover when placed back at 30°C compared to its isogenic wild type strain. A homozygous diploid strain of the same *ure2* mutant was slightly impaired in its ability to sporulate. Pseudohyphal growth of the *ure2* mutant was also impaired when grown on low concentrations of ammonia.

It is hypothesized that Ure2p has either one or two interacting domains. The first domain, situated in the N-terminus, can interact with itself or with the second domain in the C-terminus. The domain in the N-terminus is referred to as the α - domain and the second, in the C-terminus, as the β -domain (Figure 11) (Fernandez-bellot *et al.*, 1999). This was elucidated by two-hybrid analysis that also revealed that Ure2p is usually a dimer in the cell (Edkes *et al.*, 1999). Cellular extraction, followed by its analysis, revealed that Ure2p is a non-nuclear protein (Wickner, 1994). This is confirmed by the fact that GFP-Ure2p showed fluorescence only in the cytoplasm (Edkes *et al.*, 1999).

Studies to determine how Ure2p functions and with what other proteins it has interaction with, revealed nothing conclusive yet. *URE2-lacZ* fusions revealed that *URE2* expression, as well as its translation, are not regulated by the available nitrogen source (Coschigano and Magasanik, 1991). At the moment the manner in which Ure2p inhibits Gln3p is a hotly debated issue.

Epistatic effect of *URE2* and *GLN3* was first reported by Courchesne and Magasanik (1988). Coschigano and Magasanik (1991) suggested that Ure2p inhibits Gln3p by transferring a glutathione molecule to Gln3p. This is questionable because in the area of the Ure2p that is homologous to glutathione-S-transferase, it lacks an essential arginine residue. There are two areas in Ure2p which have homology to glutathione-S-transferases, amino acids 158-172 and 300-314. Ure2p shares homology to Gtt1p and Gtt2p, two glutathione-s-transferase proteins in *S. cerevisiae*, GSTD1 in *Drosophila melanogaster* and GSTT1 in humans (*Homo sapiens*) (Choi *et al.*, 1998). This homology of Ure2p with glutathione-S-transferase is highly relevant because this homology is shared in the last 300 amino acids of Ure2p. The NCR

regulation domain of Ure2p has thus far been assigned to these last 300 amino acids.

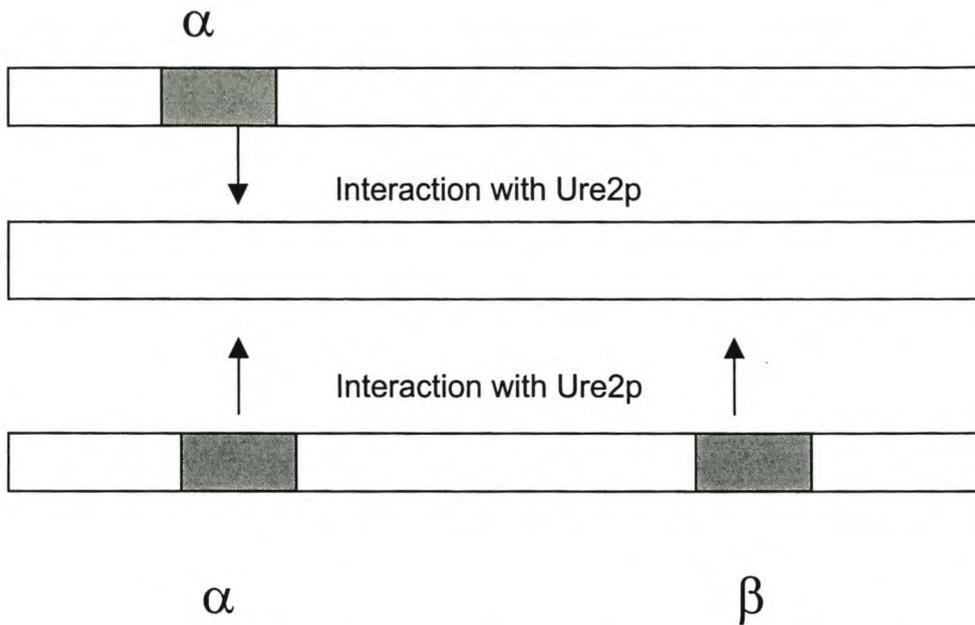


Figure 11. The domains of the Ure2p that are responsible for interaction with itself (Fernandez-bellot *et al.*, 1999).

Eaglestone *et al.*, (1999) suggested that the formation of the prion isoform ([PSI]) of Sup35p might be a mechanism by which the yeast regulates its response to environmental stress. This mechanism is however not applicable to *URE2* and [URE3] (Masison *et al.*, 1997).

Apart for *URE2*'s NCR function, it has been implicated in ion tolerance to Na⁺, Li⁺ and Mn⁺ ions. The removal of Ure2p results in a higher resistance to high concentrations of ions, however, it requires a functional Na-ATPase (PMR2/ENA1) and Gln3p (Withee *et al.*, 1998). This *ure2* phenotype broadens the functions of Ure2p in the cell. Ions have been implicated in amino acid transport, as well as regulating intracellular pH. In the presence of ethanol, increased K⁺ and Na⁺ ion concentrations resulted in increased oxygen consumption (Uribe *et al.*, 1991). In the absence of K⁺ transporters (*TRK1* and *TRK2*) a mutant of *BAP2* was found. The presence of *BAP2* relieved the cell's sensitivity to low pH. A similar mutant was

constructed in *HIP1*, the gene encoding the histidine permease. This mutant exhibited the same phenotype as the *BAP2* mutant. Increased Rb^+ uptake was also observed for both mutants (Wright *et al.*, 1997).

The *GLN3* protein

The *GLN3* gene was isolated as three different mutant phenotypes that were unable to derepress glutamine synthetase during glutamine limitation (Mitchell and Magasanik, 1984a). Gln3p is a transcriptional activator of the majority of NCR sensitive genes under conditions when NCR is not functioning (Cunningham *et al.*, 1996). These NCR sensitive genes include regulators of both NCR and catabolic genes. Regulatory genes that are positively influenced by Gln3p are *DAL80* (Coffman *et al.*, 1997), *GAT1* (Rowen *et al.*, 1997) as well as some pathway specific proteins (Torusczy *et al.*, 1984; Coornaert *et al.*, 1991; Olive *et al.*, 1991). Examples of catabolic genes that are positively influenced include *RPB1* (Naik *et al.*, 1997), *GLT1* (Valenzuela *et al.*, 1998), *ASP3* (Bon *et al.*, 1997), *GLN1*, *PUT1* and *PUT2* (Daugherty *et al.*, 1993). Gln3p is crucial for the expression of several genes encoding permeases; the allantoin permease (*DAL5*) (Cooper *et al.*, 1990), general amino acid permease (*GAP1*) (Stanbrough *et al.*, 1995) and arginine specific permease (*CAN1*) (Daugherty *et al.*, 1993).

Gln3p consists of 730 amino acids and has a molecular weight of about 55 kDa. *GLN3* transcription is minimally, if at all, regulated by a nitrogen source (Minehart and Magasanik, 1991; Blinder and Magasanik, 1995). Northern blot analysis revealed that *GLN3* expression in cells grown in glutamate was less than two-fold higher than cells grown in glutamine (Minehart and Magasanik, 1991). This was confirmed with *GLN3-LacZ* fusions that revealed its transcription, as well as its translation, was not influenced by the nitrogen source available (Coschigano and Magasanik, 1991). Gln3p has two domains: a zinc finger to bind DNA and an acidic activation domain to help initiate transcription of NCR sensitive genes.

Gln3p has an acidic activation domain. Many transcription factors are characterized by a high percentage of acidic amino acids in their N-terminal domain (Rowen *et al.*, 1997). Svetlov and Cooper (1997) identified a stretch of 13 amino acids, QQNGEIAQLWDFN, in the N-terminal domain of Gln3p that are responsible

for the activation of transcription. These 13 amino acids, with an acidic characteristic, are hypothesized to form an α -helix believed to have interaction(s) with other proteins that bind to *cis*-acting elements in promoters.

The DNA-binding domain contains a zinc-finger. The DNA-binding domain of Gln3p contains a single Cys₂/Cys₂ zinc-finger at amino acids 306 to 330 (Minehart and Magasanik, 1991). The zinc finger motif is well established as a binding domain of DNA in yeast (Bohm *et al.*, 1997). This zinc-finger is proposed to bind the 5'-GATAA-3' sequences in the promoters of NCR-sensitive genes. This is suggested by the fact that purified Gln3p was able to bind 5'-GATAA-3' -containing regions in the promoters of *DAL3*, *GLN1*, *PUT1*, *UGA4*, *CAR1* and *GDH2* *in vitro* (Cunningham *et al.*, 1996). The elements containing 5'-GATAA-3' sequences are known as UAS_{NTR} (upstream activation sequence). The minimal sequence requirement for UAS_{NTR} activity has been identified as a dodecanucleotide sequence 5'-TTNCTGATAAGG-3' (Bysani *et al.*, 1991). These UAS_{NTR} elements should not be confused with URS_{GATAA} (upstream repressor sequence) sites, since Gln3p is able to bind URS sites. The difference between a UAS_{NTR} and URS_{GATAA} element is that the UAS's contains a single 5'-GATAA-3' sequence where as the URS_{GATAA} contains two 5'-GATAA-3' sequences 15 to 30bp apart (Cunningham and Cooper, 1993) (Figure 12). One such example where Gln3p binds to a URS_{GATAA} to exert a positive effect is in the case of *CAR1* expression. Binding of Gln3p to a 5'-GATAA-3' sequence does not necessarily mean that it activates transcription. It can bind to a URS_{GATAA} site and eliminate the repression effect of Dal80p by steric hinderance.

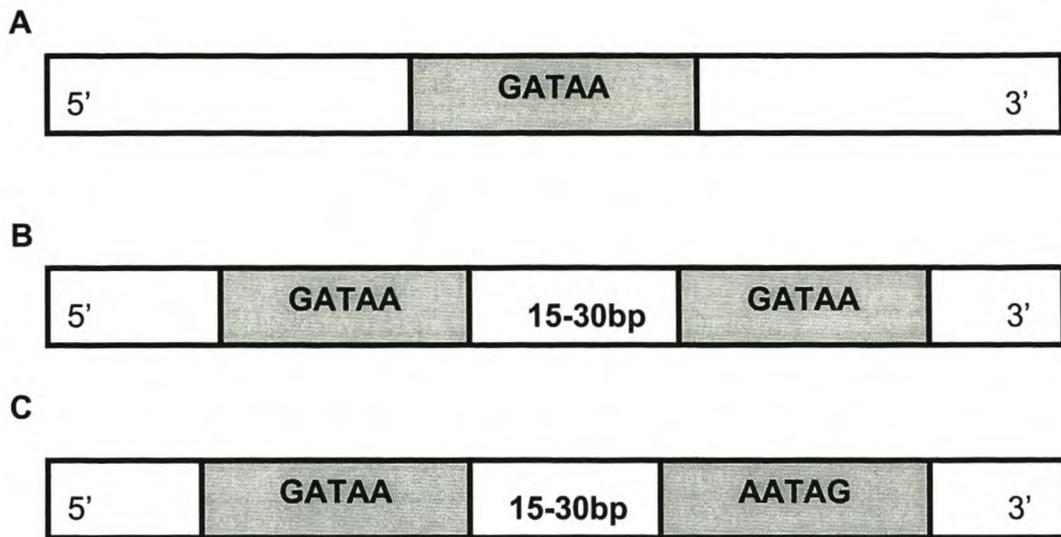


Figure 12. Organization of GATAA sequences in A) UAS_{NTR} element, B) URS_{GATAA} element and C) URS_{GATAA} element.

Phenotypes of *gln3* mutants and Gln3p's physiological significance. Studies on the physiological significance of *GLN3* revealed that if this gene is eliminated the yeast grows significantly slower than the wild type (Smith *et al.*, 1996). This is fascinating because the same effect is observed when *GLN3* is overexpressed, with the yeast having a doubling time of over 10 hours in minimal media. This slow growth of the overexpressed *GLN3* can be suppressed by simultaneous overproduction of Ure2p. This is a typical epistasis effect and indicates that Ure2p and Gln3p function closely together if not interacting with each other directly. In the presence of a good nitrogen source such as glutamine, overexpression of *GLN3* relieves the effect that NCR has on the synthesis of glutamine synthetase (*GLN1*) (Blinder *et al.*, 1996). Diploid strains with deleted *GLN3* are also defective in pseudohyphal growth when a low ammonium concentration, glutamine or proline are used as the sole nitrogen sources (Lorenz and Heitman, 1998a).

Although the *GLN1* gene is activated by Gln3p, *gln3* null mutants retain their ability to regulate synthesis of glutamine synthetase (Gln1p) in response to adenine, suggesting that Gln3p is not involved in purine control of Gln1p (Figure 8). *GLN1* expression can occur independent of Gln3p. This transcriptional regulation might be

due to the fact that glutamine, the product formed by Gln1p, is a requirement for adenine synthesis. The need for adenine drives the synthesis of glutamine synthetase. Addition of adenine to media containing glutamate as the sole nitrogen source, resulted in reduction of Gln1p levels. This purine control of *GLN1* was barely sufficient to allow growth in the absence of glutamine. This was concluded from the fact that *ADE⁺*, *gln3* strains grew much slower in the absence of adenine compared to when adenine was added (Minehart and Magasanik, 1991). Transcriptional regulation of *GLN1* is an example indicating that NCR-sensitive genes are regulated by other environmental factors as well.

Double mutations of *GLN3* with other genes give us an indication of the physiological significance of Gln3p and where it plays a role in the cells' metabolism. A double mutation of *gdh1 gln3* resulted in an inability to grow when ammonia was the sole nitrogen source. This phenotype can be complemented by the overproduction of glutamine synthetase (Gln1p) (Miller and Magasanik, 1990). This phenotype can be explained as follows; removal of Gln3p in this double mutant greatly impairs incorporation of ammonia ions to form glutamate from α -ketoglutarate in yeast cells. This is overcome to some extent however by overexpression of glutamine synthetase. Glutamine synthetase is responsible for incorporation of ammonia into glutamate to form glutamine. Deletion of *GLN3* in combination with *GAT1* results in abolishment of *DEH1* expression, but neither of the two single mutations could achieve the same effect as the double mutant (Soussi-Boudekou *et al.*, 1997). This double null mutant also failed to express *GLN1*, *GDH2*, and *GAP1* under any type of nitrogen source (Stanbrough *et al.*, 1995). Therefore, there is some functional redundancy shared between *GLN3* and *GAT1*. It was reported recently that Gan1p/Ada1p interact with both Gln3p and Gat1p as a co-activator of transcription of some genes; *GLN1*, *GDH2*, *GAP1*, *PUT4*, *MEP2* and *GDH1* (Soussi-Boudekou and Andre, 1999).

The mechanism by which Gln3p is inactivated in the presence of a good nitrogen source is a topic of dispute. It was concluded from co-immunoprecipitation studies that Gln3p and Ure2p interact; both Gln3p and Ure2p were precipitated with anti-

Gln3p antibodies. This could not be confirmed with anti-Ure2p antibodies (Blinder *et al.*, 1996; Minehart and Magasanik, 1991).

The *GAT1* protein

***GAT1* is Gln3p-dependent and Dal80p-regulated.** Gat1p functions as a weak transcriptional activator of a number of NCR-sensitive genes (Coffman *et al.*, 1996). *GAT1* influences the transcription of catabolic genes, as well as some of the other regulators in NCR such as *DAL80* and *DEH1* (Coffman *et al.*, 1997; Soussi-Boudekou *et al.*, 1997). *GAT1* itself is NCR-sensitive because it has Gln3p- and Dal80p-binding sites in its promoter (Coffman *et al.*, 1996). *GAT1* expression is almost totally eliminated in a *gln3* background (Rowen *et al.*, 1997). These findings suggest that *GAT1* transcription is Gln3p-dependent, but also Dal80p-regulated (Coffman *et al.*, 1996). In the absence of Gln3p, Gat1p is able to activate the transcription of *GAP1* and partially activate transcription of *GLN1* when *gln3* mutant cells are grown in urea (Stanbrough and Magasanik, 1995). When cells are grown in glutamine, transcriptional activation by Gat1p is antagonized by Deh1p (Rowen *et al.*, 1997).

Gat1p has homology to Gln3p. Gat1p has 29% identity to Gln3p over 217 amino acids. The sequence similarity includes the area containing the zinc finger motif, a characteristic shared with Gln3p and Dal80p (Coffman *et al.*, 1996). This implies that Gat1p could bind DNA with a zinc finger domain. The N-terminal of Gat1p also has a typical acidic characteristic shared with many other transcription activators (Rowen *et al.*, 1997; Stanbrough *et al.*, 1995).

The *DAL80* protein

In addition to the two global activator proteins of NCR that bind DNA, there are Dal80p and Deh1p which act as repressors through the binding of URS elements in promoters of NCR-sensitive genes. Dal80p only functions in an environment where NCR is uplifted. *DAL80* transcription requires both Gat1p and Gln3p (the two positive regulators in the establishment of NCR) (Coffman *et al.*, 1997). This transcriptional dependency of *DAL80* on Gln3p and Gat1p is one of many ways to

fine-tune transcription of the catabolic genes that are subject to the regulation by NCR. Not only does Dal80p down regulate NCR-sensitive genes, but it also down-regulates its own expression, and that of *DEH1* (Soussi-Boudekou *et al.*, 1997). In addition, it can also function as an antagonist to Gln3p in the nucleus (Rowen *et al.*, 1997). Genetic studies where the *DAL80* gene was deleted, revealed that in medium containing proline as a nitrogen source, elevated expression of Gln3p-dependent genes occurred (Rowen *et al.*, 1997).

Dal80p contains a zinc finger and leucine zipper motif. As with Gln3p and Gat1p, Dal80p contains a zinc finger motif that binds to URS_{GATAA} elements in the promoters of NCR-sensitive genes. The zinc finger of Dal80p is similar in structure to that of Gln3p (Stanbrough *et al.*, 1995). In addition to the zinc finger, Dal80p also contains a leucine zipper (Cunningham and Cooper, 1991; Cunningham and Cooper, 1992; Coornaert *et al.*, 1992). This leucine zipper motif is responsible for protein-protein interaction with Dal80p itself or with Deh1p (Svetlov and Cooper, 1998). Deh1p will be discussed later in this study. These protein interactions can result in the formation of three types of complexes: Dal80p-Dal80p; Dal80p-Deh1p and Deh1p-Deh1p. This homo- and heterodimerization of Dal80p with itself and with Deh1p can determine the specificity and affinity of the protein complex that binds to the promoters of NCR sensitive genes. This can result in different mechanisms of repression. There are, however, no other data available explaining this homo- and heterodimerization and its role in the yeast cell.

Suppression of slow growth by overexpression of Dal80p. As in the case with the other global regulators, *DAL80* has also been overexpressed. A strain of *S. cerevisiae* devoid of the ammonium permeases *MEP1* and *MEP2* grows slower and is unable to form pseudohyphae. Studies to explain this phenomenon revealed that if *DAL80* is overexpressed in a *mep1 mep2* genetic background, the slow growth phenotype of the double deletion was corrected. The impaired filamentous growth created by deleting *mep1* and *mep2*, however, could not be corrected by overexpression of *DAL80* (Lorenz and Heitman, 1998a).

The *DEH1* protein

The *DEH1* gene was identified by three separate research groups and therefore has three different names: *GZF3* (GATA zinc finger factor) (Soussi-Boudekou *et al.*, 1997), *DEH1* (Dal Eighty Homologue protein) (Svetlov and Cooper, 1998), and *NIL2* (Rowen *et al.*, 1997).

Deh1p is a negative regulator of NCR-sensitive genes. As in the case of *DAL80*, *DEH1* functions as a negative regulatory protein in NCR. It is hypothesized that Deh1p prevents transcriptional activation facilitated by Gat1p. Apart from regulating catabolic genes, Deh1p also regulates transcription of itself, *DAL80* and *GAT1*. Unlike Dal80p, which binds to URS_{GATAA} sites, Deh1p binds to URS sites containing a single 5'-GATAA-3' element (Rowen *et al.*, 1997). Binding of *cis*-acting elements by dimer complexes of Deh1p-Deh1p and Deh1p-Dal80p still needs to be elucidated. In the presence of repressive nitrogen sources such as glutamine and ammonia, Deh1p negatively regulates the expression of some NCR-sensitive genes such as *GAP1* and the *UGA4* gene (Coffman *et al.*, 1997).

Deh1p has a zinc finger as well as a leucine zipper. Deh1p contains two domains: a zinc finger domain in the N-terminal region that is responsible for binding DNA (Rasmussen, 1995) and a leucine zipper motif in the C-terminal region that is probably responsible for homodimer and heterodimeric formation with Dal80p. This interaction was elucidated with the two-hybrid system (Svetlov and Cooper, 1998). Deh1p has homology to other regulators of NCR, with the greatest similarity to other negative regulator Dal80p (Soussi-Boudekou *et al.*, 1997).

Physiological significance of Deh1p. Disruption of *DEH1* results in partial relief of *GAP1* repression in ammonia and glutamine, but not in proline (Soussi-Boudekou *et al.*, 1997). Furthermore, *deh1* null mutants have no detectable phenotype in an environment with proline as a nitrogen source (Coffman *et al.*, 1997). However, as in the case of the other global regulators Dal80p and Gln3p, a slow growth phenotype is detectable. The deletion of *DEH1* results in accumulation of Gat1p, resulting in increased expression of *GAP1* on repressive nitrogen sources (glutamine, ammonia). Therefore, in the presence of a good nitrogen source, the regulation of

GAP1 and *GAT1* genes are to some extent relieved by elimination of *DEH1* (Rowen *et al.*, 1997). Both Gln3p and Gat1p regulate *DEH1* transcription. The deletion of both *gln3* and *gat1* abolished expression of *DEH1*. Single deletion of *GLN3* or *GAT1* did not result in the abolishment of *DEH1* expression (Soussi-Boudekou *et al.*, 1997).

Importance of *URE2* in industrial fermentations and its role in the production of ethyl carbamate

The nitrogen content of grape must differs according to grape variety, geographical location, viticultural and enological practices. The availability and type of nitrogen source in grape must might influence formation of ethyl carbamate. Probably the biggest nitrogen-related problem in the wine industry is stuck or sluggish fermentations.

In the past, attempts to prevent or correct stuck and/or sluggish fermentations have resulted, in some cases, in an increase of ethyl carbamate in table wines. Ethyl carbamate is believed to be a possible carcinogen. The ability of yeast to utilize nitrogen in grape must is influenced by a number of factors: environmental influences; genetic makeup of the yeast, which varies between strains used in fermentations, as well as vinification practices.

The *URE2* gene has been shown to be a negative regulator of transcription of NCR-sensitive genes (Coffman *et al.*, 1994). One of the precursors of ethyl carbamate, urea, is a by-product of arginine catabolism. Arginine catabolism is regulated by *URE2*. Therefore *URE2* might play an important regulatory role in formation of ethyl carbamate. The formation of ethyl carbamate is a growing concern in the wine industry due to its potential carcinogenicity.

The occurrence of ethyl carbamate in wine

Ethyl carbamate, also known as urethane, was used in the 1940's as a hypnotic in humans and an anesthetic for laboratory animals (Zimmerli and Schlatter, 1991). Its use in animals led to the discovery that ethyl carbamate has carcinogenic abilities. Ethyl carbamate's activity against leukemia cells were described three years later. In 1971, the existence of ethyl carbamate in food products was first discovered in fruit juices that were treated with diethyl pyrocarbonate (DEPC). DEPC was used as an anti-microbial agent in fruit juices. It is now believed that DEPC reacts with ammonia to form ethyl carbamate (Solymosy *et al.*, 1978).

Sake was the first alcoholic beverage in which ethyl carbamate was detected. This was followed by the detection of ethyl carbamate in wine and distilled spirits from several countries by the Liquor Control Board of Ontario (LCBO) in 1985. The high levels of ethyl carbamate were attributed to the use of urea as an additional nitrogen source for the yeast during fermentation. Consequently, the use of urea was prohibited and guidelines were implemented to control ethyl carbamate levels in alcoholic beverages (Table 1) (Zimmerli and Schlatter, 1991). Guidelines for the maximum permitted levels of ethyl carbamate in alcoholic beverages varies from 30 ng/g in wine to 400 ng/g in fruit brandies.

Table 1. Guidelines for ethyl carbamate levels in alcoholic beverages

(Zimmerli and Schlatter, 1991)

Alcoholic beverage	Concentration (ng/g)
Table wines	30
Fortified wines	100
Distilled spirits	150
Sake	200
Fruit brandies and liqueurs	400

Formation of ethyl carbamate. Ethyl carbamate ($\text{NH}_2\text{COOCH}_2\text{CH}_3$) is the ethyl ester of carbamic acid. It has a molecular weight of 89.1, melting point of 48-50°C and a boiling point of 182-184°C (Zimmerli and Schlatter, 1991).

Carbamyl compounds react with ethanol to form ethyl carbamate (Ough, 1976; Ough *et al.*, 1988a). Monteiro *et al.* (1989) identified urea and ethanol to be the main precursors of ethyl carbamate in wine. Other possible precursors of ethyl carbamate in wine are carbamyl phosphate (CP) and hydrogen cyanide. Both these precursors occur at such low levels in wine that they are hardly significant. The reaction between urea and ethanol to produce ethyl carbamate happens spontaneously but can be induced by high temperature, light and extended periods of storage. The majority of young wines do not have ethyl carbamate or have extremely low levels

(<10ng/ml), unless exposed to 70°C for 48 hours (Zimmerli and Schlatter, 1991).

Biological activity

Mutagenicity. As early as 1948, ethyl carbamate was described as a chromosome breaking agent, able to induce the same type of mutations in *Drosophila* as high-energy radiation or mustard gas. The mutations are induced by the formation of DNA-adduct structures. Studies done with ethyl carbamate in the Bruce Ames test were surprisingly negative. Oxidation of ethyl carbamate results in the formation of the compound vinyl carbamate that gives a positive result with the Ames test. It seems that ethyl carbamate needs to be metabolically activated by oxidation to have any mutagenic capabilities (Dahl *et al.*, 1980).

Carcinogenicity. Data published thus far clearly indicate ethyl carbamate is a pluripotent carcinogen. This compound is able to induce tumors in different species, tissue types, organs and different stages of development in animals. Ethyl carbamate induces tumor formation in several organs such as lungs, lymph nodes, liver and mammary glands of adult mice and rats. Offspring of pregnant mice treated with ethyl carbamate had a remarkably high incidence of tumors, especially in their lungs (Zimmerli and Schlatter, 1991).

One single dose of 1000 mg/Kg body weight of ethyl carbamate induced tumor formation in rats, mice and hamsters (Mirvish, 1968). These doses were introduced to the laboratory animals in a number of ways: inhalation, oral, dermal, subcutaneously and intraperitoneally. It was not unusual to find 40-100% tumor incidence after administration of 100- 1000 mg ethyl carbamate/Kg body weight (Zimmerli and Schlatter, 1991).

Metabolism of ethyl carbamate. Once ethyl carbamate is in the body it is rapidly distributed, irrespective of the method of administration. Up to 90% of the substance is metabolized by the body and degraded into CO₂, H₂O and NH₃ within the first 24 hours. The remaining ethyl carbamate is excreted as ethyl carbamate, N-hydroxy ethyl carbamate, N-acetyl-N-hydroxy ethyl carbamate, ethyl mercapturic acid or N-acetyl-S-ethoxy carbonylcysteine (Mirvish, 1968). Ethyl carbamate disappears from the blood stream within 4 hours after being administered. However, administration of

a high dose of ethanol (0.5% initial blood level) leads to high ethyl carbamate levels in the blood stream for up to 8 hours. Ethanol inhibits the metabolism of ethyl carbamate by possibly blocking the metabolizing enzymes (Yamamoto *et al.*, 1988).

Urea metabolism

The enhancement of urea degradation will lower levels of urea or even eliminate it totally, thereby minimizing the possibility of ethyl carbamate formation in wine. It is therefore important to understand how the metabolic pathway, responsible for urea degradation, works to be able to eliminate ethyl carbamate in an efficient manner.

There are two sources of urea for the yeast. Urea is an intermediate of arginine and allantoin or allantoate catabolism (Figs. 3 and 13). Degradation of arginine to urea is facilitated by arginase encoded by the *CAR1* gene which has been shown to be regulated by NCR (Dubios and Messenguy, 1997; Smart *et al.*, 1996; Cooper *et al.*, 1992). Allantoate is degraded by allantoicase to ureidocycloic acid and urea (Figure 3). Allantoicase is encoded by the NCR sensitive *DAL2* gene (Yoo and Cooper, 1991).

The second source of urea might be the grape must itself in which case urea is transported into the cell via two ways: facilitated diffusion and energy-dependent transport by two permeases, i.e. Dur3p and Dur4p. The transcriptional regulation of *DUR3* is NCR sensitive (El Berry *et al.*, 1993). Once urea is in the cell, it is broken down to allophanate by *DUR1* (urea carboxylase activity) which in turn is degraded to ammonia and carbon dioxide by *DUR2* (allophanate hydrolase activity). It was later determined that these two catalytic activities are encoded by one gene, *DUR1,2* (Sumrada and Cooper, 1982). This degradation of intracellular urea is therefore facilitated by a highly inducible NCR sensitive gene product, the bi-functional urea amidolyase, (*DUR 1,2*) (Figure 13) (Genbauffe and Cooper, 1986; Whitney and Magasanik, 1973).

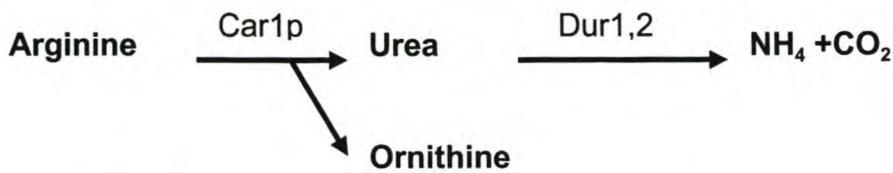


Figure 13. The degradation of arginine by arginase (Car1p) and degradation of urea by urea amidolyase (Dur1,2p) into ammonia and carbon dioxide.

Possible prevention of ethyl carbamate in wine

Supplementing grape must with nitrogenous compounds. Grape juice contains a wide variety of nitrogenous compounds consisting of ammonia, amino acids and polyamines. The largest portion of nitrogen is in the form of ammonia, arginine and proline. Ammonia can be as high as 10% of the total nitrogen. Arginine and proline represent as much as 65% of the total nitrogen available in grape juice. The yeast takes up amino acids early in the growth phase until its biosynthetic pools are filled (Boulton *et al.*, 1996). The utilization of nitrogen from the grape must is dependent on the environment, physiological and strain-specific factors (Salmon and Barre, 1998). An imbalance in the amino acid and ammonia composition has been shown to result in an increase in urea and subsequent formation of ethyl carbamate (Ough *et al.*, 1988b). Environmental factors that influence the distribution of nitrogen are grape variety, time of harvest, climate and fertilization of the soil.

As mentioned earlier, winemakers used to add urea as a nitrogen source to fermentations. This practice has been banned and replaced by the addition of di-ammonium phosphate (DAP). The current legal limit for the addition of DAP in the USA is 960 mg/L. It can be argued that the addition of DAP, a good nitrogen source, will repress the formation of urea. This is because arginine is the main source of urea and the genes responsible for the degradation of arginine are NCR sensitive. A study on the addition of DAP and its influence on two different strains, Montrachet and Prise de Mousse, study revealed that both strains had increased fermentation rates, but only Prise de Mousse increased its biomass. Addition of DAP at low levels

(0.23 g/l) had little effect on the utilization of amino acids. Only the addition of high levels (2g/l) of DAP greatly decreased the consumption and degradation of arginine (Monteiro and Bisson, 1991; 1992). Arginine degradation, however, was not totally prevented. This might be due to the fact that ornithine, an intermediate of arginine degradation, is a precursor for polyamine biosynthesis. The need for polyamines might drive the degradation of arginine to fulfill in the yeast's polyamine needs.

Fermentations with different yeast strains. Urea secretion and uptake are dependent on the yeast strain and the amount of arginine in the grape during fermentation. Studies done on the industrial yeast strains, Montrachet, Prise de Mousse and Epernay II, revealed that there was variation in the secretion and production of urea among these strains. Montrachet can metabolize all of the arginine in the must, reabsorb some of the secreted urea and metabolize it. Epernay II on the other hand, did not readily absorb urea but excreted even less, probably because this strain grew slower in comparison to the other strains (Ough *et al.*, 1991).

A survey of 22 wineries revealed that red wines with a relatively high proline content were generally low in urea regardless of the yeast strains used. The higher the content of proline in the must, the smaller the amount of urea produced. Fermentations done with Prise de Mousse in white and red wines, containing low to medium levels of proline, produced the least amount of urea. The amino acid content of the grape must be significantly higher for Prise de Mousse to produce urea (Ough *et al.*, 1990). Prise de Mousse also tends to keep its urea in the cell, whereas Montrachet secretes it to the surrounding liquid.

Genetic engineering of yeast. Attempts to construct yeast strains that would prevent the formation of ethyl carbamate, have been reported. Kitamoto *et al.* (1991) eliminated the formation of urea by successive disruption of the *CAR1* gene in sake yeast. The *CAR1* gene encodes for arginase that is responsible for the degradation of arginine into ornithine and urea. According to this group, sake yeast is diploid. They disrupted the *CAR1* gene by disrupting the first allele of *CAR1* with the *SMR1* gene, encoding for sulfometuron resistance, followed by disruption of the second allele with the geneticin resistance marker *G418*. Experimental sake fermentations

with this *car1/car1* homozygous mutant produced no urea. Testing revealed that there was no ethyl carbamate present in the sake, even after five months storage at 30°C (Kitamoto *et al.*, 1991). This strategy can also be applied to yeast used in wine fermentations. However, the presence of resistance markers such as *SMR1* and *G418* in industrial yeast is not desirable. Therefore alternatives for the disruption of *CAR1* should be found if the yeast is ever to be used on a commercial basis.

In an attempt to improve assimilation of nitrogen, a haploid *ure2* null mutant, derived from an industrial wine yeast, was constructed (Salmon and Barre, 1998). This resulted in elevated levels of proline assimilation with the incorporation of a small amount of oxygen when a large amount of proline was utilized. This might have applicability in the future for creating yeast strains that can ferment grape juice with a low nitrogen content by also utilizing proline. The deletion of the *URE2* gene can also be used to limit the production of ethyl carbamate since that the *DUR1,2* gene is regulated by the Ure2p (Genbauffe and Cooper, 1986).

Enzymatic removal of urea. The enzymatic removal of urea by urease has been explored as an option to eliminate urea from wine. However, wine has a relatively low pH and most ureases are not functional at low pH. The urease from *Lactobacillus fermentum* is, however, acid tolerant. Ough and Trioli (1988) used this urease in several varietal wines and found that the urease significantly lowered urea levels in the wine. There are some factors that influence the efficacy of this urease in wines. Urea removal was more effective in wine with a slightly higher pH. Wines with a pH of 3.0 were more resistant to removal of urea than wines with a pH 4.0. Some varieties such as Sauvignon Blanc were also more resistant to the removal of urea than Chardonnay (Ough and Trioli, 1988). A follow-up study on this urease, and its application in wine by the same group, revealed that there are a number of factors which inhibit urease activity. A number of organic acids commonly found in wine inhibit urease; these include L-malic acid, L-lactic acid, acetic acid and pyruvic α -ketoglutaric acid. L-malic acid in its unionized form is the most important inhibitor. The pH of the wine had no direct influence on the urease, but rather influenced ionization of the inhibiting organic acids. Fe^{2+} , Ca^{2+} , PO_4^{3-} , SO_2 , ethanol

and phenolic compounds all influenced urease activity to a lesser extent (Trioli and Ough, 1989).

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Deletion analysis of the Ure2p in *Saccharomyces cerevisiae* and effect of NCR on the production of ethyl carbamate during wine fermentations

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***Saccharomyces cerevisiae* has the ability to distinguish between good and poor nitrogen sources. The mechanism to discriminate between different nitrogen sources is known as Nitrogen Catabolite Repression (NCR). The Ure2p plays a central role in the regulation of genes that are NCR sensitive. Deletion analysis of *URE2* was done to determine the NCR functional domain of Ure2p. Northern blot analysis on three NCR sensitive genes encoding permeases (*DAL5*, *CAN1* and *GAP1*) revealed that the carboxyl 10 amino acids of Ure2p contain the NCR regulatory domain. The *URE2* gene also regulates the arginine catabolic pathway in *S. cerevisiae* that produces urea. High density microarray DNA analysis revealed that the regulation of arginine metabolism by NCR is functional in industrial wine yeast strains. Urea and ethanol are the precursors of ethyl carbamate, a possible carcinogen. Fermentations done with a *ure2* null mutant contained lower levels of ethyl carbamate compared to the wild type. The *ure2* mutant yeast also produced more ethanol.**

Introduction

The yeast *Saccharomyces cerevisiae* has the ability to distinguish between good and poor nitrogen sources. The classification of nitrogen sources as either good or poor depends on the relative rate of growth that the yeast maintains on a particular nitrogen source. When *S. cerevisiae* is supplied with a good nitrogen source such as glutamine, asparagine or ammonia, the genes responsible for the utilization of poor nitrogen sources are repressed. Examples of poor nitrogen sources are proline, allantoin and urea. This repression is known as nitrogen catabolite repression (NCR) (Cooper and Sumrada, 1983).

There are several global regulators of NCR including Ure2p, Gln3p, Dal80p, Gat1p and Deh1p. Ure2p is the only one of these regulators that does not function through DNA binding. Gln3p and Gat1p are positive regulators (Mitchell and Magasanik, 1984; Coffman *et. al.*, 1996), whereas Dal80p and Deh1p are negative regulators (Cunningham and Cooper, 1991; Soussi-Boudekou *et. al.*, 1997). These four proteins bind to *cis*-acting elements in the promoters of NCR sensitive genes. Gln3p binds elements known as UAS_{NTR} (upstream activating sequence) with one 5'-GATAA-3' sequence (Cunningham *et. al.*, 1996). Dal80p binds URS_{GATAA} (upstream repression sequence) containing two 5'-GATAA-3' sequences (Cunningham and Cooper, 1993). It has been suggested that Ure2p interacts directly with Gln3p by transferring a glutathione molecule to Gln3p, thereby inactivating it. This *in vivo* interaction was elucidated by co-immuno precipitation studies (Blinder *et. al.*, 1996). Ure2p has also been shown to be a prion-like protein in yeast (Wickner, 1994).

The functional domains of proteins can be determined by deletion analysis of the respective gene (Oyedotun and Lemire, 1997; Nonet *et. al.*, 1987; Meyer *et. al.*, 1997). To establish the Ure2p's NCR regulation domain, deletions were made from the 3' end of the *URE2* gene. Northern blot analysis of the NCR sensitive *DAL5*, *CAN1* and *GAP1* genes revealed that the carboxyl 10 amino acids of Ure2p are responsible for mediating NCR sensitivity in *S. cerevisiae*.

Arginine is one of the major amino acids in grape must. During the degradation of arginine, ornithine and urea are produced (Cooper, 1982). Urea can either be

secreted where it can react spontaneously with ethanol to produce ethyl carbamate, a compound believed to be a carcinogen (Zimmerli and Schlatter, 1991). Alternatively, urea can be degraded intracellularly to NH_4^+ and CO_2 . The degradation of both arginine and urea is NCR-sensitive (Dubois and Messenguy, 1997; Smart *et al.*, 1996; Coffman *et al.*, 1994; Cooper *et al.*, 1992). The absence of a functional *URE2* gene in *S. cerevisiae* might therefore lead to the reduction of ethyl carbamate produced in wine.

The phenomenon of NCR is well established in laboratory strains grown under standard laboratory conditions. High-density DNA micro-array analysis done on fermentations conducted by an industrial strain of *S. cerevisiae* revealed that NCR also functions in industrial strains of this yeast grown in grape juice. The *CAN1*, *GAP1*, *CAR1*, *DUR1,2* and *DUR3* genes involved in the transport and catabolism of arginine and urea, were all shown to be NCR-sensitive. Fermentations done with a laboratory *ure2* null mutant diploid strain and its isogenic wild type in Chardonnay grape must revealed that the *ure2* mutant produced less ethyl carbamate and more ethanol than the wild type.

Materials and Methods

Strains and culture conditions. Strains used in this study are listed in Table 1. Bacterial cultures were propagated in LB broth (DIFCO laboratories, Detroit, MI). Bacterial transformations were done using CaCl_2 (Ausubel *et al.*, 1995). Where needed, 0.1% ampicillin was added to select for clones. The media used for propagation of yeast for transformation, was YPD broth (DIFCO laboratories, Detroit, MI). Yeast strains TCY5 and JCY126 used for RNA isolations, were grown in 0.17% YNB media (without amino acids and ammonium sulphate, DIFCO laboratories, Detroit, MI) 2% glucose and 0.1% L-asparagine or 0.1% L-proline as nitrogen source, to an $\text{OD}_{600\text{nm}}$ between 0.8 and 1.0. Auxotrophic requirements were fulfilled, if necessary, at the following concentrations: uracil 20 mg/l, L-lysine 30 mg/l, L-histidine 20 mg/l, L-tryptophan 20 mg/l. Yeast transformations were done using the Lithium-acetate method (Ausubel *et al.*, 1995).

Table 1. Strains of *S. cerevisiae* and *Escherichia coli* used in this study

Strain	Description
<i>S. cerevisiae</i>	
TCY5	<i>MATα ura3 lys2 trp1::hisG</i>
JCY126	<i>MATα ura3 lys2 trp1::hisG ure2Δ::URA3</i>
MLY97	<i>MAT a/αura3-52/ura3-52 Δleu2::hisG/ Δleu2::hisG</i>
MLY140	<i>MAT a/$\alpha$$\Delta$ure2::G418/ Δure2::G418 ura3-52/ura3-52</i>
VIN13	Industrial strain used for wine fermentations
<i>E. coli</i> DH5 α	F' end A1 <i>hsdR17 (rκ^- mκ^+) supE44 thi-1 recA1 gyrA(Naf) relA1 Δ(lacZYA-argF) U169 [ϕ80dlacΔ(lacZ)M15]</i>

Construction of *URE2* deletion mutants. Deletion constructs of *URE2* were generated by PCR using genomic DNA (isolated from strain TCY5) as template. Restriction enzyme sites were introduced with the primers for cloning purposes. Primers used in this study are described in Table 2. The strategy for the cloning of these PCR-generated fragments is described in Figs. 1 and 2. The PCR products of each deletion construct were cloned into the *Bam*HI and *Sma*I sites of YCp*lac22* (Fig. 1). The native terminator of *URE2* was cloned as a *Sac*I fragment into the *Sac*I site of YCp*lac22* (Figs. 1 and 2B). The full native gene with its promoter and terminator was cloned into YCp*lac22* as a *Bam* HI/*Sac* I fragment (Figs. 1 and 2A). All constructs were sequenced to verify that there were no mutations and that the terminators were cloned in the right orientation. Plasmids used and constructed in this study are listed in Table 3. The PCR program was as follows: 94°C for 2 minutes, 29 cycles at 56°C for 40 seconds, 72°C for 90 seconds, 94°C for 40 seconds, 56°C for 2 minutes and 72°C for 2 minutes.

The *URE2* gene with a C-terminal 10 amino acid deletion (pDAN10) was generated using primers UGK10 and DE10 (Fig. 1). This fragment was then cloned into the *Eco*RI and *Bam*HI sites of pBLUESCRIPT II KS (Stratagene, La Jolla, CA), followed by subcloning of this fragment as an *Ap*al/*Sac*I fragment into plasmid pDAN5. The *Ap*al site is a native site in the PCR product downstream of primer UGK10 and the *Sac*I restriction site is situated on pBLUESCRIPT II KS. pDAN5 was pre-digested

with *Apal* and *Sacl* to remove 50 amino acid deletion construct to allow replacement of this fragment with the pDAN10 construct from pBLUESCRIPT II KS.

Table 2. Primers used in the construction of *URE2* deletion mutants

Primer	Sequence
DE1	5'-GATC GGATCC ATC TCG AGA AAT TTG ATT GAG-3'
DE5	5'-AAA TCG CGA TCA TAC GGG ATA ATC AAA G-3'
DE6	5'-AAA TCG CGA TCA ATC CGT ATA TCT TTC TAC AGC-3'
DE7	5'-AAA TCG CGA TCA TAA TGG ATT ACC AGT CTC TTT G-3'
DE8	5'-AAA TCG CGA TCA TTC GCC AAG ATT GAA ATC TAG-3'
DE9	5'-AAA TCG CGA TCA ATA CTC CAC GTG ACT CAT ATC-3'
TER1	5'-GGCC GAG CTC TAT CTA ATC GTC ATA ACT GA -3'
TER 2	5'-GGCC GAG CTC TGC TTT AAA AAC AAG AAA GAA-3'
DE10	5'-GATC GGA TCC TCA TCT CAT CAT ATG CTT CGT CCA-3'
DE20	5'-GATC GGA TCC TCA AAC TTC TGG AAA TTC AAT TTT-3'
UGK10	5'-GAT GAA TTC ATG ATG AAT AAC AAC GGC AAC-3'

Sequences in bold are restriction enzymes sites that were introduced by PCR for cloning of fragments generated by PCR onto plasmids

Table 3. Plasmids used in this study

Plasmid	DNA base pairs deleted	Number of amino acids deleted
pBLUESCRIPT	Used for cloning purposes	---
YCplac 22	---	Positive control
pDAN4	0	Negative control
pDAN10	30	10
pDAN20	60	20
pDAN5	150	50
pDAN6	300	100
pDAN7	450	150
pDAN8	600	200
pDAN9	750	250

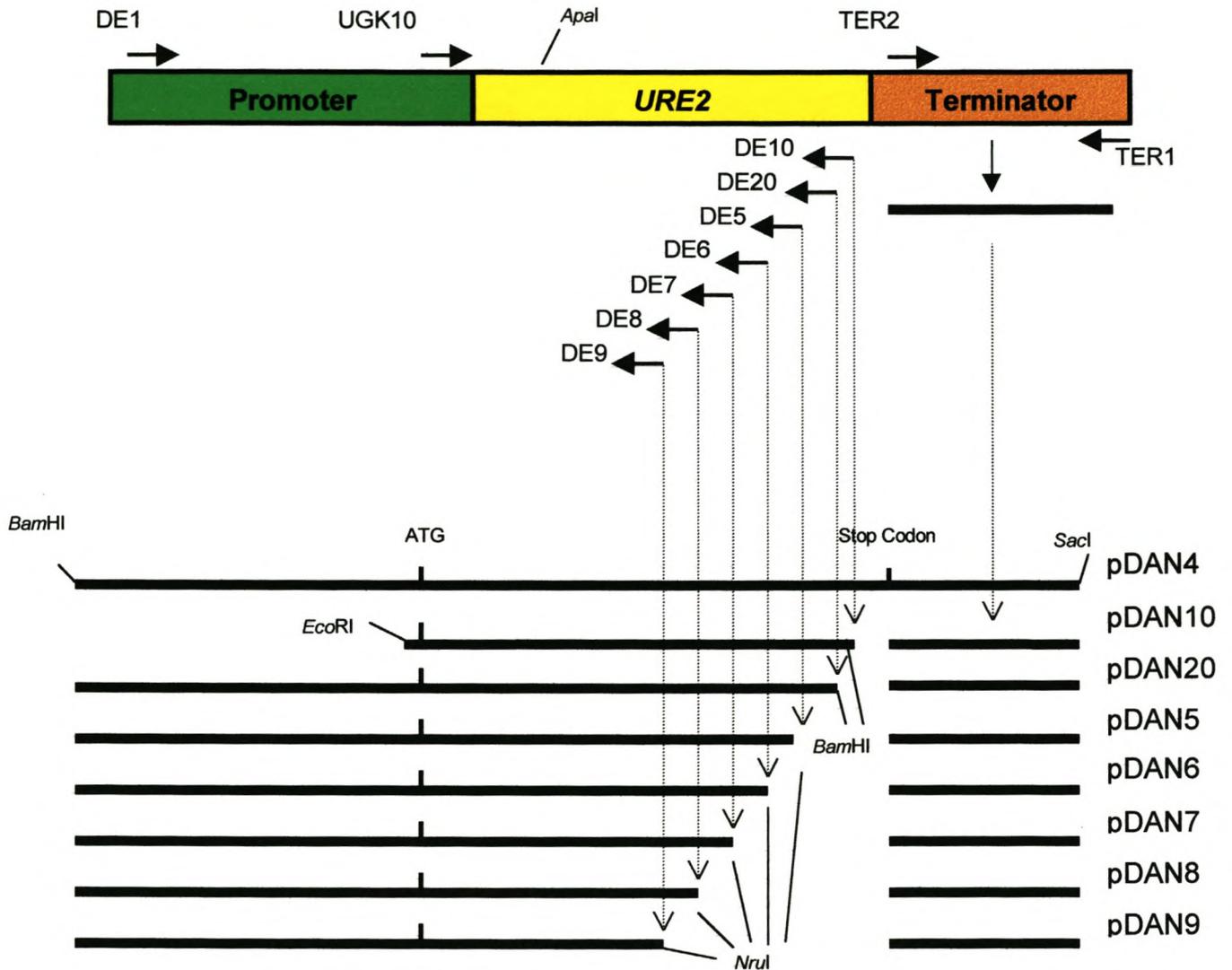


Fig. 1. Schematic presentation of *URE2* deletion mutants constructed by PCR. Restriction enzyme sites introduced for cloning purposes are indicated. Sequences of primers used are listed in Table 2.

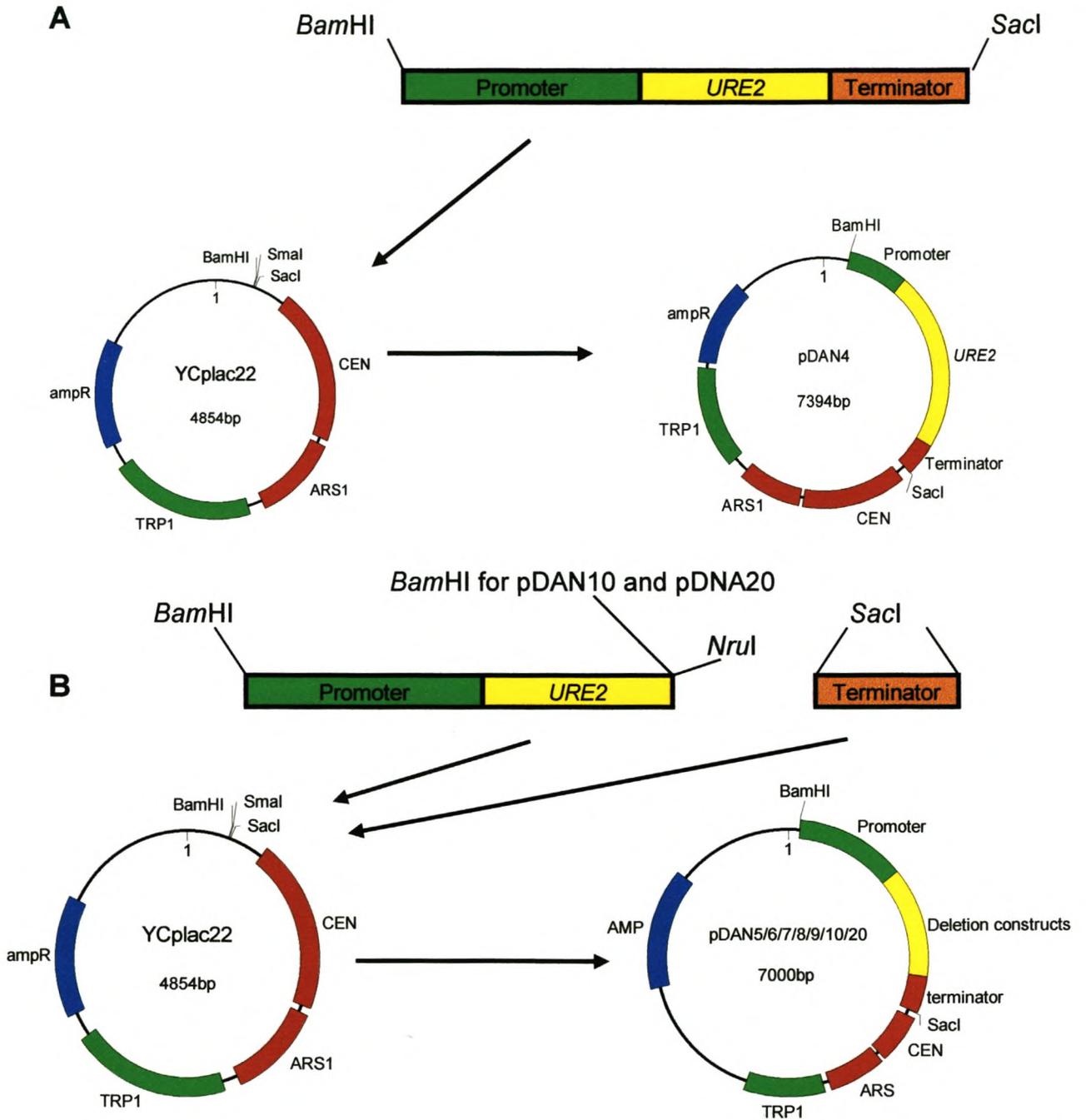


Fig. 2. A) Cloning of the *URE2* gene into the *Bam*HI and *Sac*I sites of *YCplac22*. B) Cloning of deletion constructs into the *Bam*HI and *Sma*I sites and the terminator of *URE2* into the *Sac*I site of *YCplac22*.

Northern blot analysis. Total RNA was isolated from the yeast cells by using glass beads (0.4mm in diameter, BDH laboratory supplies, Poole, England) to break cells open (Ausubel *et al.*, 1995). Total RNA was then fractionated on an 1.4% agarose gel containing 2% formaldehyde and 0.4% MOPS running buffer (Ausubel *et al.*, 1995). The RNA was then transferred overnight with 20 X SSC to a nylon membrane (Boehringer Mannheim). RNA was cross-linked to the nylon membrane by exposure to UV-light for 4 minutes. Pre-hybridization was carried out for 4.5 hours at 42°C with standard pre-hybridization solution. Pre-hybridization solution was used for hybridization for 18 hours at 42°C. Probing was done using part of the *URE2*, *DAL5*, *CAN1*, *GAP1* and *UGA1* genes respectively and the *HHF1* gene (histone 4) as control for loading efficiency. Probes were constructed with PCR (Table 4) and then labeled using the Boehringer Mannheim random priming kit and α -ATP³². Membranes were washed twice with 2xSSC/0.2%SDS for 20 minutes at room temperature followed by two washings with of 15 minutes each with 0.2xSSC/0.2%SDS at 50°C.

High-Density Micro-Array DNA analysis. Active dry yeast of an industrial yeast (VIN13, Anchor Yeast) was resuspended at 37°C in Chardonnay grape must that was diluted to 7° BRIX with sterile water. Two 1 L batches of 21.7° BRIX Chardonnay grape must were inoculated from the culture prepared at 37°C according to the yeast manufacturer's specifications (300 mg dry yeast/L of grape must) in 1 L Schott bottles and fermented at 20°C. Vapor locks similar to those used in the wine industry were used to enclose the individual fermentations. After 45 hours, 960 mg di-ammonium phosphate (DAP) was added to one of the fermentations. Samples for RNA isolation were taken 8 hours after DAP addition. RNA extraction and preparation for hybridization, genome labeling, array hybridization and scanning, and the quantitative analysis of hybridization patterns and intensities were done according to: <http://web.wi.mit.edu/young/expression> (Holstege *et al.*, 1998).

Table 4. Primers used in the construction of probes for Northern blot analysis

Primer	Sequence
H4 5'	5'-GGCC GGA TCC ATG TCC GGT AGA GGT AAA GG-3'
H4 3'	5'-GGCC GAA TTC TTA ACC ACC GAA ACC GTA TAA GG-3'
DAL5 5'	5'-CAG TAT TCA TGG GTT ACT TCC-3'
DAL5 3'	5'-TAA GGT TCT CCA GCC TTT AAT-3'
CAN1 5'	5'-ATG AGC CGG TCA CAA CCC TC-3'
CAN1 3'	5'-GAT GGA AGC GAC CCA GAA CT-3'
GAP1 5'	5'-GTT TGT TGC GTT GTT TTG GC-3'
GAP1 3'	5'-CCG ATG TAC ATA ACC ATA AC-3'
UGA1 5'	5'-GCT TTG ATC AAG GCA GCA CA-3'
UGA1 3'	5'-CGG CGT ACT CAT GAC ACC AT-3'

Fermentations with laboratory yeast. Fermentations were done in Chardonnay grape must using the wild type (MLY97- with a Σ 1278b genetic background) and its isogenic *ure2* mutant (MLY140) (Table 1) (Lorenz and Heitman, 1998). The fermentation conditions varied only in their nitrogen content (Table 5). Pre-cultures for fermentations were grown aerobically in YPD-media at 30°C until they reached the mid-log phase. The inoculum was first washed in sterile de-ionized water before the grape must was inoculated to 1.5×10^6 cells/ml. The fermentations were done in 1L batches in 1L Schott bottles with sterile filtered (0.2 micron) Chardonnay grape must at 20°C. Starting sugars of the must were measured as 21.7° BRIX. Vapor locks similar to those used in the wine industry were used to enclose the individual fermentations.

Table 5. Supplementation of Chardonnay grape must with different N-sources

Nitrogen added to Chardonnay must (mg/L)	
Control	No Nitrogen added
Arginine	1500
Asparagine	960
Glutamine	960
Arginine and Asparagine	1500 and 960
Arginine and Glutamine	1500 and 960
Di-ammoniumphosphate	960
Arginine and Di-ammoniumphosphate	1500 and 960

Determination of alcohol content: Alcohol production by the yeast was determined with an Ebulliometer. Samples taken from the wine were diluted 1:4 to eliminate the effect that residual sugar might have on the determination of alcohol concentration.

Determination of ethyl carbamate: 50 ml Samples were taken at the end of the fermentations and filtered through a 0.2 micron filter and heated at 70°C for 48 hours. This was done to induce all of the free urea to react with ethanol to form ethyl carbamate. The samples were sent to the Liquor Control Board of Ontario (LCBO) to quantify ethyl carbamate in the wine. The quantification was done with a Hewlett-Packard 5890 Series II GC with a J&W DB-FFAP column (30m x 0.25mm ID x 0.25 um film thickness) and an attached HP5971 MSD system for detection (Hom, 2000, personal communication). All samples were injected at least twice.

Results and Discussion

The focus of this study was to determine the NCR regulatory domain of the Ure2p, to establish if NCR (mainly mediated by Ure2p) is functional in wine yeasts and if the elimination of the *URE2* gene results in a decrease of ethyl carbamate production during wine fermentations.

Expression of the *URE2* gene. Northern blot analysis revealed that the deletion constructs of *URE2* as well as the plasmid-born *URE2* were functionally expressed (Fig. 3).

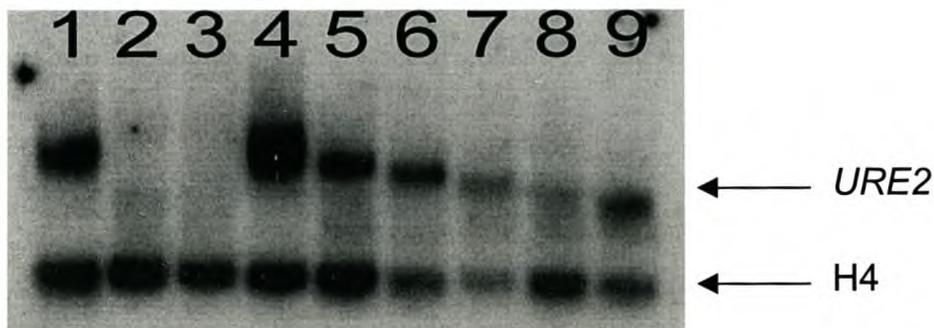


Fig. 3. Expression of *URE2* and *URE2* deletions from YCplac22. 30 μ g Total RNA was fractionated on a gel 1) TCY5 (wild type), 2) JCY126 (*ure2* mutant), 3) JCY126 with YCplac22, 4) JCY126 with pDAN4, 5) JCY126 with pDAN5, 6) JCY126 with pDAN6, 7) JCY126 with pDAN7, 8) JCY126 with pDAN8, 9) JCY126 with pDAN9.

Effect of 50 amino acid deletion from the CTD of Ure2p on NCR-sensitive genes. The *DAL5* gene has been used as an indicator gene for the study of loss of NCR in previous studies (Masison *et al.*, 1997; Coffman *et al.*, 1994). In addition to *DAL5* it was decided to include the two NCR-sensitive permease encoding genes, *CAN1* and *GAP1*. These two genes' products are responsible for the transport of arginine, the precursor of urea. The *DAL5* gene encodes for the allantoin permease (Rai *et al.*, 1987), *CAN1* encodes for the arginine permease and *GAP1* gene for the general amino acid permease (Grenson and Hou, 1972; Jauniaux and Grenson, 1990). These three genes have been shown to be NCR-sensitive via Ure2p when cells are supplied with L-asparagine as sole nitrogen source (Daugherty *et al.*, 1993; Grenson and Hou, 1972; Jauniaux and Grenson, 1990). The effect of

URE2 deletions on the expression of *DAL5* in L-asparagine containing medium is shown in Fig 4.

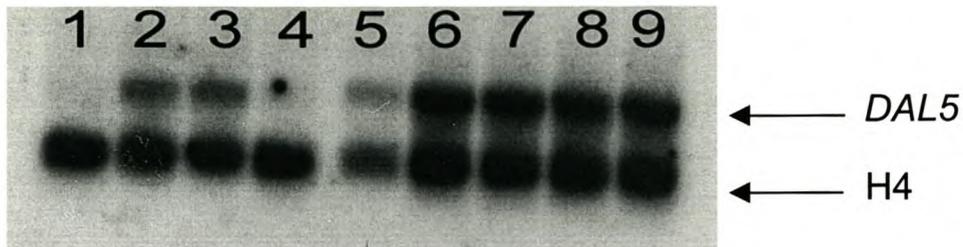


Fig. 4. Influence *URE2* deletions on the expression of *DAL5* when 0.1% L-asparagine is provided as nitrogen source. 30 μ g Total RNA was fractionated on a gel 1) TCY5 (wild type), 2) JCY126 (*ure2* mutant), 3) JCY126 with YCplac22, 4) JCY126 with pDAN4, 5) JCY126 with pDAN5, 6) JCY126 with pDAN6, 7) JCY126 with pDAN7, 8) JCY126 with pDAN8, 9) JCY126 with pDAN9.

DAL5 is expressed in lanes 2, 3, 5, 6, 7, 8 and 9 (Fig. 4), but not in lanes 1 and 4 when the cells were grown in 0.1% L-asparagine. Lanes 1 and 4 each contained a functional *URE2* gene and one would therefore not expect expression of *DAL5*. Lanes 2 and 3 are the *ure2* deletion mutant and the *ure2* deletion mutant with YCplac22 respectively, therefore *DAL5* should be expressed in these lanes. Lanes 5 to 9 contain the deletion constructs. The expression of *DAL5* in all these lanes suggests that NCR is lost with the deletion of the first 50 amino acids from the carboxy-terminus of Ure2p. The same observation is true for *CAN1* and *GAP1* transcription (Fig. 5).

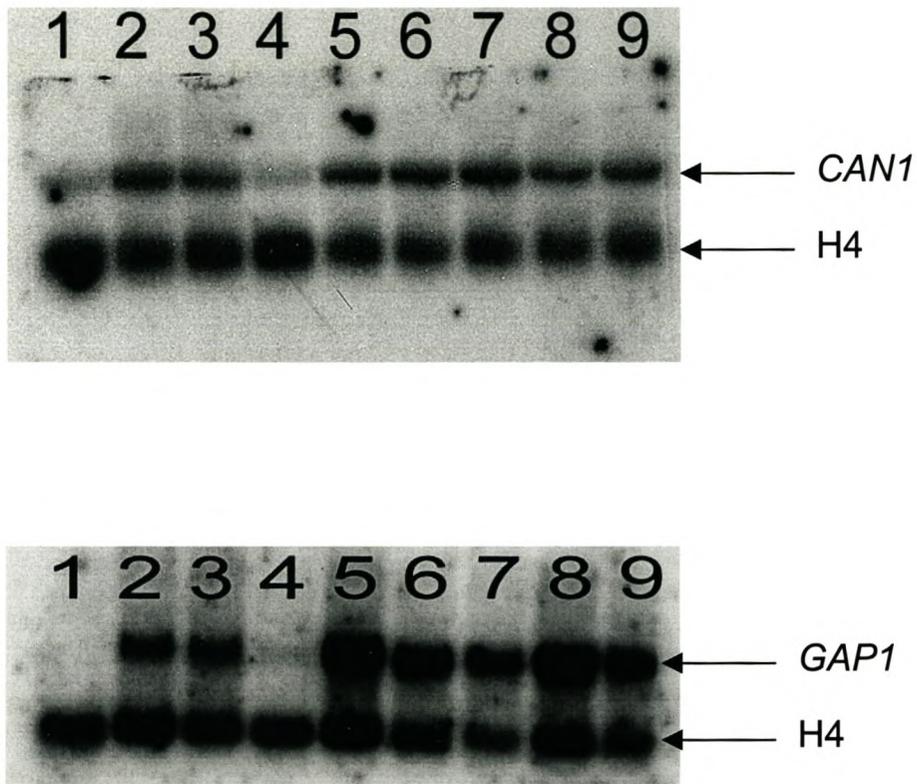


Fig. 5. Influence *URE2* deletions on the mRNA levels of *CAN1* and *GAP1* when cells are grown on 0.1% L-asparagine as nitrogen source. 30 μ g Total RNA was fractionated on a gel 1) TCY5 (wild type), 2) JCY126 (*ure2* mutant), 3) JCY126 with YCplac22, 4) JCY126 with pDAN4, 5) JCY126 with pDAN5, 6) JCY126 with pDAN6, 7) JCY126 with pDAN7, 8) JCY126 with pDAN8, 9) JCY126 with pDAN9.

Transcription analysis of NCR-sensitive but *URE2*-insensitive genes. Not all NCR-sensitive genes are regulated by Ure2p; one such gene is the *UGA1* gene (Cunningham and Cooper, 1991; Coffman *et al.*, 1995). The transcriptional regulation of *UGA1* is both *GLN3*- (Daugherty *et al.*, 1993) and *URE2*- independent (Coffman *et al.*, 1994). Therefore, the elimination of *URE2* will not relieve the repression of *UGA1*, regardless if *URE2* is genomic or plasmid-born. As a control, northern blot analysis was done on *UGA1* in cells grown on 0.1% L-asparagine. The *UGA1* gene was not expressed (Fig. 6) in the *ure2* mutant (lanes 2 and 3), the wild type (lane 1) or the plasmid born *URE2* gene (lane 4) or in any of the deletions (lanes 5 to 9). This confirms that the deletion constructs and copies of the *URE2* gene on plasmids do not affect NCR-sensitive genes that are regulated independent from *URE2*.



Fig. 6. Influence of *URE2* deletions on the expression of *UGA1* when provided with 0.1% L-asparagine as nitrogen source. 30 μ g Total RNA was fractionated on a gel 1) TCY5 (wild type), 2) JCY126 (*ure2* mutant), 3) JCY126 with YCplac22, 4) JCY126 with pDAN4, 5) JCY126 with pDAN5, 6) JCY126 with pDAN6, 7) JCY126 with pDAN7, 8) JCY126 with pDAN8, 9) JCY126 with pDAN9.

Deletion of 10 and 20 amino acids from the carboxy-terminus of Ure2p. After establishing that the deletion of the carboxyl 50 amino acids was responsible for the regulatory function of Ure2p, smaller deletions of 10 amino acids (pDAN10) and 20 amino acids (pDAN20) were constructed and assayed (Fig. 7). The expression of *DAL5*, *CAN1* and *GAP1* revealed that the regulatory function of Ure2p lies within the carboxyl 10 amino acids (Figs. 8 and 9). These two deletions also had no effect on *UGA1* expression (Fig. 10).

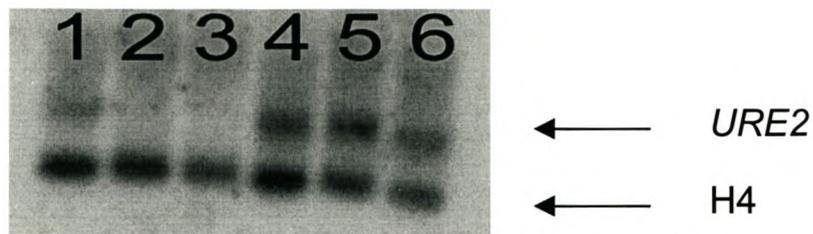


Fig. 7. Expression of *URE2* and *URE2* deletions from YCplac22. 30 μ g Total RNA was fractionated on a gel 1) TCY5 (wild type), 2) JCY126 (*ure2* mutant), 3) JCY126 with YCplac22, 4) JCY126 with pDAN4, 5) JCY126 with pDAN10, 6) JCY126 with pDAN20.

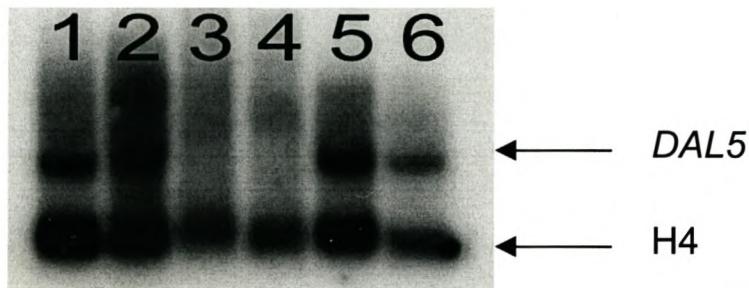


Fig. 8. Influence of *URE2* deletions on the mRNA levels of *DAL5* when cells are grown on 0.1% L-asparagine as nitrogen source. 30 μ g Total RNA was fractionated on a gel 1) JCY126 with YCplac22, 2) JCY126 (*ure2* mutant), 3) TCY5 (wild type), 4) JCY126 with pDAN4, 5) JCY126 with pDAN10, 6) JCY126 with pDAN20.

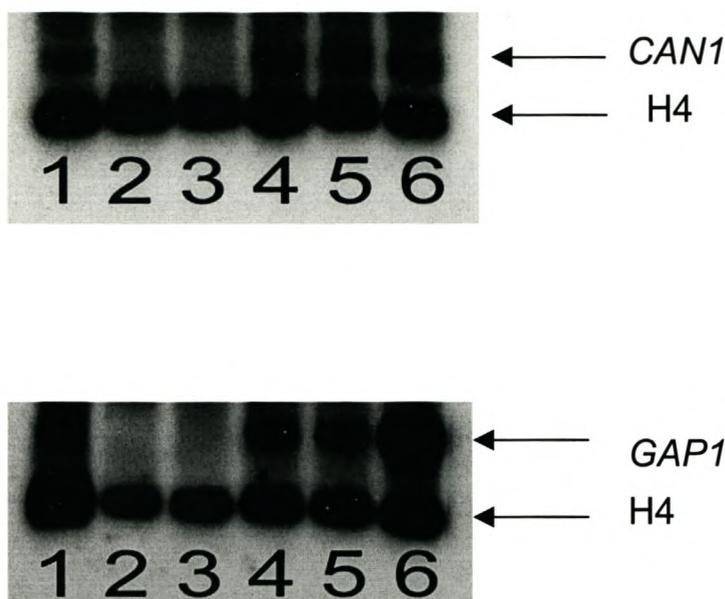


Fig. 9. Influence of *URE2* deletions on the mRNA levels of *CAN1* and *GAP1* when cells are grown on 0.1% L-asparagine as nitrogen source. 30 μ g Total RNA was fractionated on a gel 1) JCY126 (*ure2* mutant), 2) TCY5 (wild type), 3) JCY126 with pDAN4, 4) JCY126 with pDAN10, 5) JCY126 with pDAN20, 6) JCY126 with YCplac22.

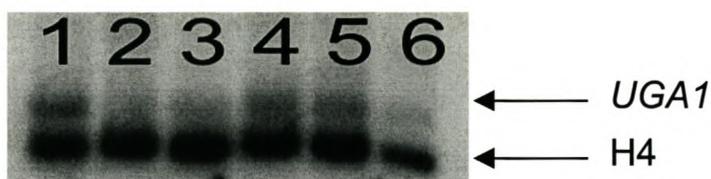


Fig. 10. Influence of *URE2* deletions on the mRNA levels of *UGA1* when cells are grown on 0.1% L-asparagine as nitrogen source. 30 μ g Total RNA was fractionated on a gel 1) JCY126 (*ure2* mutant), 2) TCY5 (wild type), 3) JCY126 with pDAN4, 4) JCY126 with pDAN10, 5) JCY126 with pDAN20, 6) JCY126 with YCplac22.

Functionality of the deletion constructs when proline is supplied as sole nitrogen source. In the presence of a poor nitrogen source the repression of *DAL5* transcription is relieved (Coffman *et al.*, 1994). To confirm that the re-introduced *URE2* gene (on plasmid pDAN4) does not function in a poor nitrogen source, northern blot analysis was done on cells grown in the presence of 0.1% L-proline as sole nitrogen source. This was done as an additional control to confirm that the *URE2* gene and its protein do not mediate NCR to sensitive genes when cells are supplied with a poor nitrogen source. *DAL5* was expressed in cells containing the plasmid born Ure2p (Fig. 11) as well as in the wild type, *ure2* deletion mutant and *ure2* deletion mutant containing plasmids with 10 amino acid and 20 amino acid deletions. These data indicate that the plasmid-born Ure2p functions the same as the genomic Ure2p in establishing NCR at transcriptional level.

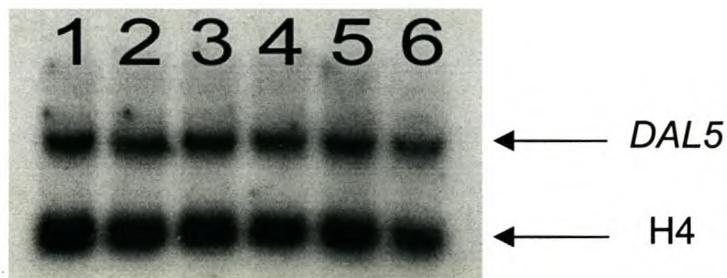


Fig. 11. Influence of *URE2* deletions on the mRNA levels of *DAL5* when cells are grown on 0.1% L-proline as nitrogen source. 30 μ g Total RNA was fractionated on a gel 1) JCY126 with YCplac22, 2) JCY126 (*ure2* mutant), 3) TCY5 (wild type), 4) JCY126 with pDAN4, 5) JCY126 with pDAN10, 6) JCY126 with pDAN20.

High-Density Micro-array DNA analysis. High-density DNA micro-array analysis revealed that the phenomenon of NCR is indeed functional in industrial yeast strains. Transcription levels of NCR-sensitive genes such as those responsible for the transport of arginine (*CAN1*, *GAP1*), and catabolism of arginine to urea (*CAR1*) and degradation of urea (*DUR1,2*) were found to be NCR-sensitive (Table 6). The addition of di-ammonium phosphate to fermentations resulted in a down regulation of NCR-sensitive genes in industrial yeast during wine fermentations.

Table 6. mRNA levels of genes involved in the metabolism of arginine and urea

Gene	No DAP	DAP	No DAP	DAP
<i>GAP1</i>	215	164	232	141
<i>CAN1</i>	141	51	161	66
<i>CAR1</i>	345	179	340	130
<i>DUR1,2</i>	96	39	122	41

No DAP- no di-ammonium phosphate added. **DAP**- di-ammonium phosphate added to a final concentration of 960 mg/l.

Deletion of *URE2* results in decreased ethyl carbamate production. The role of NCR in the regulation of the genes involved in urea metabolism (*DUR1,2*, *DUR3*) is well established (Coffman *et al.*, 1994; El Berry *et al.*, 1993; Genbauffe and Cooper, 1986). It can be hypothesized that the addition of di-ammonium phosphate (good nitrogen source) will prevent urea formation and subsequently ethyl carbamate formation in wine. Therefore, the elimination of *URE2* might relieve cells of NCR and result in the full degradation of urea and the production of less ethyl carbamate. When no nitrogen was added to the Chardonnay grape must, wild type and *ure2* mutant isogenic strains produced similar amounts of ethyl carbamate (Fig. 12). The addition of arginine alone or in combinations with good nitrogen sources resulted in elevated levels of ethyl carbamate in fermentations conducted with the wild type strain (Fig. 12). The increased levels of ethyl carbamate might be due to NCR-regulation of the *DUR1,2* gene preventing degradation of urea.

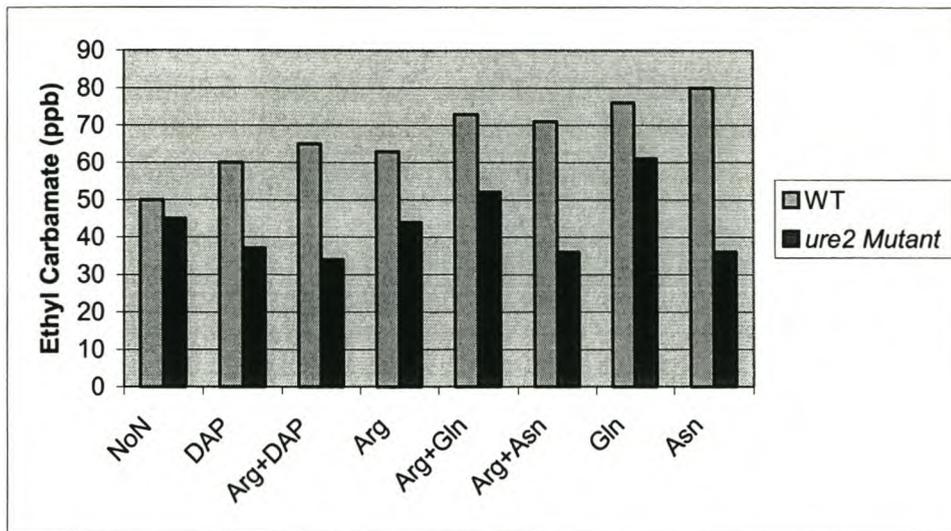


Fig. 12. Ethyl carbamate produced by a *S. cerevisiae* diploid strain and its isogenic *ure2* mutant. NoN- no nitrogen added; Arg- 1500mg/l arginine added; Asn- 960 mg/l asparagine added; Gln- 960 mg/l glutamine added; DAP- 960 mg/l di-ammoniumphosphate added; Arg+DAP- 1500mg/l arginine and 960 mg/l di-ammoniumphosphate added; Arg+Asn- 1500mg/l arginine and 960 mg/l asparagine added; Arg+Gln- 1500mg/l arginine and 960 mg/l glutamine added. Ethyl carbamate concentrations given are the mean values of at least two assays. Standard deviation was 2 ppb.

The deletion of *URE2* does not fully restore expression of *DUR1,2* in the presence of L-asparagine to the same levels as when the yeast cells are grown in L-proline as sole nitrogen source (Coffman *et al.*, 1994). It has been argued that the NCR regulation of *DUR1,2* is not solely effected by *URE2*. This might explain the increased levels of ethyl carbamate observed when glutamine was added to the Chardonnay must that was fermented with the *ure2* strain (45 ppb when no nitrogen was added and 62 ppb when glutamine was added)

The *CAR1* gene is induced by arginine and has been shown to be NCR-sensitive (Smart *et al.*, 1996). The addition of arginine will result in the production of increased levels of urea by *S. cerevisiae*. The fermentations done with the wild type showed that ethyl carbamate levels did indeed increase when arginine was added. The excess urea produced from the added arginine is probably not fully degraded by urea amidolyase (*Dur1,2p*). This might be due to regulation of the *DUR1,2* gene or

because the levels of urea were so high that some of it was secreted into the environment where it can react with ethanol. The levels of ethyl carbamate in the fermentation conducted with the *ure2* mutant did not change substantially from the fermentation with no added nitrogen. Less ethyl carbamate was produced by the *ure2* mutant strain when compared to the wild type when arginine was added to the grape juice. This may be due to derepression of the *DUR1,2* gene in the *ure2* mutant resulting in effective degradation of urea.

The data clearly showed that *ure2* mutant strains produced less ethyl carbamate in wine fermentations than the wild type strain. Due to the regulatory role of Ure2p the deletion of *URE2* may affect the physiology of the yeast which could have an influence on the formation of flavour compounds by yeast in wine. In addition, *ure2* mutants have several phenotypes that can be problematic in industrial fermentations, such as slow growth (Xu *et al.*, 1993) that can lead to stuck or sluggish fermentations. Therefore, the elimination of *URE2* might create more problems than it would solve.

The analysis of the wine revealed that the laboratory yeast did not complete the fermentation. Laboratory strains do not have the same levels of resistance, in comparison to industrial yeast, to compounds such as ethanol and other products of fermentations. With residual sugars left, the yeast probably can utilize the secreted urea as a nitrogen source thereby eliminating the major precursor of ethyl carbamate. The ethyl carbamate produced might therefore differ if the fermentations were completed by the yeast.

The fermentations conducted with the *ure2* mutant produced more ethanol.

The wild type strain seems to be sensitive to alcohol concentrations higher than 6% (v/v). The *ure2* mutant produced 1% to 2.5 % (v/v) more ethanol than the wild type strain. The nitrogen composition of the medium did not seem to play a role in the increased ethanol tolerance of the *ure2* mutant (Fig. 13). No literature has been published linking *URE2* to ethanol sensitivity in *S. cerevisiae*. The *ure2* mutant's increased ethanol tolerance in comparison to the wild type indicate that the regulatory role of Ure2p might exceed its experimentally established function.

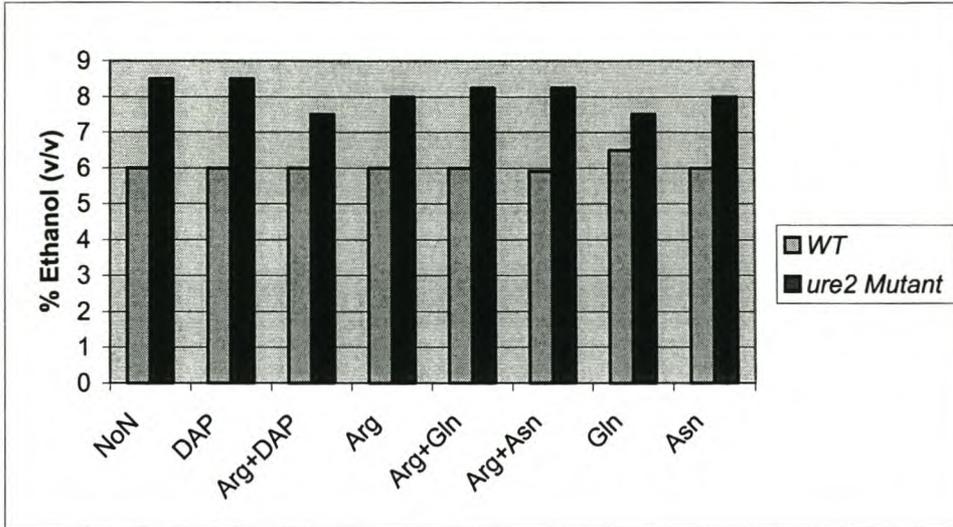


Fig. 13. Ethanol production by a diploid wild type strain and isogenic *ure2* mutant in Chardonnay grape must. NoN- no nitrogen added; Arg- 1500mg/l arginine added; Asn- 960 mg/l asparagine added; Gln- 960 mg/l glutamine added; DAP- 960 mg/l di-ammoniumphosphate added; Arg+DAP- 1500mg/l arginine and 960 mg/l di-ammoniumphosphate added; Arg+Asn- 1500mg/l arginine and 960 mg/l asparagine added; Arg+Gln- 1500mg/l arginine and 960 mg/l glutamine added.

Conclusions

1. The Ure2p in the yeast *S. cerevisiae* contains an N-terminal and a C-terminal domain. The C-terminal domain has been shown to be involved in NCR. To identify the functional region in the C-terminal domain, seven *URE2* deletion mutants were constructed by PCR. Northern blot analysis of the NCR-sensitive genes *DAL5*, *CAN1* and *GAP1* in *S. cerevisiae* revealed that the NCR regulatory activity of Ure2p was lost with the deletion of the first 30 nucleotides from the 3' end of the *URE2* gene. Therefore, it can be concluded that Ure2p requires the last 10 amino acids of the protein for regulatory function.

2. High-density DNA micro-array analysis has become a powerful tool to study metabolic and genetic control of gene expression on a genomic scale in yeast cells. This technology was used to study the effect of NCR on genes involved in the transport and metabolism of arginine and urea in an industrial wine yeast strain. The addition of DAP to fermenting Chardonnay grape must revealed that the expression

of the *GAP1*, *CAN1*, *CAR1*, and *DUR1,2* genes in the wine yeast, VIN13, was down regulated ~1.5, ~2.6, ~2, and ~2.7 fold, respectively. These genes have previously been shown to be NCR-sensitive in laboratory yeast strains. Therefore, the phenomenon of NCR is indeed functional in industrial strains of *S. cerevisiae*. Moreover, the timed addition of DAP might therefore be applied to control the production of ethyl carbamate during wine fermentations.

3. Chardonnay wine produced with a laboratory *ure2* mutant strain of *S. cerevisiae* contained less ethyl carbamate compared to wine produced with the isogenic parental strain containing a functional *URE2* gene. This could be due to the loss of transcriptional repression of the genes responsible for the degradation of urea, one of the pre-cursors of ethyl carbamate. The use of a *ure2* wine yeast strain might, therefore, result in lower levels of ethyl carbamate in industrial wine fermentations. However, *ure2* deletion strains show phenotypes such as slow growth that might result in stuck or sluggish fermentations. Furthermore, the *ure2* mutant strain produced approximately 1.5 to 2.5% more ethanol (6% vs 8.5% v/v) than the isogenic wild type strain. These data suggest that the Ure2p might have other functions in the yeast cell apart from its important role in NCR.

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