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The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*

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Abstract

Saccharomyces cerevisiae is able to use a wide variety of nitrogen sources for growth. Not all nitrogen sources support growth equally well. In order to select the best out of a large diversity of available nitrogen sources, the yeast has developed molecular mechanisms. These mechanisms consist of a sensing mechanism and a regulatory mechanism which includes induction of needed systems, and repression of systems that are not beneficial. The first step in use of most nitrogen sources is its uptake via more or less specific permeases. Hence the first level of regulation is encountered at this level. The next step is the degradation of the nitrogen source to useful building blocks via the nitrogen metabolic pathways. These pathways can be divided into routes that lead to the degradation of the nitrogen source to ammonia and glutamate, and routes that lead to the synthesis of nitrogen containing compounds in which glutamate and glutamine are used as nitrogen donor. Glutamine is synthesized out of ammonia and glutamate. The expression of the specific degradation routes is also regulated depending on the availability of a particular nitrogen source. Ammonia plays a central role as intermediate between degradative and biosynthetic pathways. It not only functions as a metabolite in metabolic reactions but is also involved in regulation of metabolic pathways at several levels. This review describes the central role of ammonia in nitrogen metabolism. This role is illustrated at the level of enzyme activity, translation and transcription.

Keywords: Nitrogen metabolism; *Saccharomyces cerevisiae*; Ammonia; Catabolite repression; Regulation

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1. Introduction

In its natural habitat the yeast *Saccharomyces cerevisiae* encounters a wide variety of nitrogen sources [1]. However, not all nitrogen sources support growth equally well. Growth on good nitrogen sources yields relatively higher growth rates than on poor nitrogen sources. Good nitrogen sources are ammonia, glutamine and asparagine whereas proline and urea are qualified as poor nitrogen sources. In order to use a molecule as nitrogen source, yeast cells have to convert this molecule into glutamate and glutamine [1–3]. These two amino acids serve as nitrogen donors for all other nitrogen containing compounds in the cell [3]. Both glutamate and glutamine can be synthesized directly using ammonia as the amino group donor. The NADPH dependent glutamate dehydrogenase (NADPH-GDH) converts ammonia and α-ketoglutarate into glutamate [4] and glutamine synthetase (GS) produces glutamine out of ammonia and glutamate [5,6] (Fig. 1). Therefore all nitrogen sources are degraded to finally yield ammonia or glutamate, the latter is eventually also converted into ammonia and α-ketoglutarate by NAD dependent glutamate dehydrogenase (NAD-GDH) [1,3].

Glutamate and glutamine might be used as nitrogen sources on their own. During growth on glutamate, GS produces glutamine using ammonia generated by NAD-GDH [3]. When glutamine is the sole nitrogen source for growth, glutamate is produced by glutamate synthase (GOGAT) or via the NADPH-GDH [3,7,8], in the former case ammonia is produced by glutaminases which degrade glutamine to glutamate and ammonia [9,10].

Other nitrogen sources like asparagine, proline or urea are also degraded to glutamate and ammonia [1]. Asparagine is converted into aspartate and ammonia by asparaginases [11,12]. Proline degradation involves three steps which are located in the mitochondria and lead to the production of glutamate [13,14]. Urea is degraded in two steps to yield ammonia. In the first step allophanic acid is produced by urea carboxylase, a biotin requiring enzyme [1]. In the second step, allophanic acid is in turn degraded to ammonia and CO_2.

Thus all nitrogen sources are converted to ammonia and glutamate. Glutamate together with glutamine plays an essential role in the nitrogen metabolism, therefore we propose to call the interconversion of ammonia, glutamate and glutamine the central nitrogen metabolism (CNM). An overview of the central nitrogen metabolism is presented in Fig. 1.

As stated above, not all nitrogen sources support growth equally well. Therefore *S. cerevisiae* selects nitrogen sources enabling the best growth by a mechanism called nitrogen catabolite repression. Two nitrogen sources eliciting nitrogen catabolite repression (NCR), ammonia and glutamine, are also encountered in the CNM.

The scope of this review is to survey the central feature of ammonia in CNM and its role in regulation of this metabolism via nitrogen catabolite repression. In order to do so, first a detailed description is given of the mechanisms known to operate in nitrogen catabolite repression. Subsequently the role of ammonia in CNM and its effect on the synthesis of key enzymes in CNM is examined. Based on genomic sequences a comparison between the CNM of *S. cerevisiae* and *Caenorhabditis elegans* is presented. Finally the central role of ammonia in CNM and NCR and hence nitrogen metabolism in the yeast as a whole is described using a mathematical model of the regulation of the transcription of the genes involved in NCR.

2. Nitrogen catabolite repression enables selection of the best nitrogen sources

When good nitrogen sources like glutamine, asparagine or ammonia are added to the medium of yeast cells growing on a poorer nitrogen source, the transcription of some genes involved in the utilization of the poorer nitrogen source is repressed, and their corresponding products are inactivated and degraded [2,3]. For example the general amino acid permease Gap1p and the proline permease

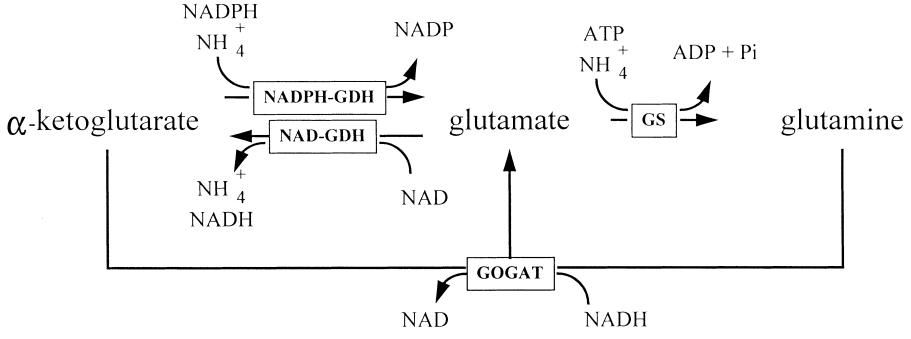

Fig. 1. Interconversion of α-ketoglutarate, ammonia, glutamate and glutamine in the CNM. Ammonia can be converted into glutamate via two pathways. It may be coupled directly to α-ketoglutarate to form glutamate by NADPH-GDH, a reaction which costs NADPH. It may also be incorporated into glutamine via GS at the expense of an ATP. Glutamine and α-ketoglutarate are converted to glutamate via GOGAT at the expense of NADH. Glutamate can be degraded to α-ketoglutarate and ammonia via NAD-GDH using NAD.

Put4p are regulated by the nitrogen source present in the medium. However, permeases like the histidine permease Hip1p, the lysine permease Lyp1p and the basic amino acid permease Can1p also exist, which are expressed constitutively [15–19,23]. The physiological response of inactivating gene expression in reaction to the nitrogen source present in the medium is called nitrogen catabolite repression [20]. The transcription repression of permease encoding genes and the selective inactivation and subsequent degradation of existing permeases upon addition of good nitrogen sources in the growth medium prevents uptake of the poorer ones [21]. These two mechanisms ensure the yeast that the best nitrogen source for growth is selected. Much work in recent decades has been directed towards the regulation of NCR, and progress has been made in understanding the underlying mechanisms of NCR.

NCR operates at different levels. The down regulation of proline metabolism by ammonia provides an excellent example of the different levels at which NCR operates. Regulation of uptake, compartmentalization of enzymatic reactions, inhibition of induction and direct repression of gene expression are all mechanisms that are encountered in this metabolism. In the following sections proline metabolism and its inactivation by ammonia will be described to illustrate the different levels of NCR. Proline is used as an example of a poor nitrogen source.

2.1. The use of proline as nitrogen source

During growth on proline this amino acid is taken up by two permeases located in the plasma membrane, i.e. Gap1p, the general amino acid permease, and Put4p the proline specific permease [22,23]. Intracellular proline is transported from the cytoplasm into the mitochondria where degradation takes place [1,13,14]. This degradation includes three steps.

At first proline is degraded to Δ-pyrroline-5-carboxylate (P5C), a reaction catalyzed by proline oxidase. Secondly P5C spontaneously converts into glutamate semialdehyde. Thirdly P5C dehydrogenase converts glutamate semialdehyde into glutamate. Biosynthesis of proline comprises reactions which are the reverse of the steps involved in degradation [1]. However, the proline biosynthetic enzymes are encoded by different genes and located in the cytoplasm instead of the mitochondria, thereby ensuring that biosynthesis and catabolism are separated [1]. This compartmentalization of metabolic pathways is one way of regulating proline metabolism but in addition there is regulation at the level of protein activity, in particular the activities of the proline permeases are regulated which will be discussed in detail.

2.2. Operation of NCR at the protein level: regulation of proline permease activity

The first step in the regulation of proline utilization is at the level of uptake. Once the permeases are properly expressed they become activated e.g. by phosphorylation and inactivated by dephosphorylation followed by degradation via the ubiquitin pathway [24–26].

2.2.1. Activation of permeases by phosphorylation

Proline grown cells express the permease encoding genes *GAP1* and *PUT4* [22,23]. Activated Gap1p is in a phosphorylated state (Fig. 2) [26]. Also other permeases, like the uracil permease Fur4p, have been shown to be phosphorylated at the plasma membrane [27–29]. In analogy with hexose transporters these observations suggest that activation by phosphorylation is a common process among nitrogen permeases. Npr1p, encoding a protein kinase homolog [30], has been shown to be involved in the activation of Gap1p [25,31]. Although *GAP1* transcription is not reduced in npr1 mutants, Gap1p activity is decreased, indicating that *NPR1* acts at the posttranslational level. However, it remains to be established whether Npr1p phosphorylates Gap1p directly.

Thus during growth under nitrogen catabolite derepressing conditions permeases are expressed and become activated due to phosphorylation probably mediated by Npr1p.

2.2.2. Inactivation, internalization and degradation of permeases

Inactivation of Gap1p is related to its phosphorylation state [26]. Inactivation of the permease by dephosphorylation is followed by a slow disappearance of the protein (Fig. 2). It is unclear whether ammonia causes dephosphorylation of the permease by inactivation of Npr1p or by activation of a yet unidentified phosphatase.

Subsequently dephosphorylated permease becomes a target for Npi1p which probably ubiquitinates the permease inducing its degradation [25]. Hein et al. [32] reported that mutations in *NPI1* suppress not only Gap1p degradation but also Gap1p inactivation whereas Stanbrough and Magasanik [26] demonstrated that the inactivation of Gap1p correlated with its dephosphorylation. The npi1 mutation also prevents inactivation of the *PUT4* and *FUR4* permeases by ammonia as well as that of the *GNP1* glutamine permease [25]. *NPI1* has been shown to encode a protein the C-terminal half of which is homologous ($>30\%$) to the human E6-AP ubiquitin ligase component, and is identical to *RSP5*, a ubiquitin-protein ligase (E3 enzyme) [32]. Deletion of *NPI1* has established that this gene is essential for cell viability, indicating that the function of this protein is not restricted to nitrogen source dependent inactivation of permeases. Somehow the C-terminus of Gap1p is involved in its inactivation and degradation. Deletion of the 11 C-terminal residues of Gap1p did not affect the Gap1p activity but prevented inactivation of Gap1p by ammonia [33].

Inactivation of the uracil permease Fur4p is followed by endocytosis and degradation in the vacuole [29]. In end3

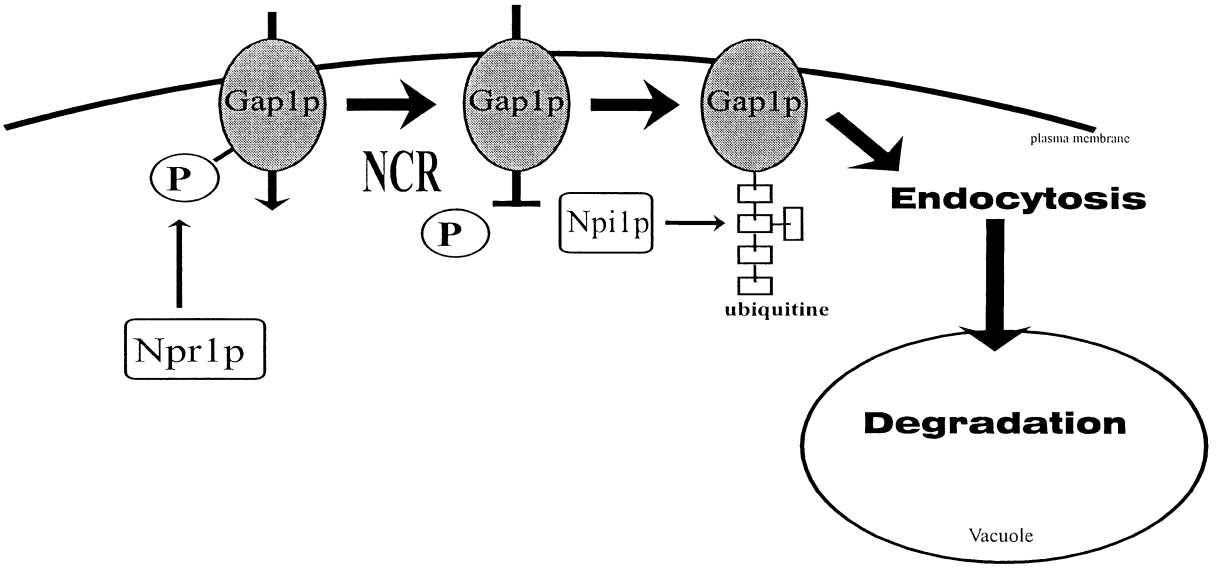

Fig. 2. Posttranslational regulation of the general amino acid permease Gap1p. Gap1p is activated due to phosphorylation probably mediated by Npr1p. NCR leads to rapid dephosphorylation resulting in inactivation of the permease which is followed by degradation via the ubiquitin degradation route, a process mediated by Npi1p.

and end4 mutants, deficient in the internalization step of receptor mediated endocytosis, degradation of the permease was strikingly slower. Moreover the permease was degraded in the vacuole, since pep4 mutants lacking vacuolar protease activities accumulate large amounts of uracil permease in the vacuole [29]. The stress induced degradation of uracil permease is prevented by a mutation in a cyclin-like 'destruction box' which is required for the ubiquitin dependent proteolysis of cyclins [34]. It might be that this holds true not only for Fur4p but that other permeases like Gap1p are also internalized and degraded in the vacuole.

Indeed the intracellular sorting of Gap1p has been shown to be dependent on the nitrogen source used for growth. For example during growth on urea, and in some strains (per1− mutants) on ammonia, Gap1p is directed towards the plasma membrane. After a shift to a medium containing glutamate the intracellular sorting of the Gap1p is re-routed via the prevacuolar compartment towards the vacuole where the protein is degraded [35]. *SEC13* together with *LST4*, *LST7* and *LST8* is required for this regulated transport of Gap1p to the plasma membrane [35,36]. Sec13p is a component of the COPII vesicle coat that acts in the endoplasmic reticulum to Golgi transport [36–38]. Perhaps Sec13p, Lst4p, Lst7p and Lst8p function as components of a post-Golgi secretory vesicle coat [36].

In summary, these data suggest that sorting of the permease depends on the nitrogen source present in the medium; moreover, addition of ammonia, or compound(s) derived from it, to nitrogen catabolite derepressed cells causes dephosphorylation and subsequent inactivation of the permease followed by ubiquitin triggered degradation in the vacuole. However, direct interaction between the permeases and the kinase as well as the permease and the ubiquitin ligase remains to be proven.

2.3. Operation of NCR at the DNA level: regulation of proline inducer exclusion

During growth on poor nitrogen sources like proline, addition of good nitrogen sources inactivates permeases transporting poor nitrogen sources [24]. In addition, the synthesis of new permeases is blocked at the level of gene expression. Compared to the rapid inactivation of the permeases, the process of transcription inhibition is a medium term effect.

Proline is transported into the cells using the *GAP1* and *PUT4* encoded permeases [25]. Intracellular proline activates a transcription factor Put3p [29–33]. This transcription factor induces the expression of *PUT1* and *PUT2*, the structural genes encoding the proline degrading enzymes proline oxidase and P5C dehydrogenase, respectively [13,44]. Subsequently these enzymes are transported into the mitochondria where they are used to degrade proline to glutamate. Thus Put3p activity is absolutely essential during growth on proline [45] (Fig. 3).

Upon addition of good nitrogen sources like ammonia or glutamine to proline grown cells, the permeases are rapidly inactivated [2,24,25]. This inactivation reduces proline uptake and thereby decreases the intracellular proline levels [35,39–43]. This will result in a decrease in the Put3p activity, and consequently the expression of *PUT1* and *PUT2* is decreased. Thus by preventing the inducer from entering the cell, the expression of the degrading enzymes is reduced.

2.4. Transcription repression

Nitrogen catabolite repression has been shown to be mediated by inhibitors of, among others, transcription factors that recognize UAS_{NTR} [46–53]. UAS_{NTR} is necessary and sufficient for NCR regulation [49]. Both *GAP1* and *PUT4* contain multiple UAS_{NTR} sites in their promoters [54]. The nitrogen regulated activation sequence UAS_{NTR} consists of two separate dodecanucleotide sites with the sequence GAT(T/A)A at their core [48,55]. In addition a single UAS_{NTR} site combined with another unrelated *cis*-acting element is able to mediate transcription as well [56]. Moreover, when this UAS_{NTR} site functions in combination with an unrelated site, the regulatory responses observed are a hybrid consisting of characteristics derived from both the UAS_{NTR} site and the unrelated site [56]. The mechanism of inducer exclusion applies to inducible genes only. In contrast, the mechanism of repression via UAS_{NTR} includes almost all NCR sensitive genes and is therefore the most important one.

Until now, five key players have been identified to participate in NCR at the level of transcription. These include four transcription factors, two positive: Gln3p, Gat1p/Nil1p and two negative: Dal80p/Uga43p, Deh1p/Gzf3p/Nil2p/YJL110c. The fifth player is the regulatory protein Ure2p [26,50,52,54,57–67].

2.4.1. Role of the positive acting transcription factors Gln3p and Gat1p

Maximal transcriptional activation mediated by UAS_{NTR} requires both a functional *GLN3* and *GAT1* gene product [51,52,55,59,62,68–72].

GLN3 is not essential for growth and it encodes a protein containing a single putative Cys_2/Cys_2 zinc finger which has homology to the *Neurospora crassa NIT2* protein, the *Aspergillus nidulans AREA* protein, and the erythroid specific transcription factor GATA-1 [59]. Immunoprecipitation experiments indicated that the Gln3p protein binds to UAS_{NTR} of *GLN1*, encoding glutamine synthetase; in addition it has been shown that in vitro Gln3p binds to UAS_{NTR} sequences [59,70,73]. The *GLN3* protein is required for expression of the NCR sensitive genes *UGA1*, *CAR1 CAN1*, *GAP1*, *PUT4, ASP3*, *GDH1, GDH2* and *DAL4*. In addition *GLN3* protein is required for expression of the *DAL1*, *DAL2* and *DAL7* genes [54,74,75,79].

Coffman et al. [71] discovered a Gln3p independent nitrogen repression pathway. The transcription factor operating in this pathway has been identified, and called Gat1p/Nil1p [26,52,65]. Gat1p is a transcriptional activator of the GATA family of DNA binding proteins. *GAT1* expression is Gln3p dependent, Dal80p regulated and nitrogen repression sensitive [52]. Consistent with this is the identification of Gln3p and Dal80p binding sites upstream of the *GAT1* gene.

It has been shown that Gln3p and Gat1p use the same GATAAG sites to activate the expression of *GAP1*. Furthermore the sensitivity towards Gln3p and Gat1p of the UAS_{NTR} elements of *GLN1*, *GDH2* and *GAP1* differed markedly [65]. All three genes can respond to Gln3p but the UAS_{NTR} of *GDH2* is unresponsive to Gat1p. Moreover, the UAS_{NTR} of *GLN1* does not respond to Gat1p as well as the two separated GATAAG elements found upstream of *GAP1*. The activity of Gat1p only becomes visible in a ure2− background [65]. So far no physiological regulatory advantage has been detected for a yeast cell expressing both Gln3p and Gat1p [52]. Full inducer independent expression of NCR sensitive genes requires both Gln3p and Gat1p. The specific contribution of each single factor to the transcription activation depends on the gene

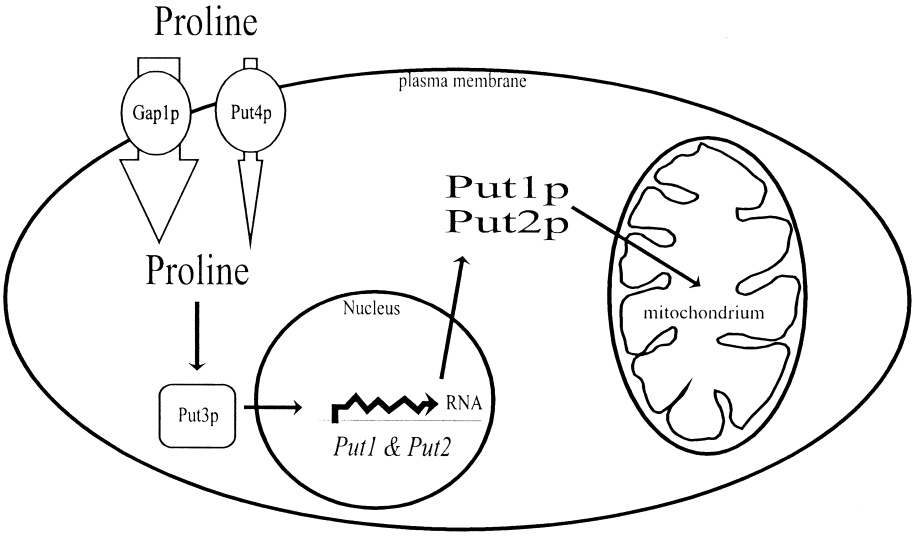

Fig. 3. Regulation of proline degradation. Intracellular proline stimulates the transcription activator Put3p, which leads to synthesis of the proline degrading enzymes Put1p and Put2p which are transported into the mitochondria where proline is converted to glutamate.

being regulated [52]. The Gln3p and Gat1p mediated transcription depends on a functional Ada1p [76]. Ada1p is part of the Ada1p/Gcn5p co-activator complex and links Gln3p and Gat1p regulated transcription to the basal transcription machinery [76–78]. The extent to which Ada1p is required, however, also varies according to the gene and to the nitrogen source available [76].

2.4.2. Role of the negative acting transcription factors Dal80p and Deh1p

Cunningham and Cooper have shown that Gln3p is not the only protein binding to sequences containing GAT-(T/A)A at their core [61]. The *DAL80* protein also binds to such sequences. Like Gln3p, the Dal80p primary structure contains a zinc finger motif and has extensive homology to the metazoan GATA-1 binding family of transcription activators [50,63]. Dal80p has been shown to contain a putative leucine zipper and can be shown to dimerize in a two-hybrid assay [63]. Moreover, the Dal80p binding site, URS_{GATA}, requires two GATAA elements for binding oriented tail-to-tail or head-to-tail, suggesting that dimerization may be involved [50,61].

The *DAL80* protein down regulates a number of inducer independent genes associated with nitrogen metabolism, which is demonstrated by the increased expression of many NCR regulated genes in a mutant carrying a disruption of *DAL80* [54]. However, Dal80p is not affiliated with NCR, NCR regulated genes remain sensitive for NCR in dal80 deletion mutants [51,52,54,71,73,76].

Not all yeast genes requiring Gln3p for expression are sensitive to Dal80p regulation and neither is the converse true [54]. These differences may originate from different association constants, or from different spacing of the GATAA elements [61]. The expression of the *DAL80* gene is sensitive to nitrogen repression, and its promoter contains 12 sequences homologous to the UAS_{NTR}. By analyzing ure2, dal80 or ure2 dal80 mutants for the expression of the Dal80p sensitive gene *UGA4*, it was found that the high level expression activating activity of the *UGA4* UAS_{GATA} is inhibited by two distinct repression systems. One system is Dal80p (Uga43p) dependent; it operates in cells grown on a poor nitrogen source. The other is the nitrogen repression system, which relies on Ure2p and glutamine and operates when a good nitrogen source is present. Nitrogen repression also blocks the synthesis of Dal80p, making the two repression systems mutually exclusive [80].

DEH1 is a fourth gene whose product displays significant homology to Dal80p, Gat1p and Gln3p [52,64,66,81]. A related shorter sequence (*NIL2*) has been reported by Stanbrough et al. [65]. The greatest homology exists between Deh1p and Dal80p, including the GATA-type zinc finger and the leucine zipper domains. Also, the upstream region of *DEH1* contains multiple GATA sequences and might therefore be regulated by the other GATA family proteins [66]. These data suggest that Deh1p might function as an additional negative regulator of NCR sensitive genes which is controlled by these factors on its own.

2.4.3. Role of Ure2p

Mutations in *GLN3* prevented normal increase in the NAD-GDH and glutamine synthetase levels in glutamate grown cells [68]. On the other hand, mutations in the *URE2* gene resulted in high levels of these enzymes during growth on glutamate and glutamine. A ure2 gln3 double mutant had low levels of glutamate dehydrogenase and glutamine synthetase under these conditions; thus Gln3p is epistatic to Ure2p [68]. These results suggest that the *URE2* product antagonizes the *GLN3* product and is situated above it in the regulatory pathway.

Ure2p is involved in the down regulation of Gln3p activity in response to ammonia, glutamine or asparagine. The *URE2* gene has been cloned and sequenced. It encodes a predicted polypeptide which has homology with glutathione *S*-transferases [60]. Deletion of the first 63 amino acids does not have any effect on the function of the protein. Expression of the *URE2* gene does not appear to be regulated [60], also the protein levels are constitutive (ter Schure, unpublished data). It has been demonstrated that Ure2p regulates NCR gene expression through the UAS_{NTR} element [51]. Recently Ure2p has been suggested to bind to Gln3p directly by coimmunoprecipitation with anti-Gln3p antibodies [82], however Gln3p could not be precipitated with Ure2p antibodies.

A particular mutation of *URE2* has received special attention. The dominant mutation of *URE2* called [URE3] was first discovered by Lacroute [83], it exhibited a non-Mendelian segregation. The phenotypes of ure2 and [URE3] are similar in both mutants. NCR sensitive genes are no longer repressed upon the addition of ammonia, glutamine or asparagine to the growth medium [84,85]. Mating a [URE3] strain with a wild-type strain resulted in diploids all being [URE3]. Cytoduction was used by Aigle and Lacroute to confirm the nonchromosomal character of [URE3]. [URE3] can be cured by growth of cells on rich medium containing 5 mM guanidine HCl [86]. But cured, purified strains again give rise to [URE3] clones. The *URE2* gene is necessary for the propagation of [URE3] and overproduction of Ure2p results in a 100-fold increase of the frequency with which [URE3] arises [84]. Thus, [URE3] satisfied all the criteria for a yeast prion. The misfolded prion conformation of Ure2p leads to constitutive nitrogen derepressed cells and the conformation of this protein may be is important for its function in NCR [84,85,87].

Recessive mutations in *URE2* show elevated expression of the *PUT1* and *PUT2* genes involved in proline degradation, when cells are grown on a nitrogen repressing medium [45]. *PUT1* and *PUT2* contain several putative UAS_{NTR} sequences in their promoter regions [54]. Coffman et al. reported that *PUT1* expression is dramatically less in a gln3Δ or gat1Δ single mutant or a gln3Δ gat1Δ

Table 1
Open reading frames of the *S. cerevisiae* genome containing six or more GAT(A/T)AG sites in 2000 bp of the 5′ untranslated regions

5′ Untranslated region of	Hit number	Information	5′ Untranslated region of	Hit number	Information
YKL070W	9	*Hypothetical ORF*	YDR023W/SES1	6	Seryl-tRNA synthetase
YKL071W	9	*Hypothetical ORF*	YDR024W	6	*Hypothetical ORF*
YIR033W/MGA2	8	Product of unknown gene	YEL010W	6	*Hypothetical ORF*
YKL031C/SPO14	8	Phospholipase D	YEL021W/URA3	6	Orotidine-5′-phosphate decarboxylase
YKR038C	8	*Hypothetical ORF*	YEL058W/PCM1	6	Phosphoacetylglucosamine mutase
YKR039W/GAP1	8	General amino acid permease	YFR050C/PRE4	6	Proteasome subunit necessary for peptidyl glutamyl peptide hydrolyzing activity
YBR261C	7	*Hypothetical ORF*			
YDL210W/UGA4	7	GABA specific transport protein			
YGL196W	7	*Hypothetical ORF*	YGL226C-A/OST5	6	9.5-kDa zeta subunit of oligosaccharyltransferase complex
YHR029C	7	*Hypothetical ORF*			
YIR027C/DAL1	7	Allantoinase	YHL036W/MUP3	6	Very low affinity methionine permease
YIR028W/DAL4	7	Allantoin permease	YHR028C/DAP2	6	Dipeptidyl aminopeptidase B (DPAP B)
YIR031C/DAL7	7	Malate synthase 2			
YJL110C/GZF3	7	GATA zinc finger protein 3 homologous to Dal80 in structure and function	YIR005W	6	*Hypothetical ORF*
			YIR032C/DAL3	6	Ureidoglycolate hydrolase
			YJL 073W/JEM1	6	DnaJ-like protein of the endoplasmic reticulum membrane
YMR108W/ILV2	7	Acetolactate synthase			
YNL128W/TEP1	7	Similar to human suppressor gene known as TEP1, MMAC1 and PTEN1. Contains sequence motifs characteristic of protein tyrosine phosphatases.	YJR152W/DAL5	6	Allantoate permease
			YKR035W-A/FTI1	6	RAD52 inhibitor (fifty two inhibitor)
			YLL065W/GIN11	6	Product of gene unknown
			YLL066C	6	*Hypothetical ORF*
			YLR081W/GAL2	6	Galactose permease
YNL142W/MEP2	7	Ammonia transport protein	YLR095C	6	*Hypothetical ORF*
YPL019C	7	*Hypothetical ORF*	YLR0226W/BUR2	6	Product of gene unknown
YPL150W	7	*Hypothetical ORF*	YLR299W/ECM38	6	γ-Glutamyltransferase homolog
YAR050W/FLO1	6	FLO1 putative cell wall glycoprotein	YLR421C	6	*Hypothetical ORF*
YBL049W	6	*Hypothetical ORF*	YMR046W-A	6	*Hypothetical ORF*
YBR122C/MRPL36	6	Mitochondrial ribosomal protein MRPL36 (YmL36)	YNL116W	6	*Hypothetical ORF*
			YNL314W/DAL82	6	Positive regulator of allophanate inducible genes
YBR124W	6	*Hypothetical ORF*			
YBR162C	6	*Hypothetical ORF*	YNL316C/PHA2	6	Prephenate dehydratase
YBR208C/DUR1,2	6	Urea amidolyase (contains urea carboxylase and allophanate hydrolase)	YNR029C	6	*Hypothetical ORF*
			YOL019W	6	*Hypothetical ORF*
YBR262C	6	*Hypothetical ORF*	YOL107W	6	*Hypothetical ORF*
YBR286W/APE3	6	Amidopeptidase yscIII	YOR219C/STE13	6	Dipeptidyl aminopeptidase
YCL064C/CHA1	6	Catabolic serine (threonine) dehydratase	YPL214C/THI6	6	TMP pyrophosphorylase hydroxyethylthiazole kinase
YDL171C/GLT1	6	Glutamate synthase (NADPH)	YPL265W/DIP5	6	Dicarboxylic amino acid permease
YDL194W/SNF3	6	Glucose sensor	YPR119W/CLB2	6	G_2 specific B-type cyclin
YDR022C/CIS1	6	Cik1 suppressor			

Total hits: 10 676
Different hit sequences: 5097
Sequences searched: 6217
Entered nucleotide pattern: GAT(A/T)AG
Dataset: 2000 bp of the 5′ untranslated regions

double mutant compared to the wild-type [52,71]. This result is clearly a different response than that observed in the strain background assayed by Xu et al. [45], in that case expression of a plasmid borne *PUT1*-lacZ fusion did not respond to deletion of *GLN3* [71]. In a ure2Δ gln3Δ double mutant *PUT1* expression is still NCR sensitive [52,71]. Thus although full expression of *PUT1* requires a functional *GLN3* and *GAT1* product, the NCR regulation of *PUT1* is not solely dependent on functional *URE2* and *GLN3* protein indicating that several repression mechanisms independent of Ure2p exist [45,52,71].

Even though *PUT3*, the proline utilization pathway transcriptional activator, is absolutely required for growth on proline, a put3 ure2 strain had somewhat elevated *PUT1* expression, suggesting an effect of the ure2 mutation in the absence of the *PUT3* product, indicating the existence of several repression mechanisms independent of Ure2p [45]. Other evidence indicating the existence of additional components was shown by the fact that NCR sensitive expression of some genes occurred in a gln3 ure2 dal80::hisG triple mutant [52,71].

2.4.4. Role of the UAS_{NTR}

Repression of NCR regulated genes involves the pro-

teins Ure2p, Gln3p, Gat1p and Ada1p. The common sequence element of nitrogen regulated genes is the UAS_{NTR} present in their promoters. The variation in regulation of different genes originates from the sensitivity of these UAS_{NTR} elements towards the different transcription factors. Ure2p is the major regulator of the expression of UAS_{NTR} containing genes, however its action is not restricted to Gln3p.

The UAS_{NTR} is characterized by the core sequence GAT(A/T)AG, this sequence is amongst others encountered in the *GAP1* promoter. These sites can be used to search the genome of *S. cerevisiae* for all open reading frames containing the same site in their promoter up to 2000 bp upstream of the putative start codon. The results of such a search using the PatMatch search program as delivered with the Stanford genomic database is shown in Table 1. In total 10 676 hits were encountered in 6217 searched sequences. Of this population a total of 5097 different genes were found. As these sequences appeared not to be selective enough arbitrary sequences containing six or more hits are presented in Table 1. This resulted in 64 open reading frames (ORFs). Of this 64 open reading frames, 23 were hypothetical ORFs. The most striking result is that the two ORFs with the highest number of hits (9) are both hypothetical. These ORFs need further investigation for their involvement in nitrogen metabolism as this search suggests they might encode proteins with a putative role in nitrogen metabolism.

The use of the 6-bp core sequence GAT(A/T)AG of the UAS_{NTR} is apparently not discriminative enough, therefore other sequences have to be used. The Gln3p binding sites in the promoters of both *GDH2* and *GLN1* have been described in more detail than the core sequence. The sequences for these sites are GATAAGATAA and GATTAGATTA respectively. Using these sites in the same search as shown in Table 1, 38 different hit sequences were found for the *GDH2* derived site and 68 different hit sequences were found for the *GLN1* derived site. These search results are shown in Tables 2 and 3 respectively. Within the population of *GDH2* and *GLN1* derived UAS_{NTR} sequences 23 and 22 hypothetical ORFs were found respectively. No ORFs were found which contained both the GATAAGATAA and GATTAGATTA in their 2000-bp upstream region.

Table 2
GATTAGATTA sites in 2000 bp of the 5′ untranslated regions of the *S. cerevisiae* genome

5′ Untranslated region of	Hit number	Information
YBL112C	1	*Hypothetical ORF*
YBL113C	1	*Hypothetical ORF*
YBR043C	1	*Hypothetical ORF*
YBR057C/MUM2	1	Muddled meiosis
YBR172C/SMY2	1	Kinesin related protein suppressing myosin defects (MYO2)
YBR175W	1	*Hypothetical ORF*
YBR272C/HSM3	1	Hsm3p may be a member of the yeast MutS homolog family
YBR274W	1	*Hypothetical ORF*
YCR020C/PET18	1	Transcription regulator
YCR020C-A/MAK31	1	MAK31 snRNP
YDL215C/GDH2	1	NAD dependent glutamate dehydrogenase
YDR140W	1	*Hypothetical ORF*
YDR380W	1	*Hypothetical ORF*
YFR031C/SMC2	1	Nuclear protein related to ScII (chicken), XCAPE (*Xenopus*), and cut14 (*S. pombe*); involved in chromosome segregation and condensation, interacts with Smc1p and Trf4p
YGL220W	1	*Hypothetical ORF*
YGL223C	1	*Hypothetical ORF*
YGL245W	1	*Hypothetical ORF*
YGR018C	1	*Hypothetical ORF*
YGR019W/UGA1	1	;gamma;-Aminobutyrate transaminase (4-aminobutyrate aminotransferase)
YHR219W	1	*Hypothetical ORF*
YIL006W	1	*Hypothetical ORF*
YIL008W	1	*Hypothetical ORF*
YJL041W/NSP1	1	Nucleoskeletal protein found in nuclear pores and spindle pole body
YJR153W/PGU1	1	Endo-polygalacturonase
YKL070W	1	*Hypothetical ORF*
YKL071W	1	*Hypothetical ORF*
YKL094W/YJU3	1	Product of gene unknown
YKL218C	1	*Hypothetical ORF*
YLR299W/ECM38	1	γ-Glutamyltransferase homolog
YLR465C	1	*Hypothetical ORF*
YML127W	1	*Hypothetical ORF*
YML128C	1	*Hypothetical ORF*
YMR088C	1	*Hypothetical ORF*
YNL026W	1	*Hypothetical ORF*
YPL019C	1	*Hypothetical ORF*
YPR167C/MET16	1	3′-Phosphoadenylylsulfate reductase
YPR174C	1	*Hypothetical ORF*
YPR175W/DPB2	1	DNA polymerase epsilon subunit B

Total hits: 38
Different hit sequences: 38
Sequences searched: 6217
Entered nucleotide pattern: GATTAGATTA
Dataset: 2000 bp of the 5′ untranslated regions

3. Regulation of ammonia metabolism

3.1. Ammonia metabolism

Ammonia uptake possibly involves at least three permeases, Mep1p, Mep2p, and Mep3p [88–90]. These permeases are expressed when low ammonia concentrations are present in the growth medium. Mep2p displays the highest affinity for NH_4^+ (K_m 1–2 M), followed closely by Mep1p (K_m 5–10 M) and finally by Mep3p, whose affinity is much lower (K_m 1.4–2.1 mM) [90]. A mep1Δ mep2Δ mep3Δ triple mutant is not able to grow on media containing less than 5 mM NH_4^+ as sole nitrogen source, while the presence of any of the single *MEP* genes restores growth on these media [90]. Growth on NH_4^+ at concentrations high-

Table 3
GATAAGATAA sites in 2000 bp of the 5′ untranslated regions of the *S. cerevisiae* genome

5′ Untranslated region of	Hit number	Information	5′ Untranslated region of	Hit number	Information
YBR208C/DUR1,2	2	Urea amidolyase (contains urea carboxylase and allophanate hydrolase)	YKL160W	1	*Hypothetical ORF*
YFL021W/GAT1	2	Transcriptional activator with GATA-1-type Zn finger DNA binding motif	YKR080W/MTD1	1	NAD dependent 5,10-methylenetetrahydrofolate dehydrogenase
YFL022C/FRS2	2	Phenylalanyl-tRNA synthetase, beta subunit, cytoplasmic	YKR092C/SRP40	1	Nucleolar protein that is immunologically and structurally related to Nopp140, a nonribosomal protein of the nucleolus and coiled bodies.
YNL142W/MEP2	2	Ammonia transport protein			
YAL062W/GDH3	1	NADP linked glutamate dehydrogenase			
YDL056W/MBP1	1	Transcription factor			
YDL122W/UBP1	1	Ubiquitin specific protease	YKR093W/PTR2	1	Peptide transporter
YDL123W	1	*Hypothetical ORF*	YLL014W	1	*Hypothetical ORF*
YDL125C/HNT1	1	Yeast member of the histidine triad protein family (HIT)	YLL043W/FPS1	1	Suppressor of tps1/fdp1, a member of the MIP family of transmembrane channels; may be involved in glycerol efflux
YDR534C	1	*Hypothetical ORF*			
YGL166W/CUP2	1	Activator of transcription			
YGL167C/PMR1	1	Ca^{2+} pump ATPase	YLL045C/RPL8B	1	Ribosomal protein L8B (rp6) (YL5)
YGR190C	1	*Hypothetical ORF*	YLL046C/RNP1	1	Ribonucleoprotein 1
YHL015W/RPS20	1	Ribosomal protein S20	YLR047C	1	*Hypothetical ORF*
YHL016C/DUR3	1	Urea transporter	YLR048W/RPS0B	1	Ribosomal protein S0B
YHR028C/DAP2	1	Dipeptidyl aminopeptidase B	YLR089C	1	*Hypothetical ORF*
YHR029C	1	*Hypothetical ORF*	YLR090W/XDJ1	1	Homolog of *E. coli* DnaJ, closely related to Ydj1p
YHR148W	1	*Hypothetical ORF*			
YHR211W/FLO5	1	Flocculin, similar to flocculation protein Flo1p	YLR091W	1	*Hypothetical ORF*
			YLR136C/TIS11	1	Zinc finger containing homolog of mammalian TIS11, glucose repressible gene
YIL090W	1	*Hypothetical ORF*			
YIR029W/DAL2	1	Allantoicase			
YIR031C/DAL7	1	Malate synthase 2	YLR138W/NHA1	1	Putative Na^+/H^+ antiporter
YIR032C/DAL3	1	Ureidoglycolase hydrolase	YLR353W/BUD8	1	Product of gene unknown
YIR033W/MGA2	1	Product of gene unknown	YMR141C	1	*Hypothetical ORF*
YJL139C/YUR1	1	Probable glycosyltransferase of KRE2/KTR1/YUR1 family; located in the Golgi	YNL144C	1	*Hypothetical ORF*
			YNL280C/ERG24	1	Sterol C-14 reductase
			YNR011C/PRP2	1	RNA splicing factor RNA dependent NTPase with DEAD box motif
YJL143W/TIM17	1	16.5-kDa inner membrane protein required for import of mitochondrial precursor proteins	YNR012W/URK1	1	Uridine kinase
			YOL052C/SPE2	1	*S*-Adenosylmethionine decarboxylase
YJL144W	1	*Hypothetical ORF*	YPL051W/ARL3	1	Similar to ADP-ribosylation factor
YJL157C/FAR1	1	Factor arrest protein	YPL052W	1	*Hypothetical ORF*
YJL172W/CPS1	1	Carboxypeptidase yscS	YPL053C/KTR6	1	Mannosylphosphate transferase
YJL173C/RFA3	1	Subunit 3 of replication factor A	YPL219W/PCL8	1	PHO85 cyclin
YJR021C/REC107	1	Meiotic recombination protein	YPL220W/RPL1A	1	Ribosomal protein L1A, forms part of the 60S ribosomal subunit
YJR023C	1	*Hypothetical ORF*			
YJR024C	1	*Hypothetical ORF*	YPL229W	1	*Hypothetical ORF*
YJR026W	1	*Hypothetical ORF*	YPL230W/USV1	1	Up in starvation
YJR027W	1	*Hypothetical ORF*	YPR035W/GLN1	1	Glutamine synthetase
YKL069W	1	*Hypothetical ORF*	YPR122W/AXL1	1	Putative homolog of human insulin-degrading endoprotease
YKL158W	1	*Hypothetical ORF*			

Total hits: 72
Different hit sequences: 68
Sequences searched: 6217
Entered nucleotide pattern: GATAAGATAA
Dataset: 2000 bp of the 5′ untranslated regions

er than 20 mM does not require any of the Mep proteins [90]. The Mep transporters seem also to be required to retain NH_4^+ inside the cells during growth on some nitrogen sources other than NH_4^+ [90]. At high ammonia concentrations the *MEP* gene expression becomes repressed [90]. On a poor nitrogen source, *MEP2* expression is much higher than *MEP1* and *MEP3* expression. High level *MEP2* transcription requires at least the presence of a functional Gln3p or Gat1p protein. In contrast, the expression of either *MEP1* or *MEP3* requires only Gln3p and is down regulated in a Gat1p dependent manner [90].

During growth on ammonia, there are two ways of producing glutamate and glutamine (Fig. 1). In the first pathway glutamate is generated via NADPH dependent gluta-

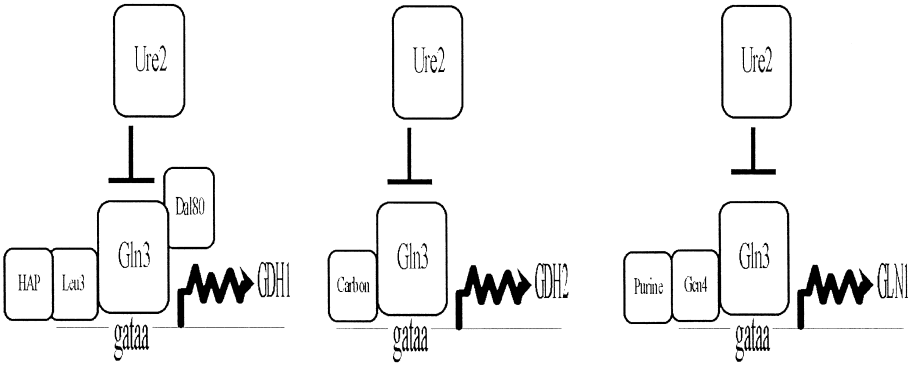

Fig. 4. Regulation of transcription of *GDH1*, *GDH2* and *GLN1* encoding NADPH-GDH, NAD-GDH and GS respectively. *GDH1* transcription is activated by the HAP complex and the transcription factor Leu3p. In addition its transcription is activated by Gln3p via UAS_{NTR} (gataa) sites. This same site is used by Dal80p to inhibit *GDH1* transcription. *GDH2* transcription is activated by Gln3 via UAS_{NTR} and via a factor in response to nonfermentable carbon sources or limiting glucose concentrations (carbon). *GLN1* transcription is regulated via three transcription factors i.e. a purine responsive element (purine), Gcn4p and Gln3p. Gln3p is inactivated by Ure2p in response to NCR.

mate dehydrogenase (NADPH-GDH) by coupling ammonia to α-ketoglutarate at the expense of one NADPH, and subsequently glutamine is generated by glutamine synthetase (GS) which converts glutamate and ammonia into glutamine at the expense of one ATP [3]. In the second pathway, glutamine is produced by GS and glutamate is synthesized via glutamate synthase (GOGAT) which converts one molecule of glutamine and one molecule of α-ketoglutarate into two molecules of glutamate at the expense of one NADH [8]. The relative importance of each individual pathway is not yet confirmed by experimental data. However, a structured, minimal parameter model of CNM predicting the fluxes through each successive pathway predicts an important role for GS/GOGAT [91].

3.2. Regulation of ammonia incorporation at the level of transcription

All nitrogen sources are ultimately converted to either ammonia or glutamate [1,3]. Since growth on different nitrogen sources yields different rates of ammonia or glutamate production, the conversion of ammonia into glutamate and subsequently glutamine must be regulated as well. When ammonia is produced NADP-GDH, GOGAT and GS are necessary to supply the yeast with glutamate and glutamine. When glutamate is an intermediate in the degradation pathway, NAD-GDH is used to produce ammonia for the synthesis of glutamine. Because all nitrogen sources are eventually degraded to ammonia, one would expect a certain constitutive activity level of the glutamate and glutamine synthesizing enzymes which can be changed rapidly due to activation and deactivation in order to adapt to changing conditions. However, this is not the case [54,70]. The activities of these enzymes are regulated depending upon the kind and the concentration of the nitrogen source present in the medium. Recent observations have shown that the regulation of these activities is mainly at the level of transcription [92]. Moreover, although these genes contain UAS_{NTR} sites their expressions do not always correlate with the NCR state of the cell [54,56].

3.2.1. Regulation of GDH1 and GDH3 encoding NADP dependent GDHs

Ammonia and α-ketobutyrate are converted to glutamate by NADP-GDH. Isoforms of this enzyme are encoded by *GDH1* and *GDH3* [93–96]. Not much is known about the regulation of *GDH3*, partial deletion results in a variety of aberrant starvation phenotypes [95]. The induction of *GDH3* upon diauxic transition is dependent upon Msn2p and Msn4p, two general transcription factors acting via the *cis* CCCCT element in response to various stresses [97]. This *cis* element is also a target for repression by the cyclic AMP signalling pathway.

The promoter of *GDH1* contains several UAS_{NTR} homologous sequences. The transcription of *GDH1* has been shown to be Gln3p dependent (Fig. 4) [54]. Moreover, the expression of *GDH1* was decreased with increasing residual ammonia concentrations in the growth medium [92]. In addition, the *GDH1* promoter contains a binding site for Leu3p, a transcriptional activator of the branched chain amino acid biosynthetic pathways [98,99]. Activation of Leu3p requires the presence of α-isopropylmalate and it has been demonstrated that *GDH1* is, in part, activated by a Leu3-α-isopropylmalate complex [100]. In addition to this UAS_{LEU} site the *GDH1* expression is also regulated by a CCAAT box [101]. The CCAAT box binding factor is required for optimal expression of *GDH1*. The CCAAT box is recognized by the HAP complex, this heteromeric complex is composed of at least four subunits named Hap2p, Hap3p, Hap4p, and Hap5p and has been shown to be involved in carbon dependent regulation of several genes involved in respiration [102–105]. Transcription of genes containing the consensus sequence ACCAATNA is activated in response to a nonfermentable carbon source by binding of four heme activated proteins Hap2p/Hap3p/Hap4p/Hap5p [104]. A hypothesis explaining the regulation of *GDH1* expression

in response to nonfermentable carbon sources via the HAP complex is that utilization of nonfermentable carbon sources requires the tricarboxylic acid cycle (TCA) and the electron transport chain (ECT). The TCA generates α-ketoglutarate, a substrate of Gdh1p, and delivers reduction equivalents to the electron transport chain which requires oxygen.

Heme is known to play a pivotal role, acting as a positive modulator for the transcription of aerobic genes and as a negative modulator for the transcription of hypoxic genes [106]. Consequently, cellular concentrations of heme, whose biosynthesis is oxygen dependent, are thought to provide a gauge of oxygen availability and dictate which set of genes will be transcribed. But the precise role of heme in oxygen sensing and the transcriptional regulation of oxygen responsive genes is presently unclear [106].

3.2.2. Regulation of GLN1 encoding GS

Glutamine is produced from glutamate and ammonia by GS, an enzyme encoded by *GLN1* [70]. The transcription of *GLN1* is controlled by three regulatory systems (Fig. 4). One system responds to glutamine levels and depends on the positively acting *GLN3* product [59,70]. The second system is general amino acid control, which couples derepression of a variety of biosynthetic enzymes to starvation for many single amino acids. This system operates through the positive regulatory element Gcn4p [107,108]. A third system responds to purine limitation; no specific regulatory element has been identified, but derepression of glutamine synthetase is observed during purine starvation in *gln3 gcn4* mutants [70]. Bas1p is a yeast transcription factor that activates expression of purine and histidine biosynthesis genes in response to extracellular purine limitation [109,110]. The sequence TGACTC is the core sequence of the Bas1p binding sites [109]. Indeed we found in a TGACTC sequence at position −268 upstream of the *GLN1* coding sequence. Genetic evidence should establish whether this site really is used by Bas1p in order to mediate purine limited induction of *GLN1* expression.

3.2.3. Regulation of GDH2 encoding NAD dependent GDH

In order to provide ammonia for the production of glutamine during growth on glutamate yielding nitrogen sources, the NAD dependent GDH, encoded by *GDH2*, degrades glutamate into ammonia [1]. The regulation of Gdh2p has been reported to occur at the level of enzyme activity and at the level of transcription [92,111–113]. It has been reported that enzyme activity of Gdh2p is controlled by phosphorylation and subsequent proteolysis during glutamate starvation; however, these results have not been confirmed by recent evidence [114,115].

The regulation of transcription of *GDH2*, the structural gene of NAD-GDH, is dependent upon six different sequence elements present in the promoter of the gene [113]. Two elements behave as upstream activation sites. The remaining four sites were found to block the effects of the two UASs. One upstream activation site (*GDH2* UASc) has been shown to be sufficient for activation of transcription during growth with nonfermentable carbon sources or with limiting amounts of glucose, even in the presence of the repressing nitrogen source glutamine [112]. In addition, the *GDH2* promoter contains a Gln3p binding site (UAS_{NTR}), hence *GDH2* transcription is lowered during growth on glutamine [54].

3.2.4. Regulation of GLT1 encoding GOGAT

GOGAT, glutamate synthase, synthesizes two molecules of glutamate out of one molecule of glutamine and one molecule of α-ketoglutarate, a reaction in which one molecule of NADH is used as cofactor [8,116,117]. GOGAT is encoded by the *GLT1* gene. The *GLT1* promoter contains one Gcn4p binding site, and three GATAA sequences which might operate as UAS_{NTR}. The *GLT1* promoter also harbors two tail-to-tail oriented URS_{GATA} sequences which might form a Dal80p binding site [117]. Glutamate rich nitrogen sources like glutamate, glutamine, or asparagine down regulate *GLT1* expression, whereas Gln3p and Gcn4p up regulate *GLT1* expression. *GLT1* is not regulated by Gat1p and *GLN3* activates *GLT1* expression with ammonia, proline, glutamate, glutamine and asparagine as nitrogen sources [117].

3.2.5. Mixed expression of genes in response to different nutritional conditions

Thus the enzymes involved in the conversion of ammonia, glutamate and glutamine are mainly regulated at the level of transcription. All five genes described contain multiple UAS_{NTR} sites [54]. It has been found that when this site functions in combination with other sites, the observed regulatory responses are a hybrid consisting of characteristics derived from both sites [56]. Because of the existence of additional regulatory sequences in all three promoters the expressions of *GDH1*, *GDH2* and *GLN1* in response to the same nitrogen source present in the medium may differ depending upon the other physiological conditions [54,119].

3.3. Ammonia acting as a repressor of gene expression

Ammonia inhibits the synthesis of a number of proteins involved in the utilization of poorer nitrogen sources at the level of transcription [2,3]. Growth on ammonia results in higher growth rates compared to growth on proline or urea, this is caused by a higher metabolic nitrogen flux towards the synthesis of glutamate or glutamine [1]. Until recently it was generally accepted that ammonia had to be converted into glutamine in order to exhibit repression of gene expression [3]. This was due to the observation that in a per1 mutant strain Gap1p and Put4p permeases are expressed at high levels when ammonia is used as a nitrogen source but are still sensitive to glutamine induced

Table 4
The entire complements of predicted proteins involved in ammonia metabolism from the budding yeast *S. cerevisiae* ('Yeast') and nematode *C. elegans* ('Worm') genomes

Yeast sequence	Function	Worm sequence	Function
GDH1/YOR375C	NADP-glutamate dehydrogenase	ZK829.4	glutamate dehydrogenase
GDH2/YDL215C	NAD-glutamate dehydrogenase	–	–
GDH3/YAL062W	NADP-glutamate dehydrogenase	ZK829.4	glutamate dehydrogenase
GLN1/YPR035W	glutamine synthetase	C28D4.3	glutamine synthase
		C45B2.5	glutamine synthetase
		T25C8.3	glutamine synthase
		K03H1.1	probable glutamine synthetase
		Y105C5zz3	Unknown
		F26D10.10	Unknown
GLT1/YDL171C	glutamate synthase	W07E11.1	glutamate synthase
		C25F6.3	unknown
GLN3/YER040W	transcription factor	C33D3.1	zinc finger protein (GATA type)
		ELT-1/W09C2.1	transcription factor ELT-1
		F52C12.5	unknown
		Y48A5B.c	unknown
DAL80/YKR034W	transcription factor	C33D3.1	zinc finger protein (GATA type)
		ELT-1/W09C2.1	transcription factor ELT-1
		F52C12.5	unknown
		Y48A5B.c	unknown
GAT1/YFL021W	transcription factor	C33D3.1	zinc finger protein (GATA type)
		ELT-1/W09C2.1	transcription factor ELT-1
		F52C12.5	unknown
		Y48A5B.c	unknown
DEH1/YJL110C	transcription factor	C33D3.1	zinc finger protein (GATA type)
		ELT-1/W09C2.1	transcription factor ELT-1
		F52C12.5	unknown
		Y48A5B.c	unknown
URE2/YNL229C	regulatory protein	–	–
MEP1/YGR121C	ammonia permease	C05E11.4	ammonium transporter
		M195.3	putative ammonium transporter
		F49E11.3	putative ammonium transporter
MEP2/YNL142W	ammonia permease	C05E11.4	ammonium transporter
		M195.3	putative ammonium transporter
		F49E11.3	putative ammonium transporter
MEP3/YPR138C	ammonia permease	C05E11.4	ammonium transporter
		M195.3	putative ammonium transporter
		F49E11.3	putative ammonium transporter

The entire complements of predicted proteins involved in ammonia metabolism from the budding yeast *S. cerevisiae* ('Yeast') and nematode *C. elegans* ('Worm') genomes were compared in a collaboration between the Saccharomyces Genome Database, Eugene Koonin's group (NCBI), and Temple Smith's group (Boston University). This analysis is described in detail in Chervitz et al. [126]. This Worm-Yeast analysis was performed at different comparison stringencies (different *P*-values (1e−100, 1e−50, 1e−20, or 1e−10) and different alignment values ($>80\%$ or $<80\%$) in order to pick out a gene's involvement in a shared or unshared cluster at each different comparison stringency.

NCR [118]. Growth on ammonia would yield high intracellular glutamine concentrations that in turn would trigger the transcription repression [2,3,92].

The NCR responses, observed during growth on ammonia, might originate from either the nitrogen flux or the nitrogen concentration. In the case of flux, higher metabolic fluxes would result in inactivation of transcription. Using continuous cultures it was shown that the expression of *GAP1* and *PUT4* was not changed by the ammonia flux but by the ammonia concentration [92,119,121].

However, the increase in the ammonia concentration also resulted in an increase in the intracellular glutamine concentration and hence ammonia repression could still be generated indirectly via glutamine [92]. Addition of ammonia to mutant cells carrying a defective glutamine synthetase, gln1–37, and hence unable to produce glutamine out of ammonia, resulted in a rapid decrease in *GAP1* and *PUT4* expression without changing the intracellular glutamine concentration either in the cytosol or in the vacuole [120,122]. This demonstrates that it is indeed the concentration of ammonia which triggers NCR. This conclusion is supported by the observation that in continuously cultured wild-type cells *GAP1* expression correlated with the ammonia concentration whereas the intracellular glutamine concentration remained constant [92].

Ammonia induced repression does not occur when cells are starved for nitrogen for more than 10 min [122]. Deletion of the *URE2* gene also prevents ammonia repression in the gln1–37 background, showing that part of the ammonia repression signal is transmitted via the Ure2p/

Gln3p pathway. These experiments demonstrate that the ammonia concentration induces nitrogen repression via Ure2p without conversion into glutamine.

3.4. Similarity between yeast and worm

S. cerevisiae is often used as model organism for other eukaryotes. The availability of the genomic sequences of *S. cerevisiae* and of the worm *Caenorhabditis elegans* provides the opportunity to ascertain whether components known to play a central role in the nitrogen metabolism of baker's yeast are also present in the worm. This gives a first glimpse of how well nitrogen metabolism and its regulation in yeast serve as model for higher eukaryotes.

In order to perform the homology search, the sequenced genes of *S. cerevisiae* were aligned to the genome of *C. elegans*. The sequences searched for are the genes encoding the enzymes in the CNM of *S. cerevisiae*, *GDH1*, *GDH2*, *GDH3*, *GLN1* and *GLT1*, and genes encoding the *S. cerevisiae* regulatory proteins *GLN3*, *DAL80*, *GAT1* (= *NIL1*), *DEH1* (= *GZF3* or *NIL2*) and *URE2*. In addition the ammonia permease encoding genes *MEP1*, *MEP2*, and *MEP3* were searched. The results obtained from the Stanford genomic database are shown in Table 4. Except for *GDH2* and *URE2*, homologs to all other sequences searched for are encountered in the worm genome. Of the 17 worm sequences found, five are of unknown function. The lack of *URE2* homologs in the worm genome suggests that the *S. cerevisiae* NCR regulation might not be present in *C. elegans*; moreover, a large number of additional GATA factors exist which might not be operative in nitrogen metabolism regulation in *C. elegans*.

4. A model describing NCR regulation on transcription

Growth on ammonia, glutamine or asparagine as the sole nitrogen source leads to repression of genes sensitive to NCR. It was assumed that nitrogen repression in yeast was triggered by high intracellular glutamine concentrations [3,119] and models in the past described a role for ammonia in NCR only by its conversion into glutamine.

Addition of ammonia to nitrogen derepressed gln1-cells demonstrated that ammonia does not need to be converted into glutamine to repress transcription [122]. Ure2p is involved in the transcription repression by ammonia. When Ure2p is stimulated this leads to the inactivation of several transcription factors, amongst others Gln3p and Gat1p, which decrease the transcription of UAS_{NTR} containing genes. Moreover, a Ure2p-Gln3p independent pathway regulating the activity of the transcription activator Gat1p is present [51,52]. In the presence of ammonia the activity of Gat1p decreases leading to the reduced activity of UAS_{NTR} containing genes. This results in a further reduction of the expression of UAS_{NTR} containing genes.

Ammonia repression affects not only genes involved in the metabolism of poor nitrogen sources, but also genes in CNM. These genes are also regulated by transcription factors operating in various other signalling pathways. The final result of the transcription activation is a hybrid between the signals of all these pathways. Therefore, different physiological conditions lead to different transcriptional outputs using the same source of nitrogen.

The transcriptional regulation of genes involved in CNM, their posttranslational modifications and the CNM as such, are very complicated processes. In the future the complexity will even increase, when biosciences will concentrate not just on the molecules but also on their intracellular localization as a function of time after a certain event. To understand the interrelation of molecules quantitatively it is necessary to develop structural and/or cybernetic models that describe even subtle differences in regulatory and metabolic processes [91,123]. Such models are a prerequisite for understanding diseases related to malfunctions of the metabolism of humans, to understand better the beneficial influence of certain components of foods on human health and reprogramming metabolisms of organisms to increase their industrial utility. The value of (still far from perfect) mathematical models was nicely demonstrated by the prediction of the structured metabolic model of the CNM, which required a significant role of GOGAT (Glt1p). To validate the model we have constructed a glt1 deletion mutant, and experiments with this mutant showed that indeed GOGAT is crucial for both the CNM and the redox regulation in *S. cerevisiae* [124]. Finally such models are necessary to compare

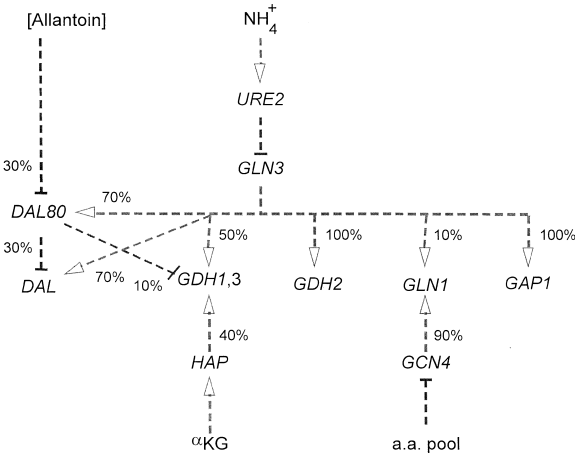

Fig. 5. Schematic overview of the regulation of transcription in CNM as included in the model. The estimated relative contribution of each factor to the activity of a gene or protein is presented. For example, the model assumes that 50% of the *GDH1* activity is regulated by Gln3p, 10% by Dal80p and 40% by the HAP complex. Thus when Gln3p is completely inactivated by Ure2p, GDH1 will only reach 10%+40% = 50% of its maximal expression.

(semi)quantitatively the regulation and metabolic processes of different organisms.

These mathematical models can also be used to describe quantitatively the expression of proteins involved in major parts of the metabolism. Such a model, describing the expression of the genes involved in CNM, has been developed considering different types of nitrogen sources, each type yielding different intracellular concentrations of ammonia glutamate and glutamine (Fig. 5). The model was validated using the experimental data of ter Schure et al. [92], and it was shown that the expression levels of genes involved in CNM could be predicted reasonably well under several different conditions based on the generation of intracellular ammonia and glutamate levels [125]. Although the model is still far from perfect it clearly demonstrates that complicated processes like regulation of expression in CNM can be predicted accurately using a quantitative approach and computational methods.

The sensor for ammonia remains obscure but continuous culture experiments indicate that the concentration of ammonia is sensed rather than its metabolic flux. It might be possible that Ure2p is able to recognize ammonia on its own, it is also possible that an unidentified protein senses ammonia and delivers its signal to Ure2p. Additionally, ammonia inactivation of permeases is independent of glutamine. For this mechanism also a protein sensing ammonia has to exist. Subsequently this protein triggers signal transduction pathways that will lead to transcription repression and permease inactivation. The gene containing the per1 mutation might encode such a sensing molecule [118]. As a role for glutamine and asparagine has not been eliminated, it might be that more than one signal exists.

In conclusion, the role of ammonia in the regulation of nitrogen metabolism is more important than only its conversion into glutamate and glutamine. Ammonia regulates enzyme activities, permease activities and transcription of nitrogen regulated genes. The pathways underlying these regulations are complex. It is a challenge for the future to determine the components of these pathways, including the sensor, and to unravel the molecular mechanisms. Such studies will preferably use molecular biological and physiological approaches like continuous culturing.

5. Conclusions

The function of ammonia in the nitrogen metabolism of the yeast *S. cerevisiae* is not restricted to its use as a nitrogen source. Whereas the yeast can use ammonia as sole nitrogen source, ammonia also functions as an important intermediate connecting degradation and biosynthesis routes. Its important role in nitrogen metabolism is further illustrated by its role in the conversion of glutamate to glutamine and vice versa. The conversion of these three nitrogen metabolites in the CNM is highly regulated, as is the expression of the genes encoding the enzymes of the CNM. The role of ammonia in regulating this expression is becoming clearer. However, the sensing mechanism remains to be established.

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