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Regulation of Nitrogen Metabolism and Gene Expression in Fungi

GEORGE A. MARZLUF

Department of Biochemistry and Program in Molecular, Cellular and Developmental Biology, The Ohio State University, Columbus, Ohio 43210

INTRODUCTION

Nitrogen is a major element found in many of the simple compounds and nearly all of the complex macromolecules of living cells. Proteins and nucleic acids are especially rich in nitrogen. Thus, it should not be surprising that a substantial cellular investment is made in the metabolic machinery comprising nitrogen catabolic pathways to ensure a constant nitrogen supply for growth. Extensive studies of nitrogen metabolism and its control have been carried out in three fungi, *Neurospora crassa*, *Aspergillus nidulans*, and *Saccharomyces cerevisiae*. In each of these organisms, major control systems exist to regulate nitrogen metabolism. In this review, certain nitrogen catabolic pathways will be briefly described, and the regulatory mechanisms which control nitrogen metabolism in these fungi will be considered in depth. A number of excellent reviews concerning nitrogen metabolism and various related topics in fungi are available (23, 29, 30, 59, 60, 65, 66, 84, 88, 99). Although certain compounds, particularly ammonia, glutamate, and glutamine, are favored nitrogen sources, these fungi are capable of utilizing many diverse secondary sources, including nitrate, nitrite, purines, proteins, numerous amino acids, acetamide, and even acrylamide. The use of these secondary nitrogen sources invariably requires the synthesis of catabolic enzymes or, in some cases, an activation of previously existing enzymes. De novo synthesis of many of the nitrogen-regulated enzymes requires that two conditions be met. First, there must be a lifting of nitrogen catabolite repression (also called ammonium repression); second, in many cases, specific induction of the enzymes of a particular catabolic pathway by a substrate or intermediate of the pathway must also occur. A major goal is to gain an understanding of the genetic and metabolic signals that are responsible for the separate steps of repression and induction.

The majority of the available information and evidence concerning the regulation of nitrogen metabolism in the above fungi is derived from genetic analysis of various structural and control mutants. Thus, it is appropriate to summarize certain concepts relevant to such genetic studies. Genetic evidence alone is usually (perhaps always) insufficient to establish the level at which the product of a regulatory gene acts; in fact, it is even difficult to unequivocally identify a regulatory gene with only genetic evidence, since other types of mutants can yield a phenotype similar to that expected for mutants of control genes. The ability to obtain different mutants of the same gene which yield opposite phenotypes (constitutive versus null) provides at least strong indirect evidence for a true regulatory gene. However, the inability to find mutants with opposite phenotypes does not exclude a regulatory role for a particular gene.

Most of the regulatory genes of the fungi are not linked to their structural genes, although certain exceptions to this rule exist. A regulatory mutant has two possible phenotypes: it may

cause the constitutive expression (partial or complete) of the relevant enzymes, or it may lead to the absence (or reduced levels) of these same enzymes (null mutant). It is diagnostic that mutation within positive-acting control genes will frequently give rise to null mutants and only rarely yield constitutive mutants. Conversely, mutants of negative-acting control genes are usually constitutive, and only infrequently are null phenotypes obtained. We will encounter examples of both positive and negative nitrogen control genes in the fungi. Another important feature is the presence of "major" and "minor" control genes; the major nitrogen control genes integrate the expression of numerous enzymes of nitrogen metabolism, whereas the minor control genes are pathway specific and affect only the enzymes of a particular catabolic pathway. Finally, we can anticipate identification of control regions adjacent to structural genes (which may serve as recognition sites for regulatory proteins) as *cis*-acting mutants, which yield either a constitutive or a null phenotype. As will be seen, these types of mutants can be particularly difficult to define with genetic evidence alone.

It is occasionally necessary to be reminded that changes in the amount of enzyme activity observed in mutants and in various strains grown under different nutrient conditions can result from effects at many different levels of control. These include transcription (and possibly processing and secretion of messenger ribonucleic acid [mRNA]), translation, mRNA stability, enzyme maturation (from an inactive precursor), enzyme turnover, and enzyme inhibition (by low- or high-molecular-weight inhibitors). Since many studies of nitrogen regulation in fungi involve measurement of enzyme activities in crude extracts, it is virtually impossible in such cases to know which of the above steps are implicated. Accordingly, an attempt will be made to weigh the strength of evidence as it is presented and to discuss any available information which implies control at certain levels, e.g., de novo enzyme synthesis or mRNA synthesis. Finally, the cellular location of enzymes, substrates, effectors, and regulatory components is of paramount importance to regulation as it occurs in vivo; however, since this itself is a comprehensive area in which excellent reviews are available (28–30), no attempt will be made to cover this subject here.

NITRATE REDUCTASE

Inorganic nitrate serves as an excellent nitrogen source for both *Neurospora* and *Aspergillus*. Nitrate is enzymatically converted to nitrite in a two-electron transfer reaction; nitrite is then converted to ammonium in a complex reaction involving the transfer of six electrons. Nitrate reductase of *Neurospora* is a dimeric enzyme of molecular weight 228,000 and is composed of two identical polypeptide subunits (11). Nitrate reductase possesses a molybdenum-containing cofactor, which is also found in other molybdenum enzymes, including xanthine dehydrogenase (XDH) (84). Nitrite reductase also appears to be a homodimer of molecular weight 290,000 and contains an unusual siroheme prosthetic group. The synthesis of both nitrate reductase and nitrite reductase requires induction (by nitrate) and is completely repressed by ammonium.

Mutants in a large number of unlinked genes in *Neurospora* lead to the inability to use nitrate as a nitrogen source (Table 1). The *nit-3* gene apparently encodes the major polypeptide of nitrate reductase and is unlinked to *nit-6*, which specifies nitrite reductase (94). By contrast, *niaD* and *niiA*, which encode nitrate and nitrite reductase, respectively, in *Aspergillus*, are tightly linked (93). In *Aspergillus*, an additional five or six loci, known as the *cnx* genes, together specify a molybdenum-containing cofactor shared by nitrate reductase and XDH I and II (Table 1). This cofactor consists of a polypeptide component encoded by *cnxH* and a molybdenum ligand group whose formation is apparently instructed by the other *cnx* genes (23, 84). A similar situation exists in *Neurospora*; at least four genes, *nit-1*, *nit-7*, *nit-8*, and *nit-9*, seem homologous to the *Aspergillus cnx* genes and are responsible for synthesis and assembly of the molybdenum cofactor (94). The *nit-9* locus is complex and contains three complementation

TABLE 1. *Genes in Neurospora and in Aspergillus which affect the nitrate assimilatory pathway*

Function	Genetic locus	
	Neurospora	*Aspergillus*
Structural gene for nitrate reductase	*nit-3*	*niaD*
Structural gene for nitrite reductase	*nit-6*	*niiA*
Genes which specify a molybdenum cofactor	*nit-1*	*cnxABC*
	nit-7	*cnxE*
	nit-8	*cnxF*
	nit-9	*cnxG*
		cnxH
Pathway-specific control gene (mediates induction)	*nit-4*	*nirA*
Major nitrogen regulatory genes (mediates nitrogen catabolite repression)	*nit-2*	*areA*
		tamA

groups, indicating that it may comprise three closely linked genes, similar to the complex *cnxABC* locus of *Aspergillus*.

In addition to the genes encoding structural components of the nitrate assimilatory enzymes, at least two types of regulatory mutants are found in *Neurospora* (and *Aspergillus*) which cannot use nitrate or nitrite for growth and lack both nitrate and nitrite reductase. One control gene of *Neurospora*, *nit-4*, is pathway specific, and mutants of it lack nitrate reductase and nitrite reductase but do not affect any other nitrogen-related enzymes. All mutants of *nit-4* are null, which implies that this gene is a positive-acting regulatory locus. Although originally another pathway-specific control gene, designated *nit-5*, was reported in *Neurospora*, it was recently demonstrated *nit-4* and *nit-5* are actually representatives of a single locus, hereafter referred to as *nit-4* (94). This control gene is apparently responsible for mediation of the induction of nitrate and nitrite reductase; it has been postulated that the *nit-4*$^+$ gene product is a regulatory protein which, upon binding the inducer nitrate, binds at recognition sequences adjacent to the *nit-3* and *nit-6* structural genes to "turn on" their expression (59, 94). A second positive-acting regulatory gene of *Neurospora*, designated *nit-2*, controls nitrate and nitrite reductase along with many other nitrogen-related enzymes and mediates nitrogen catabolite repression (36, 59, 78).

In *Aspergillus*, a pathway-specific control gene (*nirA*) for induction and a major control gene (*areA*) which mediates nitrogen catabolite repression appear to be analogous to the *Neurospora* regulatory genes described above. The fact that constitutive *nirA*^{c} mutants do not require induction but still are nitrogen repressible for nitrate reductase, whereas constitutive-type *areA* mutants (*areA*^{d}) still require induction (but are nonrepressible), supports the suggested roles postulated for these control genes.

The structural genes which encode nitrate reductase (*niaD*) and nitrite reductase (*niiA*) are very closely linked and probably contiguous in *Aspergillus*. Tomsett and Cove (93) have accomplished a detailed genetic analysis of the *niaD-niiA* region by deletion mapping. They found that approximately 1% of spontaneous chlorate-resistant mutants behaved as *niaD-niiA* double mutants, which in every case were shown to be deletions. The deletion mapping confirmed the extremely tight linkage of these two genes and, moreover, permitted localization of a *cis*-acting regulatory mutant which affects *niiA* gene expression within the region between *niiA* and *niaD* (93).

The tight linkage of *niiA* and *niaD* obviously brings up the question of whether they may be regulated as a unit in an operon type of arrangement. Both genes require the product of *nirA*$^+$, the pathway-specific positive control gene, and of *areA*, a positive-acting major nitrogen control gene, for expression. However, similar control genes regulate the analogous but unlinked structural genes in *Neurospora*; moreover, synthesis of nitrate and nitrite reductase in *Aspergillus* is not strictly coordinately regulated, and *cis*-acting mutants which may define promoter or control elements are found which are specific for just one or the other of these genes. In addition, all *nirA*^{c} mutants studied to date lead to much higher constitutive expression of nitrate reductase than nitrite reductase (9); this result is most easily interpreted to suggest that *niiA* and *niaD* each possess a recognition site for the *nirA* gene product, and that they differ sufficiently to have markedly different affinities for the altered product specified by the *nirA*^{c} mutant.

Arst et al. (9) have completed a detailed study of *nis-5*, which behaves as a tightly linked *cis*-acting constitutive mutant that controls *niiA* expression. The *nis-5* mutant permits about 8% constitutive expression of just nitrite reductase, which is independent of any *nirA* gene product and is not ammonium repressible nor inducible; upon induction, the *nis-5* mutant strain produces about 40% of the wild-type level of nitrite reductase (77). It was recently determined that the *nis-5* phenotype actually resulted from a nonreciprocal translocation in which a segment of chromosome II was inserted between the *niaA* and *niiA* genes (9). It was suggested that the inserted segment contains a low-level promoter which is responsible for the low constitutive expression of *niiA* and that a natural promoter specific for *niiA*, which resides between *niaD* and *niiA*, was still present in the translocation mutant and was responsible for the inducible expression. The reduced expression upon induction could be explained by the presence of neighboring chromosome II sequences which place the regular promoter in a new environment. It perhaps deserves emphasis that the constitutive phenotype of *nis-5* suggested that it was a mutant of a control gene when, in fact, it resulted from a translocation. The available information is consistent with the existence of two such different promoters for *niiA* in the *nis-5* strain, but the evidence is not compelling. It does seem clear that separate promoters exist for the *niaD* and the *niiA* genes, which suggests that these linked genes are controlled separately although in a parallel manner. Arst et al. (9) speculated that the *niiA* gene is normally ex-

pressed in two ways, first as a bi- or polycistronic mRNA which initiates at the *niaD* gene and reads through into *niiA* sequences, and second by transcription of *niiA* as a monocistronic mRNA from a promoter lying between *niaD* and *niiA*. Evidence necessary to establish this possibility should include a demonstration that *niiA* coding information occurs on transcripts of radically different sizes. However, since only the first coding sequence in a polycistronic mRNA can apparently be translated in eucaryotic systems (70), it seems probable that *niiA* and *niaD* are transcribed separately, each from its own promoter-control region.

Garrett and his colleagues have presented convincing evidence that induction of nitrate reductase in *Neurospora* involves de novo enzyme synthesis. When noninduced cultures were transferred to medium containing 90% deuterium oxide, induction by nitrate yielded nitrate reductase of uniformly heavy density (11). This result argues for de novo enzyme synthesis and rules out the possibility that an inactive precursor or even any major component of nitrate reductase existed before induction. Furthermore, Amy and Garrett (1) used highly specific immunoelectrophoretic techniques to detect nitrate reductase protein, even partial chains, independent of any enzymatic activity. The antibodies used inhibited all known activities of nitrate reductase and did not display any cross-reaction with XDH or nitrite reductase. They found that uninduced wild-type cultures which lack nitrate reductase, as well as nitrogen-repressed cultures, lack any cross-reacting material (CRM). Moreover, *nit-1*, *nit-3*, and *nit-6* mutants contain CRM when induced, as expected, but the two regulatory mutants *nit-2* and *nit-4* lack any detectable CRM (1). These results convincingly demonstrate that nitrate reductase is not controlled by activation of a preexisting precursor protein or by some form of inhibition of a cryptic enzyme, but involves de novo synthesis.

Premakumar et al. (72) have used a novel approach to study the synthesis and stability of *Neurospora* nitrate reductase mRNA. Tungsten, a molybdenum analog, inhibits the development of nitrate reductase activity because it is actually incorporated into nitrate reductase (in place of Mo), yielding an inactive enzyme. This feature permits experimental approaches in which nitrate reductase mRNA can be accumulated in the absence of synthesis of any active enzyme. Uninduced mycelial pads are transferred to "transcription medium" which contains nitrate as the inducer plus tungsten, so that mRNA can accumulate but any nitrate reductase synthesized will be inactive (72). The pad is subsequently moved to translation medium containing glutamine (to prevent any additional mRNA synthesis) plus Mo, so that any accumulated mRNA can then be translated into active nitrate reductase. Cultures are harvested and assayed for their content of nitrate reductase activity, with the assumption that the capacity to synthesize the enzyme is equivalent to the cellular content of mRNA (72). This approach permitted several tentative conclusions. Glutamine prevents the accumulation of nitrate reductase mRNA but does not affect mRNA translation nor enzyme activity, indicating that nitrogen repression occurs at transcription. It is important to note that presumptive mRNA accumulated under inducing conditions, and that both nitrogen derepression and nitrate induction were required for mRNA accumulation (73). Induced cultures which contain such accumulated mRNA begin synthesizing active nitrate reductase immediately upon transfer to translation medium, whereas uninduced cultures display a 20-min lag before the onset of nitrate reductase synthesis; this difference also argues that translatable mRNA can accumulate under conditions in which active nitrate reductase cannot be made. The results strongly argue that control of nitrate reductase, at least in *Neurospora*, occurs at the transcriptional level and that both induction and nitrogen catabolite repression control this step, or a closely related one, such as mRNA processing or transport. If any control of this enzyme occurs at the translational or post-translational level, it apparently makes only a minor contribution to the overall regulation.

NITRATE REDUCTASE AND AUTOGENOUS CONTROL

In *Aspergillus nidulans*, synthesis of nitrate reductase and nitrite reductase is controlled both by nitrate induction and by ammonium repression. Furthermore, nitrate reductase has been postulated to play an autogenous regulatory role, controlling its own synthesis and that of nitrite reductase. Indeed, Cove and Pateman (24), who suggested such a control function for nitrate reductase, may have been the first to propose autogenous gene regulation. The key observation that suggests this hypothesis is that many mutants which lack nitrate reductase activity nevertheless display constitutive synthesis of nitrite reductase and of nitrate reductase CRM. Cove (23) found that 17 *niaD* mutants, including one deleted for a large segment of the *niaD* gene (out of a total of 27 examined), produced nitrite reductase and nitrate reductase CRM constitutively. Moreover, certain *cnx* mu-

tants also resulted in the constitutive synthesis of nitrite reductase. Thus, it appears that the nitrate reductase protein, probably in at least a near-native conformation, is needed for normal regulation. The results indicate that synthesis of the nitrate pathway enzymes normally requires nitrate induction, but that when nitrate reductase is missing, their synthesis still requires a *nirA*$^+$ protein but not nitrate. Thus, nitrate reductase may itself provide the nitrate recognition site, whereas the *nirA*$^+$ product may be responsible for the specific recognition of the structural genes. A simple interaction suggested by these considerations is that nitrate reductase itself binds to the *nirA*$^+$ protein when nitrate is absent, thereby leading to a conformation of the *nirA*$^+$ product which is unable to initiate *niiA* and *niaD* expression. Upon binding nitrate, the reductase may no longer bind to the *nirA*$^+$ protein (or its conformation changes) so that the *nirA* protein can turn on expression of the structural genes. According to this suggestion, a mutant completely lacking nitrate reductase, or possessing an altered form incapable of binding the *nirA* protein, should no longer require nitrate induction for synthesis of nitrite reductase (or nitrate reductase CRM if relevant). Another suggested mechanism is that nitrate reductase is a repressor and directly binds to deoxyribonucleic acid (DNA) recognition sequences to repress structural gene expression unless the inducer nitrate is present (84). In this view, however, it is not clear what function the *nirA* protein would have.

Although the autoregulatory role proposed for nitrate reductase stems entirely from genetic evidence, and thus must remain speculative, it seems very important that future work be directed at providing additional evidence for such a function and elucidating the molecular mechanism for autogenous control. Although many possible models can be suggested, each of them should permit specific predictions for in vitro experimentation. Thus, the first model suggested above predicts that nitrate reductase should bind to (or alter in some other way) the *nirA*$^+$ product and, moreover, that the binding should be abolished in the presence of NO_3^-. This, in fact, could provide a direct means to search for the postulated *nirA*$^+$ gene protein via affinity chromatography techniques, since highly purified nitrate reductase is available. One might also expect that nitrate reductase might, in part, be localized within the nucleus for its regulatory function. Alternatively the *nirA*$^+$ product could be a DNA-binding protein found in the cytoplasm (complexed with nitrate reductase) and able to enter the nucleus only upon nitrate induction.

TURNOVER OF NITRATE REDUCTASE

Attention has been given to the inactivation of nitrate reductase, which occurs both in vivo and in vitro, and the possibility that turnover of this enzyme could have a regulatory function (91, 100). In *Neurospora*, two different decay mechanisms for nitrate reductase can be distinguished in vivo. One decay system, designated "N," is especially prevalent in nitrogen-starved cells, is very sensitive to ethylenediaminetetraacetic acid and cycloheximide, and decreases with mycelial age. The second system, termed "A," is relatively insensitive to ethylenediaminetetraacetic acid and cycloheximide and increases with mycelial age. The N system for nitrate reductase decay is apparently a general turnover system which appears during nitrogen starvation. The post-translational effect of nitrate probably results from its stabilization and partial protection of the enzyme from system N.

The existence of two systems for inactivation of nitrate reductase has also been demonstrated in vitro (91). Inactivator I was present in all mycelia tested, regardless of nitrogen growth conditions, and was present in the *nit-2* mutant; it may correspond to system A. Inactivator I is inhibited by a specific thermostable inhibitor present in boiled crude extracts of *Neurospora*. Inactivator II, also studied in vitro, is nitrogen repressible and is missing in *nit-2* mutants; it is phenylmethylsulfonyl fluoride sensitive and very active at pH 5. This inactivator is apparently a serine protease and may be the one responsible for rapid turnover of nitrate reductase in young, nitrogen-starved cells (system N). Inactivator II is also found in association with a specific thermostable inhibitor. The inhibitors are excluded by Sephadex G-25, and both appear to be small, thermostable proteins (91). The two inactivators are separable on Sephadex G-150, and both have properties which indicate that they are proteases. The results presently available suggest that neither of these decay systems is specific for nitrate reductase; they both probably play only a general role in protein turnover rather than any special regulatory function.

PURINE CATABOLISM

Another area of nitrogen metabolism which has been extensively studied in *Aspergillus*, *Neurospora*, and yeasts is purine catabolism (Fig. 1). Although the pathway itself and its regulation are very similar in *Aspergillus* and *Neurospora* (79, 86), striking differences are present in yeasts (21).

The conversion of hypoxanthine to xanthine and then to uric acid in *Aspergillus* has been studied in detail recently (89, 90), and several

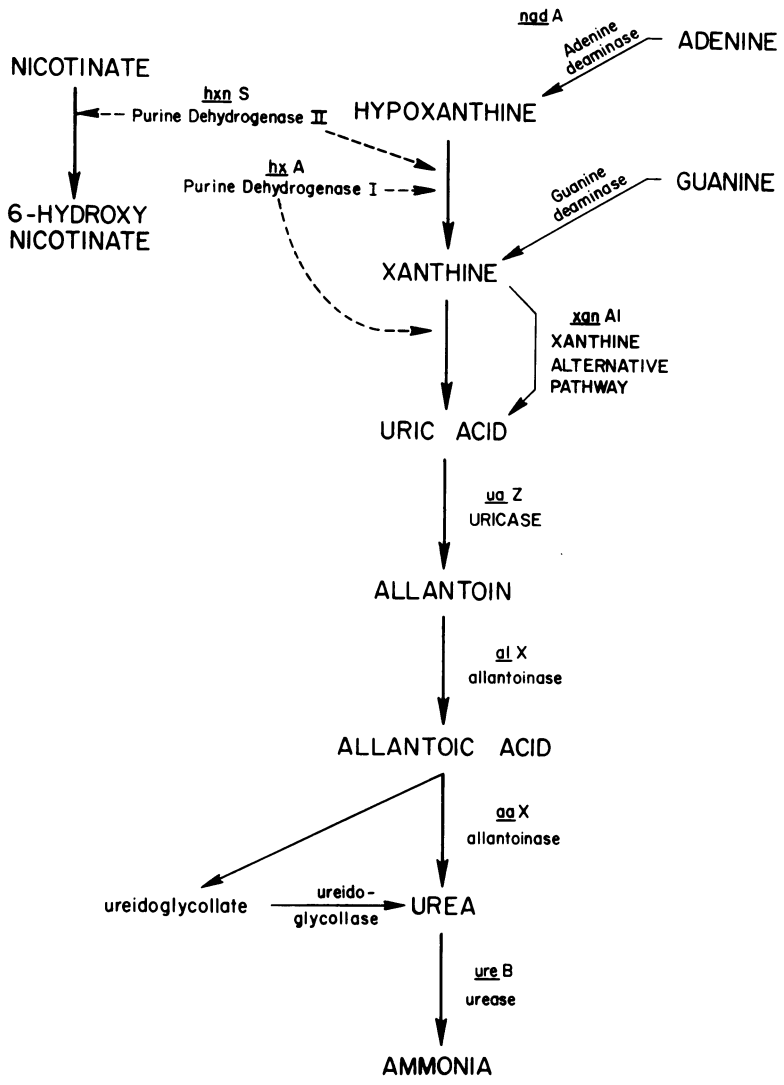

FIG. 1. *Pathway for purine catabolism in Aspergillus. The structural genes which specify most of the catabolic enzymes are given, but regulatory genes are omitted. Neurospora possesses an identical pathway, except that the xanthine alternative pathway and purine dehydrogenase II have not been examined. Both the pathway and its regulation differ significantly in S. cerevisiae.*

earlier uncertainties are now largely resolved. *Aspergillus* possesses two enzymes which catalyze the hydroxylation of hypoxanthine (90). Purine dehydrogenase I, the physiologically significant enzyme for purine catabolism, catalyzes the conversion of hypoxanthine to xanthine and of xanthine to uric acid; this enzyme is specified by the *hxA* gene and is induced by the product uric acid. Purine dehydrogenase II is induced by 6-hydroxynicotinic acid, the product of its activity upon nicotinic acid, its primary substrate, but it also has strong activity toward hypoxanthine but not xanthine (90); it is encoded by the *hxS* structural gene. Both purine dehydrogenase I and II activities are missing in all *cnx* mutants, which also lack nitrate reductase, indicating that all three of these enzymes share a common molybdenum-containing cofactor. Moreover, purine dehydrogenase I and II activities are both missing in a mutant designated as *hxB*, although CRM corresponding to each enzyme is present (90), suggesting that they further share some common subunit or perhaps require a common post-translational modification specified by hxB^+.

It is interesting that each of these purine

dehydrogenase enzymes requires induction by the product of its physiologically relevant activity. Such product induction seems to occur when the substrate is an essential metabolite but the product is metabolically dispensible. The *hxB* mutant, which lacks both purine dehydrogenases, is nevertheless still capable of metabolizing xanthine to produce uric acid. This reaction has been shown to take place by the "xanthine alternative pathway," which is absent in a mutant designated *xanA1* (89). Since the alternative pathway is active in all *cnx* mutants, it is obvious that no Mo-containing cofactor is required. As one would predict, the double mutant *hxB xanA1* completely blocks all uric acid production.

Eight structural genes of *Aspergillus* which encode the purine catabolic enzymes are all unlinked to one another, but are controlled as a group by the pathway-specific control gene uaY^+, which mediates uric acid induction of this entire set of enzymes (86, 87). Uric acid is the only physiological inducer of purine dehydrogenase I, adenine deaminase, and uricase, although the 2- and 9-thio analogs of uric acid are effective as gratuitous inducers (95). By contrast, the other purine enzymes are induced by both uric acid and allantoin. Allantoinase and allantoicase are inducible in the wild type by both uric acid and allantoin (86). The fact that these two enzymes are inducible by allantoin alone in *uaY* mutants suggests that allantoin induction is mediated by another mechanism (29). This entire set of enzymes is also subject to nitrogen catabolite repression, mediated by the *areA* control gene in *Aspergillus*. The last two enzymes of the pathway, ureidoglycollase and urease, appear to be constitutive or only slightly inducible in *Aspergillus* (86) and in *Neurospora* (79).

A preliminary fine-structure map of the *uaY* control gene and the adjacent related *oxpA* gene has been constructed by Scazzocchio et al. (87). Ten different alleles including one deletion mutant are nonleaky and cannot be induced for any of the enzymes tested (uricase, adenine deaminase, and purine dehydrogenase), whereas two other well-separated *uaY* alleles (*uaY109* and *uaY110*) are leaky and only slightly inducible for purine dehydrogenase and adenine deaminase but highly inducible for uricase. Since all 12 mutants, including the 2 leaky ones, fail to display intragenic complementation in any pairwise combination, the *uaY* product may not be a homomultimeric protein.

No uaY^c (constitutive) mutants have yet been found, and the frequency of mutation to the uaY^- null phenotype and reversion pattern are compatible only with a positive mode of action. The uaY^- phenotype derives from a loss of function; i.e., *uaY* deletions have a null phenotype, further indicating that the wild-type uaY^+ gene actively turns on expression of the structural gene set. In agreement, the catabolic enzymes are all synthesized in uaY^+/uaY^- diploids, although the amount of *uaY* product is apparently not sufficient to evoke full expression of two sets of structural genes; thus, the enzyme levels are slightly less than half that found in uaY^+/uaY^+ diploids. It is particularly interesting that the expression of uaY^+ is nucleus limited and that uaY^+ can turn on the expression of $uapA^+$ in the same, but not a different, nucleus. This result is especially convincing since complementation occurred in diploids where all genes were in the same nucleus but failed in heterokaryons in which $uapA^+$ and uaY^+ were in separate nuclei (85).

One of the structural genes controlled by the uaY^+ product, $uapA^+$, encodes a uric acid-xanthine permease. A tightly linked, *cis*-dominant mutant, called *uap*-100, is constitutive for the permease and does not require inducer, although it is still fully dependent upon a functional *uaY* product (10). This constitutes good evidence that the *uaY* product controls the *uapA* gene and, by analogy, obviously also the other structural genes of this same set. The *uap-100* mutant has at least three related effects: (i) it shows a 2.5-fold "up promoter" effect beyond that of the wild-type level; (ii) it is strongly constitutive (62%) in the absence of inducer; and (iii) it responds to the *areA102* mutant product as well as the $areA^+$ product (85). These properties imply that the *uap-100* mutation somehow alters the binding sites for both the *uaY* product and the *areA* product, suggesting these binding sites may partially overlap each other. The *uap-100* mutant almost certainly alters a control recognition site which serves the $uapA^+$ structural gene. The *uap-100* alteration of the recognition site apparently allows binding of a different conformational form of the *uaY* protein than that recognized by the wild-type recognition sequence, which presumably can bind only a uaY^+ product (whose conformation has been changed after binding inducer). Another mutant, *oxpA5*, defines a gene located adjacent to *uaY*, but is believed to be separate because it complements all *uaY* mutants, including a deletion in *uaY* (87). The *oxpA5* mutant displays oxypurinol resistance and is also partially constitutive for adenine deaminase, purine hydrogenase I, and uricase. It has been speculated (87) that the *oxpA* gene codes for a protein which somehow limits the flow of inducers into the nucleus; however, this must be regarded only as an inter-

esting possibility, since the evidence for it is virtually nonexistent.

Using immunoprecipitation of uricase and purine dehydrogenase I, Winther et al. (103) established that induction of these enzymes involves de novo protein synthesis and that the enzyme protein increases in proportion to enzyme activity during induction. They also measured purine dehydrogenase I- and uricase-specific mRNA by in vitro translation of RNA extracted from wild-type and mutant strains under different conditions of induction. The subunits of uricase (32,000 daltons) and purine dehydrogenase I (150,000 daltons) were identified as products in the translation mixture by immunoprecipitation and subsequent sodium dodecyl sulfate-gel electrophoresis. Induction of these two enzymes, both under control of the *uaY* gene, was shown to occur at the level of production of translatable mRNA.

Urease, the last enzyme of the purine catabolic pathway in *Neurospora* and *Aspergillus*, is a constitutive enzyme, which is perhaps not surprising since urea is also derived from arginine metabolism. Mutations at four genetic loci prevent the utilization of urea as a nitrogen source (58); *ureA* specifies a urea permease, whereas mutants of the other three, *ureB*, *-C*, and *-D*, all lack urease activity. The *ureB* locus is thought to be the structural gene, and although the function of *ureC* is unknown, it is interesting that *ureD* apparently has a role in the production or assembly of a nickel cofactor required for urease activity. Addition of nickel ions to *ureD* restored both urease activity and the ability to utilize urea (58).

Considerable evidence was presented above that the uaY^+ gene of *Aspergillus* encodes a positive-acting regulatory product which mediates uric acid induction and turns on the transcription of at least eight unlinked structural genes (86, 87). The *cis*-acting *uap-100* mutant (described above) is believed to define a specific DNA recognition sequence for the uaY^+ product. Uric acid and its 2- and 9-thio analogs are inducers and thus should all bind to the uaY^+ product if the proposed mechanism for its action is correct. Philippides and Scazzocchio (70) have attempted to identify the *uaY* product by direct examination of whole cell extracts for the presence of a protein with the predicted characteristics, namely, an ability to bind both inducer and DNA. In one approach, inducer-binding proteins were separated by phosphocellulose chromatography and then identified by binding to [^{14}C]2-thiouric acid. In a second technique, proteins were first resolved by DNA-cellulose chromatography and then identified by their ability to bind ^{14}C-labeled uric acid. Both methods detected and permitted purification of two binding proteins. Both of these binding peaks are missing in mutants with a putative deletion in the *uaY* gene, whereas *uaY109*, a leaky mutant, shows only one peak, which elutes at a different position from that of either of the wild-type peaks. This result suggests that one or both of these protein peaks may represent a *uaY*-coded protein. However, other proteins in a total cell extract might also be expected to bind uric acid. Two obvious candidates, uricase and purine dehydrogenase, do not correspond to either of the protein peaks detected in this work.

One interesting possibility is that one protein peak is the *uaY* product and that the second peak found in the wild type is one of the proteins under *uaY* control. A *uaY* deletion would be expected to lack both proteins, whereas other *uaY* mutants might possess a qualitatively different *uaY* product (but should be missing the protein controlled by the *uaY* gene). Philippides and Scazzocchio (70) have purified sufficient amounts of the major protein, which they believe to be the *uaY* product, to study its binding of the three inducers by equilibrium dialysis. These results are provocative and may represent a first instance of identification of a genetically defined regulatory gene product in a eucaryote. However, considerable work remains to be completed, first to demonstrate unequivocally that the major binding protein is indeed encoded by uaY^+, and then to characterize its properties and regulatory behavior.

The pathway of purine catabolism and its regulation in *Neurospora* appears to be very similar to that just described in *Aspergillus*. No pathway-specific gene analogous to the *uaY* gene of *Aspergillus* has yet been identified in *Neurospora*. Structural gene mutations identifying the major enzymes have been found and are unlinked; they are controlled both by uric acid induction and nitrogen repression (59, 78, 79). Synthesis of a transport system for uric acid and xanthine is subject to nitrogen repression but does not require uric acid induction (95). A separate hypoxanthine-adenine-guanine permease is not regulated by either induction or repression (95). Induction of uricase apparently occurs at the level of transcription, since uricase-specific mRNA could be accumulated under inducing conditions in the presence of cycloheximide and subsequently translated to yield active enzyme (101). Two purine catabolic enzymes of *Neurospora* have recently been purified and partially characterized; Lyon and Garrett (57) used a powerful immunoabsorption technique to purify XDH, which was found to be a dimeric enzyme composed of subunits of molecular weight 155,000. Using imunoelectro-

phoresis to detect XDH, they demonstrated that the induced increase in XDH activity resulted from an equivalent increase in enzyme protein (57), indicating that induction involves de novo enzyme synthesis. Wang and Marzluf (102) recently purified *Neurospora* uricase to homogeneity and found that the protein appears to be a tetramer composed of subunits having a molecular weight of approximately 33,000. Uricase is a stable enzyme and is not subject to feedback inhibition by ammonia, glutamate, or glutamine. Reinert and Marzluf (78) studied the in vivo stability of several of the purine catabolic enzymes; both uricase and allantoicase are stable enzymes, whereas allantoinase is quite labile both in vivo and in vitro, with a half-life in vivo of approximately 20 min. It is not clear whether allantoinase turnover has physiological significance, e.g., by contributing to the control of flow along this pathway. Allantoinase appears to be extremely sensitive to endogenous proteases, although it may be purified by using steps to quickly separate and protect it from proteases.

Superimposed upon the more familiar pattern of nitrogen control of enzymes and permeases may be interesting developmental regulation. An instance occurs in *Neurospora*, in which freshly harvested conidia possess a general system for purine transport; upon germination, this system increases and a second, adenine-specific permease also develops (68). It is obviously of great interest to understand such developmental-stage-specific regulation and how it may interact with the usual nitrogen control signals.

Allantoin can serve as a nitrogen source in *S. cerevisiae*, and its catabolism requires five enzymes. The pertinent structural genes are located in two unlinked clusters; the final two enzymes, urea carboxylase and allophanate hydrolase, were thought to be encoded by contiguous genes (*dur1* and *dur2*) of one cluster and to comprise a multienzyme complex (55). R. Sumrada, C. Lam, and T. G. Cooper (unpublished data) have recently found that the *dur1* and *dur2* genes actually represent a single structural gene whose product is a bifunctional protein (molecular weight of 202,000) that possesses both urea carboxylase and allophanate hydrolase activity (probably in two different globular domains). The second cluster is composed of three adjacent genes, *dal1*, *dal4*, and *dal2*, which encode allantoinase, allantoin permease, and allantoicase, respectively (21); these enzymes exist as discrete polypeptides. Moreover, the fact that the central gene, *dal4*, is regulated differently than the outside genes clearly indicates that they are expressed as separate units.

Allophanate, the last intermediate of the pathway, induces the synthesis of allantoinase, allantoicase, urea carboxylase, and allophanate hydrolase. Another compound, oxalurate, acts as a non-metabolizable inducer of these same enzymes. All of these enzymes are also nitrogen repressed; serine and certain other amino acids are more active as repressors than is ammonia, suggesting that ammonia itself must be metabolized to an amino acid or other metabolite to cause repression (13). The fact that oxalurate, the gratuitous inducer, is transported into the cells by a constitutive, energy-dependent permease is instructive, since it implies that the failure of oxalurate to induce allophanate hydrolase during nitrogen repression does not result from inducer exclusion. Apparently induction and repression are independent, and both directly affect gene expression. G. Chisholm and T. G. Cooper (unpublished data) have recently isolated mutants which define a new control gene, *dur5; the dur5* mutants produce the five activities required for allantoin catabolism in high constitutive levels. Furthermore, *dur1 dur5* double mutants, which cannot form any allophanate, the inducer of the pathway enzymes, are still constitutive for the remaining activities, implying that no type of internal induction is taking place. Thus, the $dur5^+$ gene apparently encodes a repressor active in negative control to stop gene expression unless inducer is present. Mutants in *dur5* are recessive to wild type, as expected for a repressor gene. It should be emphasized that this negative control by $dur5^+$ over these yeast catabolic enzymes is in sharp contrast to the well-documented positive control (by uaY^+) of the related genes in *Aspergillus*.

Yeast allantoin permease, at least one component of which is coded for by *dal4*, is controlled quite differently than are the other pathway components and is not induced by allophanate (92); rather, allantoin itself, as well as two allantoin analogs, induce the allantoin permease. Allantoin in the medium can enter cells by way of a basal level of allantoin transport activity that is normally present, and once inside, it can induce the allantoin permease to full capacity (45). Allantoin permease synthesis is also repressed by nitrogen catabolite repression. Finally, transinhibition of allantoin permease activity is exerted by intracellular asparagine, aspartic acid, and lysine; this type of feedback inhibition may play a regulatory role by excluding allantoin from the cell when preferable nitrogen sources are available (92). Turoscy and Cooper (96) have also examined the transport of allantoic acid in yeasts, which occurs by a constitutive, energy-dependent system, distinct from the allantoin permease system. During the course of this work, the allantoinase reaction

was discovered to be readily reversible, but by using *dal1 dal2* double mutants (lacking allantoinase and allantoicase), transported allantoic acid accumulated without change. No efflux of allantoate from preloaded cells could be detected, which may be due at least in part to the sequestering of intracellular allantoate in a vacuole.

Lawther and Cooper (54) studied the kinetics of induction of allophanic hydrolase and observed that enzyme activity increased within 3 to 4 min when inducer (allophanate) was added to cells growing at 36°C. Its specific mRNA, measured as the capacity for enzyme synthesis, also decayed very rapidly, with a half-life of only 3 min. Other yeast enzymes required about 10 min for induction, which suggested the possibility that that allophanic hydrolase and the other three related enzymes might be controlled at a post-transcriptional step. To investigate this possibility, lomofungin and cycloheximide were used to specifically interfere with transcription and translation, respectively. The results showed that: (i) induction occurred at the transcriptional level and required mRNA synthesis; (ii) synthesis of hydrolase mRNA began immediately upon induction and could occur in the absence of protein synthesis; (iii) the mRNA half-life was increased when protein synthesis was blocked; and (iv) the enzyme itself was not degraded if inducer was removed. It was concluded that both induction and repression of allophanate hydrolase synthesis occurred at the level of transcription, although inducer may also influence the rate of mRNA translation (54).

The sequence of molecular events involved in the induction of allophanate hydrolase has been studied by working with yeast cultures at 22°C, because at this temperature a 12-min lag occurred before enzyme activity increased (12). It was clear from prior results that induction required both RNA and protein synthesis, and it was of interest to determine what time periods were devoted to each during the lag phase. Two yeast mutants were helpful since they interfere with specific aspects of macromolecular synthesis; *rna1* is a temperature-sensitive mutant believed to be defective in the processing or transport from the nucleus of mRNA and other RNAs. The *prt1* mutant is also temperature sensitive and is defective in initiation of protein synthesis at the restrictive temperature. Since no yeast mutants are yet available which specifically block initiation of mRNA synthesis, lomofungin was used to inhibit this step. The results of this series of experiments indicated that upon induction, mRNA synthesis was initiated within 1 to 1.5 min after inducer was added, nuclear secretion of the mRNA occurred within 4 min, protein synthesis began at about 10 min, and active enzyme appeared at 12 min (12). Thus, a major part of the lag period, about 6 min, appeared to occur as a delay between the time transcription occurred and the enzyme synthesis began. One simple yet interesting conclusion is that inducer uptake and all nuclear events required for turning on gene expression occur very rapidly, even at 22°C.

Cooper and his colleagues have also studied the functional half-life of allophanate hydrolase mRNA and several other yeast mRNA's, measured as capacity for subsequent enzyme or protein synthesis (22). The observed mRNA half-life was found to be strongly influenced by various experimental variables; e.g., mRNA turnover was increased at higher temperatures, whereas inhibition of protein synthesis could increase mRNA stability dramatically, in some cases up to 15-fold. Changes in nitrogen source did not influence the functional half-lives of any of the mRNA's examined. Clearly, comparison of messenger stability is meaningful only if measurements are conducted under highly controlled conditions. In a single comparative study, Lawther and Cooper (54) found that the half-lives of mRNA's for invertase, α-glucosidase, and gross protein synthesis were all about 20 min, compared with 3 min for allophanate hydrolase mRNA. The tentative conclusion from these results is that, at least in yeasts, mRNA's which encode regulated enzymes may, as a class, turn over considerably faster than do mRNA's which specify constitutive enzymes. Obviously, measurement of a number of mRNA's representative of each class will be necessary to judge the correctness of this hypothesis.

PROLINE CATABOLISM

Enzymes for the utilization of compounds that contain carbon but not nitrogen (e.g., acetate) are controlled simply by carbon catabolite repression. Similarly, enzymes involved in the metabolism of compounds containing just nitrogen (e.g., nitrate) are controlled only by nitrogen catabolite repression. However, the metabolism of compounds that can be utilized as both nitrogen and carbon sources may be subject to both nitrogen and carbon regulation. Thus, the structural genes encoding certain enzymes may be subject to multiple regulatory signals, which implies that a complex control region containing two or more recognition sites may be situated next to them.

A gene cluster (in *Aspergillus*) comprised of one pathway-specific regulatory gene and three structural genes which specify proline catabolic enzymes is apparently interrupted by a central

regulatory recognition region in which *cis*-acting mutants can arise (Fig. 2). Proline is a carbon and nitrogen source for *Aspergillus*, and its catabolic enzymes are indeed subject to both nitrogen and carbon catabolite repression (5, 6).

The *prnA* gene is believed to encode a positive-acting product which mediates proline induction of the three structural genes, *prnD*, *prnB*, and *prnC*, which specify proline oxidase, proline permease, and pyrroline 5-carboxylate dehydrogenase (PCD), respectively. *prnA* mutants lack all three enzymes. Since *prnD*$^-$ mutants (which lack proline oxidase) show induction by proline of proline permease and PCD, proline itself appears to be the genuine inducer.

A *cis*-acting regulatory mutant *prn*^{d}, which maps in the central regulatory region, shows a strong up-promoter effect and also derepresses the expression of *prnB*-coded proline permease (*prn*^{d} was isolated as a suppressor of an *areA*^{r} mutant which restores the ability to utilize proline as a nitrogen source). The *prn*^{d} mutant also leads to a derepressed synthesis of proline oxidase and PCD, which led to an early suggestion (4) that a common internal control region regulated both leftward and rightward transcription. It now appears that the *prn*^{d} mutant directly affects only *prnB* expression and that the resulting elevated level of proline permease indirectly increases *prnC* and *prnD* expression, simply because it overcomes inducer exclusion. The fact that *prn*^{d} affects *prnB* only when in *cis*, but *prnC* and *prnD* in *cis* or *trans*, is in agreement with this suggestion (7). The *prn*^{d} mutant represses proline synthesis and bypasses the requirement for an *areA* product, but still requires a *prnA*$^+$ product and induction by exogenous proline, indicating that the pathway-specific control mechanism is still intact (7).

One can suspect that at least four types of recognition sites must lie in the regulatory region adjacent to *prnB*, those for the *prnA* inducer signal, *areA* product, and *creA* product (carbon catabolite repression), as well as a binding site for RNA polymerase. Since proline permease is subject to both carbon and nitrogen repression, it is not clear whether the *prn*^{d} mutant relieves carbon or nitrogen repression, or even both. The promoter apparently overlaps with control sites, since the derepression in *prn*^{d} is accompanied by a marked increase in the level of expression. The fact that this *cis*-acting control mutant relieves repression (carbon, nitrogen, or both) but still requires the *prnA*$^+$-mediated induction is a strong indication that expression from the natural promoter has been altered.

Arst and MacDonald (6) have recently reported another interesting observation, that deletions which extend from within *prnD* to within *prnB*, which remove the putative central control region, lack not only proline oxidase and proline permease (as expected) but show a decided reduction in levels of PCD, encoded by the distal *prnC* gene (see Fig. 2). Since an absence of proline permease would be expected to diminish the ability of exogenous proline to induce *prnC* expression, this observation might seem reasonable. However, they found that a

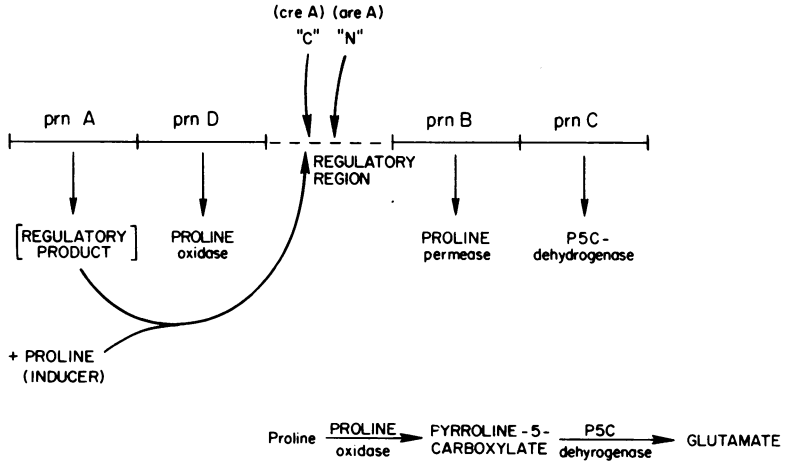

FIG. 2. *Gene cluster and pathway for proline catabolism in Aspergillus. The prnA gene appears to encode a regulatory product responsible for induction of prnB, prnC, and prnD. A central region which separates prnD and prnB apparently has a regulatory function and controls prnB, which is subject to both nitrogen and carbon catabolite repression. The central regulatory region is defined in part by prn*^{d} *mutants which map in it (and affect expression of prnB) and by deletion mutants. It has been suggested that prnB and prnC may be transcribed together as a polycistronic mRNA (see text).*

prnD prnB double mutant displayed normal *prnC* expression. Moreover, *prnC* mutants complement *prnD prnB* double mutants, but show reduced complementation with the central deletion mutant (overlapping *B* and *D*). This argues that the *prnC*$^+$ gene next to the deletion is not expressed efficiently rather than that there is a lack of sufficient proline for induction. It would seem imperative to accurately measure the level of proline transport and the amount of intracellular proline which accumulates in these various strains before dismissing the possibility that the observed differences simply result from differences in induction. Arst and MacDonald (6) interpreted their results to mean that the central deletion removes a regulatory region between *prnD* and *prnB* which includes a promoter from which a dicistronic *prnB-prnC* mRNA is transcribed. Since the deletion strains do synthesize some *prnC*$^+$-coded PCD, it suggested the presence of another origin specific for just *prnC* (presumably at the *prnB-prnC* border). Although this interpretation is consistent with the available information, the evidence is insufficient to establish the existence of polycistronic mRNA; it will be necessary to directly demonstrate the presence of two distinct size classes of mRNA's corresponding to *prnC*, including the postulated *prnB-prnC* dicistronic mRNA. In other cases, similar genetic studies have suggested the presence of polycistronic mRNA's in lower eucaryotes; e.g., the *argB-argC* gene cluster of yeasts has genetic properties (as polarity) which are suggestive of a dicistronic mRNA for these genes (61). The two best-studied cases in which an operon type of arrangement was suspected are the *his4* gene cluster of yeasts (39) and the *arom* gene cluster of *Neurospora*. Each of these cases proved to actually represent a single gene which encodes a large polypeptide with multiple catalytic activities (38, 39, 56). The review by Giles (39) should be consulted for additional examples and an excellent discussion of gene clusters. At present, there exists no well-established case of genuine polycistronic mRNA's in any eucaryotic organisms, and considerable evidence, particularly direct biochemical identification, will be required to establish their existence in eucaryotes.

Very recent studies of proline utilization in *S. cerevisiae* permit a comparison with the detailed results obtained with *Aspergillus* (14). In yeasts, a specific proline permease (different from the general amino acid-permease) is subject to nitrogen catabolite repression but does not require induction. The structural genes for the catabolic enzymes, proline oxidase (*put1*) and PCD (*put2*), are unlinked; these enzymes are induced by proline but are not ammonium repressed (14). A mutation, designated *put3*, results in partial constitutive expression of the two catabolic enzmes, and both enzymes are hyperinducible (two to three times the normal level) when *put3* is grown in medium containing proline. This pathway-specific gene (*put3*) apparently is responsible for mediating proline induction. One possible explanation, internal induction by endogenous proline, was eliminated by showing that the partial constitutive phenotype of *put3* persisted even in mutant strains incapable of forming proline (14). The regulation of proline degradation may have some similarities in *Aspergillus* and yeasts, but significant differences are also evident; the structural and regulatory genes in yeasts are scattered, whereas they are tightly clustered in *Aspergillus*. More significantly, the *Aspergillus* pathway-specific control gene, *prnA*$^+$, appears to act in a positive fashion, whereas in yeasts the corresponding *put3*$^+$ gene may be a typical repressor gene active in negative control.

ACETAMIDE UTILIZATION AND INTEGRATOR GENE

Acetamide can be used by *Aspergillus* as a sole nitrogen source and also as a sole carbon source. Its metabolism requires the enzyme acetamidase, specified by the *amdS* structural gene, in which at least 14 mutant sites have been recognized; *cis*-acting control-type mutants all map at one end of *amdS*. Regulation of acetamidase synthesis is unexpectedly complex, and yet much insight has been gained by Hynes (46, 47). At least three unlinked genes affect *amdS* expression: the familiar *areA* gene, which mediates nitrogen catabolite repression; *amdA*, which specifically leads to high acetamidase levels in an unknown manner; and *intA* (integrator A, also referred to as *amdR*). Acetamidase is induced by three separate classes of compounds: (i) sources of acetyl-coenzyme A (CoA) such as acetate and acetamide; (ii) beta-alanine and other ω-amino acids; and (iii) benzoate and benzamide (46). The physiological significance of acetamidase induction by ω-amino acids and by benzoate is unknown. The effects of these three groups of inducers is approximately additive, indicating that they may act in independent ways. Moreover, the behavior of several *cis*-acting control mutants closely linked to *amdS* also suggests independent means of induction, as well as a separate nitrogen repression control. Thus, the *cis*-acting mutants *amdI9* and *amdI18* increased acetamidase induction by sources of acetyl-CoA, whereas another tightly linked mutant, *amdI93*, abolished induction by ω-amino acids but not the other two classes of inducers (48). These results suggest that there are at least

three separate ways to turn on expression of *amdS* via induction in addition to the positive *areA* signal indicating nitrogen derepression. There may in fact be as many as five functionally independent, separate recognition sites adjacent to *amdS* (Fig. 3), each capable of binding one of the control signals; the total expression of this structural gene may be increased stepwise by binding one, two, or more of these effectors. This picture, although provocative, must be regarded as tentative since it is derived from circumstantial evidence, and several of the postulated positive-acting genes have not even been identified. Nevertheless, it seems almost certain that a complete picture of *amdS* gene expression, which must include molecular studies of the structural gene and its postulated adjacent control region, will be extremely interesting and may provide new insight concerning how a single gene can be multiply regulated.

The integrator gene (*intA*) described above is of special interest because it has been suggested to integrate expression of several seemingly unrelated genes (2, 3, 8, 9). The *intA* gene was so named because of its formal resemblance to the "integrator gene" of the well-known Britten-Davidson model for eucaryotic gene control (15). The *intA* gene is believed to mediate induction by beta-alanine and other ω-amino acids of at least four separate structural genes, *amdS* (acetamidase), *gabA* (γ-aminobutyrate [GABA] permease), *gatA* (GABA transaminase), and *lamA* (lactamase, or possibly lactam permease) (3, 9). It is not yet clear why these various enzyme activities should all be subject to an integrated expression. The properties revealed thus far of *intA* suggest that it is but another example of a minor positive-acting regulatory gene which mediates induction by a key metabolite (in this case, ω-amino acids). There seems to be no reason why it should be considered as a special type of control locus nor assigned such a suggestive name as an "integrator gene."

Another interesting case in which a single structural gene is controlled in a complex manner is provided by an extracellular protease in *Neurospora* and *Aspergillus* (18–20, 43). *Neurospora* secretes an alkaline protease into its growth medium in response to the presence of an exogenous protein and a limitation for either nitrogen, sulfur, or carbon (19, 48). Thus, the single structural gene which encodes this protease is postulated to be served by a complex regulatory region near its 5′ end which presum-

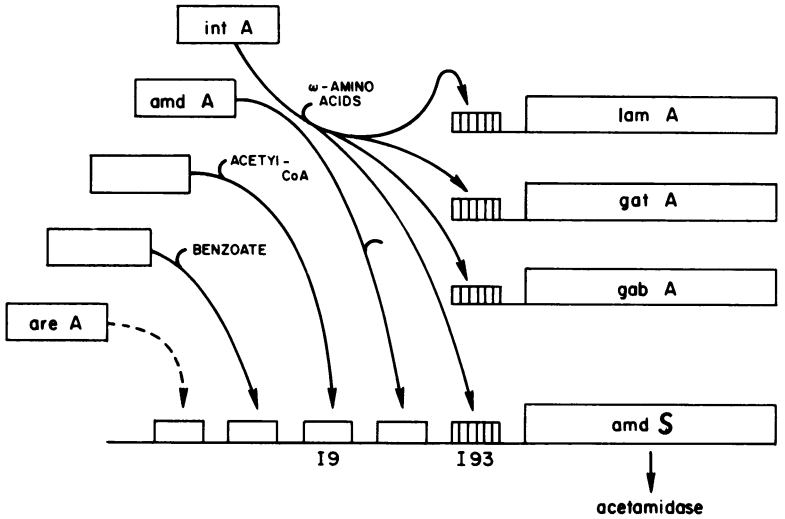

FIG. 3. *Speculative model for the complex regulation of the acetamidase structural gene (amdS) and related genes. Acetamidase is independently induced by ω-amino acids, acetyl-CoA, and benzoate, each of which is believed to interact with a specific positive-acting gene product, although only intA, which mediates induction with ω-amino acids, has been identified. Another unlinked gene, amdA, also controls amdS and could mediate induction by some other unknown compound. The site of action of intA is identified by the cis-acting I93 mutant; similarly, the I9 mutant defines the site for induction by acetyl-CoA. The amdS structural gene is also subject to nitrogen catabolite repression (by areA gene) as well as carbon repression. The intA gene also controls at least three other genes, lamA, gatA, and gabA. Acetyl-CoA is an inducer of amdS and also of glyoxylate bypass structural genes. The arrangement of the postulated recognition elements adjacent to amdS is completely arbitrary. The empty boxes represent possible additional regulatory genes which have not yet been identified.*

ably contains individual recognition sequences for positive signals arising independently from the nitrogen, sulfur, or carbon regulatory circuits (43). The *nit-2* gene is responsible for turning on protease gene expression during nitrogen limitation. A primary goal for studying protease gene expression (and acetamidase gene expression) must be to clone the structural genes as a prelude to directly identifying and sequencing the postulated adjacent control sites.

NITROGEN CATABOLITE REPRESSION: GENETIC STUDIES

A regulatory gene of *Aspergillus*, which is central to nitrogen catabolite repression, has been identified and designated *areA* (for ammonium repression). Hundreds of *areA* mutants have been isolated and display a variety of phenotypic effects (4, 17, 23, 49, 67). One type which is most commonly found is termed $areA^r$ (for repressed) and results in the inability to utilize a wide variety of nitrogen sources, such as purines, amino acids, amides, nitrate, and nitrite. These same *areA* mutants cannot be derepressed for the synthesis of certain nitrogen-related enzymes. Different alleles of *areA* each show individual patterns of enzymes and of nitrogen source utilization. One allele, $areA^r217$, which was isolated secondarily from a parental strain already carrying an *areA* mutant (*areA102*), grows very poorly on all nitrogen sources except NH_4^+. Still others grow even better than the wild type on some nitrogen sources but fail to use others.

The second type of mutants, $areA^d$ (for derepressed), lead to the loss of ammonium repression for certain nitrogen-related enzymes; one $areA^d$ cannot be repressed by ammonium for synthesis of nitrate reductase, XDH, uricase, acetamidase, an extracellular protease, and distinct permeases for glutamate and urea (17, 49, 67). All of these enzymes still require induction, however, indicating that the *areA* gene is concerned only with nitrogen catabolite repression. The distinction between $areA^r$ and $areA^d$ alleles is far from absolute, since a particular *areA* mutant may be derepressed for one ammonium-repressible activity, be normally repressible for another, and lead to abnormally low levels for a third. Consequently each *areA* mutant really has its own highly specific phenotype. It is rather unusual, furthermore, that *areA* mutants not only affect ammonium repression but can also increase the maximal level of enzyme(s) above that achieved in the wild type. For example, *areA102* can utilize both acetamide and histidine much better than the wild type and has enhanced levels of acetamidase and histidase (71). These characteristics suggest a functional overlap between the promoter and a binding site for the *areA* regulatory product. It is of interest that the expression of acetamidase and histidase is normally so severely limited that their substrates cannot be efficiently used as nitrogen sources.

It is believed that the *areA* locus encodes a protein that serves as a positive control element and is essential for the synthesis of various nitrogen-related enzymes. Its role seems to be to monitor the nitrogen status of the cell, and the *areA* protein is presumably inactive in the presence of sufficient levels of nitrogen, reflected in the cellular concentration of a key nitrogen metabolite (see below). The heterogeneity of phenotypes exhibited by different *areA* alleles (and revertants) strongly supports the suggestion that this locus encodes a macromolecule, probably a protein, rather than specifying an enzyme responsible for the synthesis of a small molecule involved in derepression. Scazzocchio (unpublished data) has recently isolated a putative chain termination (nonsense) mutant of *areA* which has a null phenotype, thus providing definitive evidence that *areA* encodes a protein and works in a positive fashion. The available evidence, although still incomplete, suggests that *areA* acts at the transcriptional level, although other levels as translation cannot yet be excluded.

Another locus, known as *tamA*, which also seems to have a major role in nitrogen regulation, was identified by Kinghorn and Pateman (50). The first *tamA* mutants were isolated as simultaneously resistant to thiourea, aspartate hydroxamate, and chlorate and had low but detectable levels of a number of nitrogen-related enzymes, including nicotinamide adenine dinucleotide phosphate (NADP)-linked glutamate dehydrogenase (GDH) (NADP-GDH) and nitrate reductase (23).

Although *tamA* and *areA* are unlinked, they share many similar properties; indeed, one allele ($tamA^dI$) results in the derepression of a number of normally ammonium-repressible enzymes, whereas $tamA^r50$ is unable to grow on all nitrogen sources examined and is missing various nitrogen-regulated enzymes (50), thus resembling the contrasting phenotypes also known for *areA* mutants. Thus, it seems probable that the *tamA* and *areA* gene products both have central roles in nitrogen catabolite repression. They do not represent separate or alternative control mechanisms, but both are needed for proper regulation of the same large group of nitrogen-related enzymes. They might encode different subunits of a multimeric regulatory protein; alternatively, they might act at separate steps in

a regulatory sequence or even at different synthetic levels, e.g., transcription and translation.

In *Neurospora*, a single major regulatory gene, *nit-2*$^+$, appears to mediate nitrogen catabolite repression and has many similarities with the *areA* locus of *Aspergillus* (16, 36, 59, 78). All *nit-2* mutants isolated so far have the null phenotype, and no constitutive *nit-2* mutants or revertants have yet appeared, despite attempts to isolate them (59). Moreover, the presence of a second regulatory gene (analogous to *tamA*) has not been detected even though dozens of *nit-2* mutants have been obtained. It is not clear whether the mutant types which might be expected simply have remained undiscovered so far, or whether there occurs a fundamental difference in the mechanism of nitrogen regulation between *Aspergillus* and *Neurospora*. Before considering the mechanism of nitrogen catabolite repression or the identity of the catabolite responsible for nitrogen repression, we must first examine pathway-specific control genes and the properties of two key enzymes of nitrogen metabolism, GDH and glutamine synthetase (GSase).

PATHWAY-SPECIFIC CONTROL

Many of the structural genes of the nitrogen control circuit in *Aspergillus* and in *Neurospora* require the simultaneous presence of two positive control signals for their expression. One such signal is the product of the major regulatory gene which mediates nitrogen catabolite repression; the second is the product of a pathway-specific control gene, such as *nit-4*, *nirA*, *uaY*, *prnA* or *amdR* (described above) which mediates induction by signaling the presence of the substrate or intermediate of the particular catabolic pathway. What is the relationship of the specific gene products involved in induction and the *areA* or *nit-2* gene product? In general these two signals seem to act independently, since *areA*^{d} mutants are nitrogen derepressed but still require induction, and *nirA*^{c} mutants synthesize nitrate reductase without nitrate induction but still are repressed by ammonia or other good nitrogen sources. The interaction of various *areA* alleles and *nirA* alleles, when present together in double mutants, also points to their independence. These results thus favor a mechanism in which a regulatory product of the *areA*$^+$ gene and of the *nirA*$^+$ gene (or other pathway-specific control genes) are both required to turn on structural gene expression. The available evidence suggests that both act at the transcriptional level, although additional proof is needed to establish this point.

An interesting new constitutive *nirA* mutant of *Aspergillus*, designated *nirA*^{d}*101*, was obtained in two mutational steps, and somehow its altered pathway-specific positive gene product also alleviates the usual requirement for the general-acting *areA* product (77). Thus, *nirA*^{d}*101*, unlike other constitutive *nirA* mutants, can suppress *areA*^{r}*18*, which is believed to represent a loss-of-function mutant of the *areA* locus, indicating that the *nirA*^{d}*101* product alone is sufficient to turn on expression of *niaD* (77). We should inquire what type of molecular mechanism could satisfactorily explain this set of results. The simplest explanation (although clearly speculative) perhaps is that RNA polymerase II cannot by itself open the promoter region of the *niaD* structural gene, nor can either positive-acting protein alone normally facilitate melting of the duplex DNA sufficiently for transcription, whereas the combined activity of the polymerase and of the *areA*$^+$ and *nirA*$^+$ gene products permits transcription. According to this view, the altered product of the *nirA*^{d}*101* mutant may somehow be capable of unwinding (or stabilizing) a longer stretch of DNA at the promoter region than does the *nirA*$^+$ product.

REGULATORY RECOGNITION SEQUENCES?

Traditionally, *cis*-dominant mutants, tightly linked to a structural gene, which yield a constitutive phenotype for the corresponding enzyme have been regarded as defining a control site; such constitutive mutants may fail to bind a repressor protein, or could act by making expression of the adjacent gene independent of a positive-acting gene product, or perhaps capable of using a mutant form of the positive control gene product. Such *cis*-acting mutants tightly linked to structural genes are of considerable importance in nitrogen regulation because they may identify a recognition site for regulatory proteins, such as the *areA* gene product or the *nirA* gene product. *Cis*-acting constitutive mutants can be isolated as suppressors of null-type regulatory gene mutants (such as *areA*^{r}, *nit-2*, and *nirA*$^-$) which restore the ability to use a certain secondary nitrogen source and the relevant enzyme missing in the parental strain. Some of these *cis*-acting mutants may be "initiator constitutive" and render the structural gene expression independent of any *areA* gene product, whereas others represent changes in the recognition sites which permit utilization of a mutant *areA* product (and which are simultaneously insensitive to nitrogen catabolite repression). This latter class probably constitutes the clearest cases which imply mutational alteration within true regulatory DNA sequences, rather than other trivial changes. It is important to note that *cis*-acting mutants with a constitutive

phenotype could also arise from other alterations, which have little to say about regulation. Thus, mutation could create a new, perhaps inefficient, promoter which is not subject to any control and thus would mimic a constitutive mutation in a control sequence. The *nis-5* mutant of *Aspergillus* (described above) is of interest in this regard; it is tightly linked to the *niiA* structural gene and causes a low constitutive expression of nitrite reductase, which does not require induction or derepression. One cannot decide from such information whether *nis-5* represents a true regulatory defect or arises from another cause such as mutational formation of a new promoter. In fact, *nis-5* resulted instead from a translocation which apparently placed a foreign promoter close to *niiA*. This example must warn us to be cautious when interpreting constitutive mutants on the basis of limited genetic information.

Another expected class of *cis*-acting control-type mutants are null, in which the adjacent structural gene in question can no longer be expressed (or only at a reduced level). It is obvious that this class would be easily confused with promoter-down mutations unrelated to control and even CRM^- structural gene mutants, situated near the 5′ end. Perhaps the only *cis*-dominant null-type mutants which can be relatively safely regarded as control in nature would occur in cases where a single structural gene is responsive to more than one regulatory signal (such as carbon and nitrogen). If the mutant should render the structural gene silent in response to one signal, e.g., the $areA^+$ product, but it still responds normally with the second signal (e.g., for carbon catabolite derepression), it would seem probable that the mutational alteration has occurred in a regulatory recognition sequence. The *amdI93* mutant, which is tightly linked to the structural gene for acetamidase in *Aspergillus*, abolishes enzyme induction by ω-amino acids but not by other inducers (48) and thus is an example of a *cis*-acting null mutant which tentatively can be concluded to be regulatory. In the case of negative control systems, operator constitutive mutants should arise and apparently have been found in yeast (61).

GLUTAMATE DEHYDROGENASE

The enzyme GDH occupies a strategic position in nitrogen metabolism and an important branch point between nitrogen and carbon metabolism, since it catalyzes either the reductive amination of α-ketoglutarate to yield glutamate or the oxidative deamination of glutamate, which provides ammonia.

Neurospora crassa, *Aspergillus nidulans*, and other microorganisms possess two GDHs (65). NADP-GDH appears to function in a biosynthetic role for glutamate formation, whereas nicotinamide adenine dinucleotide (NAD)-GDH is responsible for the reverse catabolic reaction in which ammonia and α-ketoglutarate are products.

The structural gene for NADP-GDH has been identified in *Neurospora* (*am*), *Aspergillus* (*gdhA*), and yeasts (*gdhA*). Mutants in *Aspergillus* at the *gdhB* locus (unlinked to *gdhA*) apparently identify the structural gene for NAD-GDH (58), although no such mutants are yet available for *Neurospora*. The *Neurospora* NADP-GDH is one of the most studied and best understood fungal enzymes. This enzyme is a hexamer, comprised of six identical subunits, each of which is a polypeptide chain of 452 amino acid residues. The complete amino acid sequence of the *Neurospora* NADP-GDH is known (44). Many pioneering studies concerning allosteric behavior (53), intragenic complementation, and osmotically reparable mutant enzymes (37) have been done by Fincham and his colleagues. A fine-structure map of the *am* locus is available, plus a large number of different *am* mutants which affect structural, kinetic, and allosteric properties of the enzyme; the amino acid replacements are known for nearly a dozen mutants and include nonsense, frameshift, and missense mutants (53). Kinsey and Fincham (52) recently reported a highly unstable *am* mutant (*am126*) which reverts at a frequency at least 40 times greater than that of the next most revertible *am* mutant. Should the basis for this highly revertible mutant be due to the presence of a small transposable element or insertion sequence, as found in the yeast *his4* gene (81), it could provide the basis for new molecular approaches to study the *am* locus.

Despite the fact that *Neurospora* NADP-GDH is very well characterized biochemically, our understanding of the regulation of this enzyme and its NAD counterpart is only fragmentary. Since the NAD and NADP forms of GDH seem to function differently and catalyze opposing reactions, they may be subject to some form of concurrent regulation. Another complication which makes their study difficult is that these enzymes appear to be controlled by both the nitrogen and carbon circuits. High activity of NADP-GDH is found in *Neurospora* (25) and *Aspergillus* (51) when wild-type cells are grown with a limited amount of inorganic nitrogen source, such as nitrate or ammonia. Increasing amounts of inorganic nitrogen compounds repress NADP-GDH but cause an increase in NAD-GDH. By contrast, a rich carbon source

(such as glucose) leads to an increase in NADP-GDH but represses NAD-GDH, which suggests that the NAD enzyme is controlled in large part by carbon catabolite repression. Thus, *nit-2* mutants of *Neurospora* (defective in nitrogen control) seem to possess only basal levels of NADP-GDH during growth on 2% sucrose, but can synthesize relatively high levels of NAD-GDH when the carbon source is limited (25). This result clearly shows that the NAD-GDH enzyme can be turned on by carbon limitation, independent of any positive activation from the nitrogen circuit. The *am* mutant also shows decreased levels of NAD-GDH, similar to that observed with *nit-2*; however, in this case the effect could be secondary due to the accumulation in *am* of a carbon catabolite (e.g., α-ketoglutarate) which represses NAD-GDH synthesis (25).

Although NAD-GDH is obviously subject to carbon control and may be primarily concerned with the utilization of glutamic acid (and other amino acids which can be converted into glutamic acid) as a carbon source, this enzyme is also regulated by the nitrogen circuit. Thus, even in the presence of a rich carbon source (2% sucrose), NAD-GDH is increased approximately 10-fold during growth of wild-type, *nit-1*, or *nit-3 Neurospora* strains on 150 mM urea compared with growth on 25 mM NH_4Cl; by contrast, three different *nit-2* mutants possessed only the basal level of NAD-GDH under either condition. It therefore appears that NAD-GDH can also be derepressed by the nitrogen source under certain conditions and that the *nit-2* control mutant is defective in this nitrogen response, but can derepress NAD-GDH via the carbon signal (25). Considerable work is needed to understand the mechanisms, which appear to be complex, that control NADP-GDH and NAD-GDH; one particularly interesting question which needs careful study is to determine whether there is any absolute connection which links the synthesis of these two enzymes, such that they are necessarily formed in a reciprocal fashion.

When an adequate carbon source is available, *gdhB* mutants (NAD-GDH$^-$) of *Aspergillus* nevertheless grow extremely poorly with glutamate, aspartate, proline, alanine, phenylalanine, isoleucine, and ornithine as sole nitrogen sources (51); this result argues that the NADP-GDH can not substitute adequately for the NAD-GDH for glutamate catabolism and also suggests that each of these amino acids is metabolized via glutamate, most likely by transamination. Other amino acids (e.g., asparagine, glycine, and serine) are utilized by *gdhB* mutants at a wild-type rate, demonstrating that ammonia can be derived from them independently of any NAD-GDH activity. Similarly, *gdhB* mutants are incapable of utilizing many amino acids as a carbon source, which again suggests that their metabolism involves glutamate as an intermediate, implying that an initial transaminase step is followed by use of the resultant α-keto acid.

It has been generally assumed that the growth, after an initial lag, of *am* mutants of *Neurospora* and the homologous *gdhA* mutants of *Aspergillus* on low ammonium concentrations is due to low levels of the NAD-GDH activity. However, the fact that the catabolic NAD-dependent enzyme has a very low affinity for ammonia, plus the finding that a *gdhA gdhB* double mutant of *Aspergillus* grows as well as *gdhA*, indicates that some other enzyme system is responsible for the growth response. It now appears that an enzyme system consisting of glutamine synthetase and glutamate synthase (GOGAT) is the responsible one (33, 45).

$$\text{Glu} + \text{ATP} + \text{NH}_3 \xrightarrow{\text{GSase}} \text{Gln} + \text{ADP} + \text{P}_i$$

$$\text{Gln} + \alpha\text{-KG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GOGAT}} 2\ \text{Glu} + \text{NADP}^+$$

where ATP is adenosine triphosphate, ADP is adenosine diphosphate, P_i is inorganic phosphate, and α-KG is α-keto glutarate.

The overall reaction leads to the incorporation of ammonia into the α-amino group of glutamate: Glu + NH_3 + α-KG → 2 Glu. The view that this system provides an alternative pathway for glutamate synthesis is strongly supported by the demonstration that the *en-2(am)* mutant of *Neurospora*, which enhances the phenotypic effect (glutamate auxotrophy) of *am* mutants, is devoid of GOGAT activity and may be the structural gene for glutamate synthase (33). The biochemical basis for a second mutant, designated *en-1(am)*, which also enhances the mutant phenotype of *am*, is unknown (33).

GLUTAMATE DEHYDROGENASE: A REGULATORY PROTEIN?

In several bacterial systems, certain enzymes appear to serve a regulatory function in addition to their familiar catalytic role (40). However, caution is required in interpreting such results. In several cases, considerable evidence suggested that the first enzyme of a biosynthetic series also had a regulatory function; nevertheless, when deletion mutants lacking the entire first structural gene (and thus the first enzyme) were examined, they displayed normal regulation for the remainder of the pathway enzymes.

It appears that in both *S. cerevisiae* and *A. nidulans*, the enzyme NADP-GDH may, quite independently of its catalytic function, serve as a regulatory protein for control of many nitrogen-related enzymes. The key evidence is that certain mutants within *gdhA*, the structural gene for NADP-GDH in both of these fungi, relieve the usual ammonium repression of many nitrogen-related enzymes (31, 41, 67). The regulatory defect of the *gdhA* mutants could not be accounted for as a simple deficiency for glutamate. The nature of GDH involvement in control is still uncertain, however, and strongly contrasting suggestions have been proposed. Thus, NADP-GDH might act directly as a repressor to preclude structural gene expression or, alternatively, it could provide the ammonium recognition site for nitrogen repression; in the latter case, a positive-acting signal (such as a *tamA-areA* multimeric protein complex in *Aspergillus*) might turn on nitrogen gene expression unless rendered inactive by binding to an ammonium-GDH complex. Loss of NADP-GDH would, according to this suggestion, lead to nonrepressible expression of the various nitrogen genes, although they would still require an active *areA* and *tamA* gene products, in agreement with observed results. Other possibilities remain open, however, and NADP-GDH might have only an indirect role; e.g., Arst and Cove (4) suggested that NADP-GDH might only affect the intracellular distribution of ammonia, perhaps lowering its concentration within a special compartment in which it must be localized to effect repression.

It would be of interest to examine the regulatory effects of a mutant deleted for the entire *gdhA* gene and to carefully study such a deletion strain for any abnormal distribution of metabolites such as ammonia, glutamate, or glutamine. Since the NADP-GDH could have a direct regulatory role, it would be of considerable interest to determine whether any of this enzyme is located within the nucleus or involved in a complex with other proteins.

A related phenomenon is the finding that sporulation in yeasts can be blocked in its early stages by the addition of ammonia salts. Newlon (62) recently reported experiments using ocher nonsense mutants of *gdhA* of *S. cerevisiae* which indicate that NADP-GDH is not essential for ammonium repression of yeast sporulation. Since a small amount (about 1%) of GDH activity was still present due to the NAD-GDH enzyme, it was not absolutely certain that ammonium itself was the repressor; however, the finding that methylamine, a non-metabolizable analog of ammonia, also prevented sporulation (62) suggests that ammonia may be an active repressor of yeast sporulation.

GLUTAMINE SYNTHETASE

GSase is an enzyme of central importance in nitrogen metabolism not only because of its function to produce glutamine, an essential amino acid, and its role as amino donor for synthesis of other compounds, e.g., tryptophan, but also because this enzyme has been directly implicated as a regulatory macromolecule in bacteria (97), and a similar role in control has been suggested for GSase in *Neurospora* (32).

Mora et al. have studied GSase of *Neurospora* in considerable depth. GSase has been extracted from *Neurospora* cells grown with 5 mM glutamate as a nitrogen source and purified to homogeneity (63). A key step used was chromatography on an anthranilate-Sepharose affinity column, which provides a simple, rapid purification. Since only about 100-fold purification is required to achieve a pure enzyme, it can be concluded that GSase is a very abundant enzyme and, in fact, must represent approximately 1% of the protein in the initial cell extract. GSase prepared in this way is an octameric enzyme composed of identical subunits of molecular weight 47,000 (63).

Since GSase is a key enzyme, one might expect that its concentration or activity would be regulated. In fact, GSase activity is modulated by widely different mechanisms in different organisms, which include modification by adenylylation, enzyme inactivation and degradation, and alterations in its synthetic rate. In *Neurospora*, the apparent activity of GSase varies considerably when wild-type cultures grown under different conditions are compared. Enzyme activity is high when glutamate is the sole nitrogen source and much lower when cells are grown with excess ammonia. Immunochemical titration and also sucrose gradient sedimentation experiments revealed that the differences in enzyme activity are strictly reflected in the amount of enzyme protein, and it was concluded that in *Neurospora* this enzyme is controlled primarily, if not exclusively, by changes in its rate of synthesis (77, 98). No evidence for modulation of activity by enzyme modification, feedback inhibition, or related mechanisms was found. Moreover, the rate of enzyme synthesis observed in vivo was directly reflected in the amount of GSase-specific mRNA, measured by in vitro translation of polysomal polyadenylated RNA [poly(A)$^+$ RNA] (64, 83), which strongly suggests that GSase synthesis is controlled at the transcriptional level or by a closely related step such as RNA processing or transport. Sodium

dodecyl sulfate-gel electrophoresis demonstrated that the product synthesized in vitro from poly(A)$^+$ RNA migrated identically as did authentic GSase monomers; furthermore, the in vitro product also chromatographed as authentic GSase on an anthranilate-Sepharose affinity column (64).

There is at least one condition, however, under which the activity of GSase is controlled by means beyond that of synthesis rate. When *Neurospora* cultures are limited for carbon, GSase is degraded, and this occurs in addition to preventing its de novo synthesis (35). The degradation of GSase presumably occurs from attack by endogenous proteolytic enzymes, although this has not yet been demonstrated. It has been suggested that GSase turnover during carbon limitation has a physiologically meaningful purpose to prevent the wasteful use of carbon skeletons to synthesize glutamine when the cell is facing a carbon emergency (22). However, it needs to be demonstrated that GSase is particularly sensitive to turnover, as opposed to a general elevated attack by endogenous proteases on cell proteins during conditions of carbon limitation.

Several complications have hindered our complete understanding of the status of GSase and its regulation in *Neurospora*. First, it has been consistently observed that the enzyme multimeric state varies under different growth conditions (26, 82); thus, in wild-type cells grown in medium containing excess ammonia, the enzyme possesses an octameric form, but has a tetrameric structure if grown with limited ammonia. Second, a glutamine auxotroph, *gln*, cannot synthesize glutamine and cannot grow without glutamine supplementation on medium containing high ammonia or glutamic acid (26, 27). However, the *gln-1a* mutant can grow (after a lag) on medium containing low ammonia and is found to have active GSase in a tetrameric form; another allele (*gln-1b*) has similar properties, although it has both a tetrameric and dimeric form of GSase. Both mutants lack the octameric form of GSase. These complications are at least partially resolved by the discovery that the octameric and tetrameric enzymes are actually composed of very similar but distinct subunit polypeptides which can be resolved by electrophoresis in sodium dodecyl sulfate–7 M urea polyacrylamide gels (82). The tetrameric form of GSase is composed predominantly of α subunits, which appear to be slightly larger than the β subunits, that comprise the octameric enzyme. The *gln-1* mutants are devoid of the β subunit, and this locus is probably its structural gene. The α and β subunits are believed to be two different gene products and not to have any precursor-product relationship (82).

This tentative conclusion is probably correct, since both α and β subunits can be detected as products after in vitro translation of polysomal RNA (R. Palacios, unpublished data), indicating that they are encoded by distinct mRNA's. This could be confirmed by northern blot analysis and hybrid-arrested translation of the distinct mRNA's when clones containing the GSase structural genes become available. The presently available evidence strongly suggests the presence of two different GSase enzymes, composed of different subunits and regulated in opposite directions, the octameric form being predominant during growth on excess ammonia and the tetrameric form being found with growth on limited ammonia. These different GSase forms presumably have different functions. It would be useful to isolate mutants defective in the tetrameric enzyme; they could perhaps be isolated by starting with *gln-1* and selecting for mutants unable to grow on low-ammonia medium without glutamine. It will, of course, be of much interest to compare these two GSase enzymes for similarities, including possible immunochemical cross-reaction, and to study in more detail the separate control of each form and any possible reciprocal control of their synthesis.

MECHANISM OF NITROGEN CATABOLITE REPRESSION

A major question which has confronted those studying nitrogen metabolism in fungi is the true identity of the nitrogen metabolite which leads to the well-established nitrogen catabolite repression. Numerous metabolites could have this function, including the ammonium ion, various amino acids, and purines; more than a single compound might be active. Although this issue has not been settled, certain evidence suggests that in *Neurospora* glutamine is the effector metabolite. Premakumar et al. (73 ,75) have examined the effect that various nitrogen metabolites have on the induced rate of appearance of nitrate reductase. Such studies are complicated, since many points exist at which a metabolite might have an effect on nitrate reduction expression. Thus, in addition to directly altering the rate of nitrate reductase mRNA or enzyme synthesis, a metabolite may have possible effects upon inducer (nitrate) uptake, inhibition of nitrate reductase activity, and mRNA and enzyme decay. Ammonia and several acids (e.g., glycine, serine, asparagine, arginine, and glutamine) prevent the induction of nitrate reductase activity in wild-type cultures. However, ammonia by itself is ineffective as a repressor metabolite in *am* mutants (which lack GDH), although ammonia plus glutamate cause strong nitrogen repression

in these mutants. This result argues that ammonia is not itself the repressor metabolite but must be metabolized before repression occurs. Similarly, in *gln* mutants (which lack GSase), neither ammonia nor glutamate represses nitrate reductase synthesis, but glutamine is very effective for repression. Moreover, glutamine also causes nitrogen repression in mutants which are incapable of further conversion of glutamine in the synthesis of arginine, histidine, tryptophan, or pyrimidines, which argues that metabolism of glutamine is not required but that glutamine itself, or a very close relative, is the true repressor metabolite.

A closely related study which supports these views involved the use of L-methionine-DL-sulfoximine (MSX) which is phosphorylated in vivo and then binds tightly to the active site of GSase, completely inhibiting this enzyme. Under conditions where GSase of wild-type strains was inactivated by MSX, only glutamine could repress nitrate reductase synthesis. Ammonia was transported and accumulated intracellularly but could not evoke nitrogen repression in wild-type cells lacking GSase activity (75).

Glutamine does not prevent the uptake of nitrate and thus does not act by inducer exclusion, nor does glutamine increase the enzyme turnover rate or inhibit the activity of nitrate reductase (73). All of these studies point to a central role of glutamine in mediating nitrogen catabolite repression in *Neurospora*. Various nitrogen compounds such as ammonia, glutamate, and other amino acids can be converted to glutamine and thus indirectly cause repression but themselves seem incapable of evoking repression. Glutamine apparently exerts its repressing effect at the transcriptional level, since its addition prevents the accumulation of translatable nitrate reductase mRNA (73) and uricase mRNA (101). A major uncertainty is the nature of the macromolecules which play a direct role in nitrogen catabolite repression. It seems well established that the *nit-2* gene product of *Neurospora* and the *areA* (and *tamA*) gene products of *Aspergillus* function in nitrogen repression, but it is unclear whether other elements (e.g., GHD) may also be involved. It is also not yet certain what regulatory component directly senses the cellular glutamine concentration.

The enzyme GSase has a central role as a regulatory protein for nitrogen metabolism in certain bacteria (104). It thus is not surprising that GSase has also been implicated as a regulaory element in fungi (32, 34). As described above, it has been demonstrated repeatedly in *Neurospora* that neither ammonium or glutamate causes nitrogen repression of nitrate reductase (34, 73, 75) or uricase (108) in *gln* mutants (which lack GSase). These results suggest that the catalytic activity of GSase to provide glutamine is required for proper nitrogen repression (in *Neurospora*) but that GSase itself is not necessary. On the other hand, Dunn-Coleman and Garrett (32) found that a particular *gln* mutant (known as *gln-1b*) cannot establish nitrogen repression even when exogenous glutamine is supplied, suggesting that the GSase protein may be directly involved in regulation. They have presented a detailed model (32) which seems compatible with most available information and proposes that an octameric form of GSase is strongly favored in the presence of glutamine (otherwise a tetrameric form predominates) and that the octameric form itself is a repressor of *nit-2*$^+$ gene expression. Thus, during nitrogen limitation, an insufficiency of glutamine would lead to the disappearance of octameric GSase, permitting *nit-2*$^+$ gene expression, whose product would in turn turn on the expression of the various nitrogen-related genes. One difficulty with this detailed model is the finding that the octameric and tetrameric forms of GSase actually represent separate enzymes composed of distinct subunits (as described above), so that the conversion of tetrameric GSase into the octameric forms seems improbable. Although the specific model is speculative, a possible regulatory role for GSase deserves careful attention. Deletion mutants of the *gln* gene and mutants for the second GSase would be useful in assessing the control role of the enzyme. Direct biochemical studies are necessary both to firmly establish a control role for GSase and to reveal the molecular mechanism by which it might act. If GSase has the regulatory role postulated above, certain specific predictions arise which merit careful testing; e.g., GSase may display DNA-binding activity and may be found within the nucleus, at least during conditions of nitrogen repression; alternatively, glutamine synthetase may directly bind the *nit-2* gene product. It is also plausible that GSase plays an important but indirect role in nitrogen repression, perhaps by formation of a specific intracellular glutamine pool which is active in repression and cannot be readily substituted for by exogenously supplied glutamine.

Another mechanism proposed for nitrogen catabolite repression in *Neurospora* assumes that glutamine is the repressor metabolite and that it interacts directly with the *nit-2*$^+$ gene product, converting it into an inactive form (59, 78). When the cellular glutamine concentration is diminished, according to this view, the *nit-2*$^+$ product assumes an active conformation and binds at recognition sequences adjacent to each nitrogen structural gene, thus turning on their expression.

This proposed mechanism allows the prediction that the *nit-2*$^+$ gene may specify a nuclear DNA-binding protein, whose affinity for DNA would be markedly decreased in the presence of glutamine. Grove and Marzluf (42) directly examined nuclear nonhistone proteins for the presence of a DNA-binding protein with such characteristics. Radioactively labeled nuclear nonhistone proteins were applied to a *Neurospora* DNA-cellulose affinity column, and after being washed well to remove all loosely bound proteins, the column was specifically eluted with glutamine. Elution of a parallel DNA-cellulose column with asparagine was used as one control to demonstrate specificity. Proteins eluted were separated by sodium dodecyl sulfate-acrylamide gel electrophoresis and visualized by fluorography. Wild-type nuclei possessed a single DNA-binding protein (molecular weight 22,000) which was eluted by glutamine but not by asparagine; this same protein was severely reduced in quantity in nuclei of two *nit-2* mutants. Furthermore, a revertant of one of the *nit-2* mutants (which had regained the ability to utilize various secondary nitrogen sources) possessed approximately 20 times more of this protein than did the parental *nit-2* strain, an amount essentially identical to that found in wild-type cells (42). These results suggest that the *nit-2*$^+$ protein has been identified, although additional support is needed to establish this point; moreover, the results also imply that the *nit-2* protein may directly interact with glutamine, thereby lowering its affinity for DNA.

One may anticipate that glutamine enters the nucleus for its regulatory role and that the nuclear concentration of glutamine should increase significantly during nitrogen repression conditions. It would be exceedingly interesting to ascertain the amount of key nitrogen metabolites, such as glutamine, glutamate, and ammonium, in the nucleus under repression and derepression conditions, although such information may be difficult to obtain because of the possibility of rapid loss of metabolites from nuclei during their isolation. Another interesting question is to determine whether the putative *nit-2*$^+$ protein is made constitutively and whether it is restricted to the nucleus under all metabolic conditions.

Sorger's laboratory has isolated a new mutant, MS5, which fails to repress nitrate reductase and related nitrogen enzymes in the presence of sufficient glutamine to lead to full repression in wild-type cells (74). The identity of this mutant is unknown except that it is not allelic to *nit-2* or to *nit-3*. The failure of nitrogen repression in MS5 is not due to cellular impermeability to glutamine nor to a change in the rate of nitrate reductase turnover (74). It is clearly important to ascertain the action of the MS5 gene, whose very existence argues that the mechanism of nitrogen catabolite repression may be more complicated than anyone presently conceives. One possibility is that MS5 could contribute to the transport of glutamine into the nuclei. Premakumar et al. (74) have suggested that there may also exist negative effectors for control of nitrogen-related genes, in addition to the positive control exerted by *nit-2*$^+$, and that MS5 could be a lesion in the negative system thus providing a constitutive phenotype.

CLOSING REMARKS AND FUTURE PROSPECTS

Our present understanding of the regulation of nitrogen metabolism in fungi is encouraging and suggests that considerable progress at the molecular level can now be anticipated. The now classic approaches of biochemical genetics have provided much insight concerning the control of nitrogen metabolism in *Aspergillus*, *Neurospora*, and yeasts. The elements which comprise the nitrogen regulatory circuit have been defined by genetic analysis and shown to include an entire set of predominantly unlinked structural genes and both major and minor regulatory genes, plus both nitrogen repressor and inducer metabolites. Nitrogen catabolite repression and pathway-specific induction clearly seem to be mediated by distinct regulatory genes, which act independently of one another. Each structural gene has been postulated to be served by one or more adjacent recognition sites, which are presumably the binding sites for the pertinent regulatory gene product; some of these recognition sites have apparently been identified as tightly linked, *cis*-acting mutations, although caution is necessary when interpreting their mode of action. In *Aspergillus* and in *Neurospora*, the regulatory genes appear to act in a positive manner, probably by encoding a nuclear protein which actively turns on expression of the structural genes. By contrast, most of the yeast regulatory genes appear to act in a negative fashion by specifying a repressor which binds at a classical operator site. It is not at all clear why this apparent fundamental difference in the mode of nitrogen regulation occurs, if indeed it is real.

Genetic approaches can still provide invaluable guidance in our dissection of the nitrogen regulatory circuits of these fungi. Specific types of mutants, particularly nonsense mutants, deletions, and temperature-sensitive alleles, are needed for many of the regulatory genes. There is also a real need in many cases to obtain *cis*-acting control mutants, because they can help to define recognition sites and will be of great value when such recognition regions become available

for DNA sequencing. Moreover, certain types of other mutants could be of much service as "reagents," e.g., conditional mutants which prevent the initiation of transcription or of translation and others which block messenger RNA processing and secretion. However, substantial progress in our understanding of nitrogen regulation clearly now requires direct analysis with molecular approaches.

It is necessary to unequivocally identify the nitrogen repressor metabolite (which seems to be glutamine) and to assess its distribution throughout the cell, including the nucleus, under conditions of nitrogen repression and derepression. Another major objective is to identify the genetically defined regulatory gene products, perhaps by the use of affinity chromatography techniques, such as those which have permitted the tentative identification of the Aspergillus *uaY*-coded regulatory protein (70) and the *Neurospora nit-2*-coded protein (42). It will be important to discern the cellular distribution of these regulatory proteins and to isolate sufficient amounts of them to study their properties and to reconstitute in vitro systems. It is also necessary to ascertain whether certain key nitrogen-related enzymes, such as GDH and GSase, play any direct role in nitrogen regulation; both genetic and biochemical evidence will be required for this task. Perhaps the greatest advances will be achieved by cloning various structural and regulatory genes of the nitrogen circuit. Such cloned genes will obviously provide the DNA probes needed to directly examine regulation of transcription and translation in vivo and to eventually develop in vitro systems with which to study regulation with purified components. An interesting question which can be resolved once cloned genes are availabie is whether tightly linked structural genes, such as the *argB-argC* cluster in yeast (61), are expressed as a single polycistronic mRNA. An important goal must be to use cloned nitrogen-related structural genes to directly identify the recognition sites postulated to lie in the 5′ flanking region and to determine the DNA sequence of several such recognition sites which genetic evidence suggests bind the same regulatory protein. The *cis*-acting mutants will be of invaluable assistance in this work. Promising results in this area can be expected soon; indeed, Cooper (unpublished data) has now cloned 11 different yeast genes which include both structural genes and nitrogen regulatory genes. We can anticipate that considerable progress will be achieved soon by direct molecular approaches and should, in fact, amount to a revolution in our understanding of the mechanisms of nitrogen regulation in fungi. It promises to be an exciting period.

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