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REGULATION OF URICASE GENE EXPRESSION UPON INDUCTION

IN

NEUROSPORA CRASSA

DISSERTATION

Presented in Partial Fulfillment of the Requirements
for the Degree Doctor of Philosophy
in the Graduate School of the Ohio State University

By

Baek Hie Nahm, B.S., M.S.

* * * * *

The Ohio State University
1986

Dissertation Committee:        Approved by
George A. Marzluf
David H. Ives
Lee F. Johnson

Adviser
Department of Biochemistry
"Having been justified therefore by faith, let us have peace with God through our Lord Jesus Christ, through whom we also have access by faith unto that grace in which we stand, and exult in the hope of the glory of the sons of God. And not only this, but we exult in tribulations also, knowing that tribulation works out endurance, and endurance tried virtue, and tried virtue hope. And hope does not disappoint, because the charity of God is poured forth in our hearts by the Holy Spirit who has been given to us."

(Romans 5: 1 - 5)
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I also want to give deep appreciation to my parents who gave strength and endurance during my study.

Finally, I want to give my love to my family, my wife, Sangyoon and our baby who will be born this fall.
VITA

March 23, 1953 .................Born - Seoul, Korea

1975 .............B.S.
Department of Agricultural Chemistry
College of Agriculture
Seoul National University
Suwon, Korea

1977 .............M.S.
Department of Biological Science
Korea Advanced Institute of Science
Seoul, Korea

1977-1980........Research Scientist
Dept. of Food and Biological Science
Korea Institute of Science and Tech.
Seoul, Korea

1980-1983........Teaching Associate
Department of Biochemistry
Ohio State University
Columbus, Ohio

1983-Present......Research Associate
Department of Biochemistry
Ohio State University
Columbus, Ohio

PUBLICATIONS

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FIELDS OF STUDY

Major Field: Molecular Biology

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Neurospora crassa as an Organism

*Neurospora crassa* is an aerobic filamentous fungus classified as Ascomycetes, sub-class Pyrenomycetes. It is a simple eukaryote in terms of cellular structure and genetic organization. It has been examined genetically and cytologically as a model system for study of the regulation of gene expression in eukaryotic cells because of its simple and well-defined nutritional requirements, rapid growth rate, short generation time, and excellent genetics (Fincham and Day, 1965).

*Neurospora crassa* grows well on a fully defined simple medium that contains sugar as a carbon source, inorganic salts, and one vitamin, biotin (Vogel, 1956). Inorganic sulfate is a good sulfur source and phosphate is a phosphorous source. Ammonium ion is the most commonly used nitrogen source, but nitrate, urea, various amides, purines and a number of amino acids can also be used. Inorganic ions such as $K^+$, $Mg^{2+}$, and a variety of trace elements are required.

The growth of *Neurospora* occurs at the tip of branched filaments called hyphae. As hyphae grow, side
branches are formed and each one has the same potential for growth as the primary hypha. The whole hyphal system, called mycelium, is subdivided by crosswalls called septa. Each resulting compartment contains numerous nuclei and the septa have central pores which allow the passage of streaming protoplasm as well as nuclei (Shatkin and Tatum, 1959).

The fungus is propagated principally by means of asexual spores, conidia, which are developed from aerial branches, although it can undergo sexual reproduction by the mating of two different mycelia (Fincham and Day, 1965). There are two types of asexual spores. One is the microconidia which are formed at the aerial hyphae and the other is the macroconidia which are extruded directly from the stationary phase mycelial cells. The macroconidia are quite variable in size and may contain one to ten or more nuclei, whereas the uninucleate microconidia are only about 1 μ in diameter and are usually far outnumbered by the macroconidia. When supplied with an adequate nutrient, both kinds of conidia germinate readily to form a hyphal system.

In sexual reproduction, the sexual fruiting body (perithecia) is only formed when two mycelia of different mating types, designated A and a, are brought together. There is no morphological difference between
A and a strains, and both can form abundant female reproductive structures, protoperithecia, when grown on solid medium of suitable composition (Westergaard and Mitchell, 1947). A protoperithecium consists of the ascogonium, which is a coiled multicellular hyphae enclosed in a knot-like aggregation of hyphae. Fertilization occurs when a cell of the opposite mating type comes into contact with a part of the trichogyne, which is a branching system of very slender hyphae extruded at the tip of the ascogonium, resulting in the formation of ascospores.

Regulation of Nitrogen Metabolism

*Neurospora crassa* can utilize a variety of compounds as nitrogen sources. Ammonia, glutamate and glutamine are utilized as primary nitrogen sources. It can also utilize as secondary nitrogen sources many other nitrogen compounds as nitrate, nitrite, purines, proteins, numerous amino acids, and even acrylamide. It has been observed that a primary nitrogen source represses the synthesis of the nitrogen-related enzymes required for the utilization of secondary nitrogen sources. This phenomenon is known as nitrogen catabolite repression (Marzluf, 1977). The utilization of secondary
nitrogen sources requires the synthesis of a series of catabolic enzymes or an activation of preexisting enzymes. The synthesis of a series of catabolic enzymes to utilize secondary nitrogen sources requires two conditions: namely, (1) the release of catabolite repression caused by primary nitrogen sources, and (2) the induction of enzymes with an inducer such as a substrate or an intermediate in the catabolic pathways (Marzluf, 1981).

In the regulation of nitrogen metabolism, two levels of control genes have been observed. A major nitrogen control gene integrates the expression of numerous nitrogen-related structural genes, and minor control genes are pathway-specific and control the expression of the genes of a particular catabolic pathway. As a major control gene, nit-2, a positive regulatory gene, is known to control many nitrogen-related enzymes and mediates nitrogen catabolite repression in Neurospora (Reinert and Marzluf, 1975a). A minor control gene, nit-4, controls the specific pathway for nitrate assimilation. Nit-4 is a positive regulatory gene for the induction of nitrate reductase and nitrite reductase encoded by nit-3 and nit-6, respectively (Tomsett and Garrett, 1980, Marzluf, 1977). Nit-4 mutants cannot utilize nitrate or nitrite as a
Figure 1. The regulation mode of nitrogen metabolism in *Neurospora crassa*. The nit-2 gene product regulate the expression of many structural gene related to nitrogen metabolism in a positive fashion. The nmr-1 gene is proposed to regulate the expression of the nit-2 gene. As a minor control gene, nit-4 gene regulates the expression of nit-3 and nit-6 which encodes nitrate and nitrite reductase, respectively. Another minor regulatory gene which specifically control purine catabolic pathway has not been yet identified in *Neurospora crassa*. 
nitrogen source, but still can utilize other secondary nitrogen sources.

Two mechanisms of nitrogen catabolite repression have been proposed, although a detailed mechanism has not yet been established. It has been suggested that among the various metabolites which have been known to effect repression, glutamine may be the true repressor for nitrogen catabolite repression (Wang and Marzluf, 1979; Premakumar et al., 1979). In the regulation of nitrogen-related structural gene expression, Reinert and Marzluf (1975a) have proposed that glutamine interacts directly with the nit-2 gene product, converting it into an inactive form. Grove and Marzluf (1981) demonstrated that the nit-2 gene product is a nuclear DNA binding protein, whose affinity for DNA is markedly decreased in the presence of glutamine. Chambers et al. (1983) also showed the synthesis of L-amino acid oxidase was regulated by trans-nuclear action of the the nit-2 gene product in various heterokaryons which have nit-2 mutant and wild type nuclei. The data support the possibility that the nit-2 gene product is a protein, which may be synthesized in the cytoplasm and imported into the nuclei. These results suggest that when the glutamine concentration is lowered, the nit-2 protein assumes an active conformation and binds at
recognition sites adjacent to each nitrogen-related structural gene, and thus turns on their expression. However, at high concentration of glutamine, the nit-2 protein may bind glutamine and adopt a conformation that cannot bind to recognition sequences and thus fails to express the nitrogen-related structural genes.

A second model proposes that the enzyme glutamine synthetase has a central role as a regulatory protein for nitrogen metabolism (Dunn-Colmann and Garrett, 1980). A particular mutant of glutamine synthetase (gln-lb) is known to be incapable of nitrogen repression. It was suggested that the octameric form of glutamine synthetase is strongly favored in the presence of glutamine and that in this form it acts as a repressor of nit-2 gene expression. Under nitrogen limitation, an insufficiency of glutamine may lead to a change in conformation of glutamine synthetase from octamer to tetramer which turns on nit-2 gene expression.

In addition to the nit-2 control gene, another control gene, MS5, is known to relieve the nitrogen catabolite repression. The MS-5 gene (allelic to nmr-1), which may encode a negative regulator. The MS-5 mutant fails to repress nitrogen related enzymes in the presence of glutamine such as nitrate reductase (Premakumar et. al., 1980) and L-amino acid oxidase...
Premakumar et al. (1980) and Chambers et al. (1983) suggested that the M55 gene might encode a repressor that controls the nitrogen-related genes negatively in addition to the positive control by the nit-2 protein. However, De Busk and Ogilvie (1984) proposed that the MS-5 gene product may bind glutamine and then act by binding to a recognition sequence adjacent to the nit-2 gene, blocking the expression of the nit-2 gene, which is required to express various nitrogen related structural genes.

These models differ from each other in their views of the interactions between the repressor metabolite, glutamine, and regulatory proteins. However, it is well established that glutamine is a true repressor metabolite and the nit-2 gene product is a major regulatory protein which acts positively in the expression of nitrogen related structural genes.

The regulation of purine metabolism

*Neurospora crassa* can utilize purine bases as a secondary nitrogen source. In the purine catabolic pathway a series of enzymes are required to fully degrade either guanine or adenine into usable ammonia. Reinert and Marzluf (1972a,b) showed that structural
yenes of the major enzymes in the purine catabolic pathway are unlinked and are controlled by uric acid induction and nitrogen repression. They demonstrated that the final two enzymes of this pathway, ureidoglyoxylate hydrolase and urease, are constitutive, whereas three enzymes, uricase, allantoinase, and allantoicase, are controlled by both induction and nitrogen catabolite repression. In the induction of uricase and allantoicase, uric acid acts as a true inducer. However, allantoinase is induced by either uric acid or allantoin. In addition to these enzymes, synthesis of a transport system for uric acid and xanthine is subject to nitrogen repression but does not require uric acid as an inducer (Tsao and Marzluf, 1976). A separate hypoxanthine-adenine-guanine permease is not regulated by either induction or repression.

Among purine catabolic enzymes of Neuropora, xanthine dehydrogenase (XDH) has been purified and characterized. Lyon and Garrett (1978) used an immunoabsorption technique to purify XDH. They demonstrated that it is a dimeric enzyme composed of subunits of molecular weight of 155,000 and its induction results from an equivalent increase in enzyme protein, indicating that induction involves de novo enzyme synthesis.

The purine catabolism pathway and its regulation
are very similar in Aspergillus and Neurospora. Eight structural genes of Aspergillus which encode the purine catabolic enzymes are all unlinked to one another (Scanzocchio and Darlington, 1968). Also these genes are known to be controlled as a group in a positive fashion by the pathway-specific control gene uaY in Aspergillus, although a similar pathway-specific control gene has not been identified in Neurospora (Scanzocchio et al., 1981). The entire set of enzymes is also subject to nitrogen catabolite repression by the areA control gene in Aspergillus as the nit-2 gene in Neurospora. The last two enzymes of the pathway, ureidoglycollase and urease, appear to be constitutive or slightly inducible as in Neurospora.

By immunoprecipitation of uricase and purine dehydrogenase I in Aspergillus, Winther et al. (1980) established that induction of these enzymes involves de novo protein synthesis and that the enzyme protein increases in proportion to enzyme activity during induction. By measuring these enzyme specific mRNAs with in vitro translation of RNAs followed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis, they showed the induction of these two enzymes, both under the control of uaY gene, occurred at the level of production of translatable mRNA.
Uricase as an enzyme

Uricase is an enzyme (urate:U2 oxidoreductase, EC 1.7.3.3) which catalyzes the oxidation of uric acid in the presence of oxygen and results in the formation of allantoin and hydrogen peroxide (Vogels and Van Der Drift, 1976). It is located in the peroxisomes in eukaryotic cells (Tolbert, 1981). The enzyme exhibits a stringent specificity in terms of both the electron donor and electron acceptor. Only oxygen is used as an electron acceptor; and, therefore, no organism is known to metabolize the uric acid under anaerobic conditions. Uricase is absent in humans and most animals and therefore, uric acid is excreted as a waste product. Edozien et. al. (1970) observed that the feeding of single cells containing nucleic acid to humans increased urinary uric acid excretion. The result suggests that there may be precipitation of uric acid crystals in joint (gout), in soft tissue (tophi) or in the formation of stones in the urinary tract in individuals with a genetic tendency to overproduce uric acid. Microbial uricases have been applied in the treatment of hyperuricemia (Dumas et. al., 1973) and primary gout (Kissel, et. al., 1968). Wang and Marzluf (1980) have purified and characterized uricase from Neurospora
crassa. The molecular weight of the enzyme was estimated to be 123,000 daltons. The enzyme is composed of 4 identical or similarly sized subunits having a molecular weight of 33,000 daltons. The $K_m$ value of the enzyme for uric acid is estimated to be $4.2 \times 10^{-5}$ M. Oxonic acid was shown to be a competitive inhibitor of uricase with a $K_i$ value of $6.7 \times 10^{-7}$ M. The enzyme appears to be a metalloenzyme with no essential sulfhydryl group and is stable in vivo and in vitro. It is also insensitive to feedback inhibition by ammonia, glutamate, or glutamine. The synthesis of the enzyme was regulated by nitrogen catabolite repression via the nit-2 protein, a positive regulatory protein in nitrogen metabolism. Wang and Marzluf also demonstrated that synthesis of uricase messenger RNA could be induced even when its translation was blocked by cycloheximide, and the accumulated mRNA could in turn be translated after removal of cycloheximide under conditions which precluded any further message synthesis (Wang and Marzluf, 1979). These results suggested that uricase synthesis is controlled at the level of transcription. However, the accumulated results were not sufficient to establish the regulation mode of uricase gene expression.
Intracellular protein topogenesis

It is well known that a eukaryotic cell has many internal organelles such as nuclei, mitochondria, chloroplasts, lysosomes, and peroxisomes. These organelles, as well as the cell itself, are surrounded by single or double layers of membrane structures which primarily consist of lipids. Each compartment has a different series of proteins in order to maintain its own morphology and function in the cell.

Most proteins, however, are synthesized on ribosomes in the cytoplasm. Therefore, some proteins remain in the same compartment in which they are synthesized, whereas others have to be either translocated across or integrated into the cellular membranes in order to reach their intracellular compartment; other proteins are secreted into the environment. As a result, the location of proteins can be broadly categorized into four sites i.e., in the cytoplasm (endoplasm), inside the organelles (ectoplasm), outside the cell, and within the membrane structure itself.

It is very important to know how proteins reach their compartments to establish a relationship between a protein structure and its function and to trace the phylogeny of membrane structures. Recently, Blobel
(1980) proposed a mechanism for the translocation and integration of proteins, related to protein structure and to the evolution of membrane structures. To provide background, this hypothesis is reviewed and the mechanism for translocation of uricase, a peroxisomal protein, into the peroxisomes is presented to explain the origin of peroxisomal membranes.

Blobel refers to the intracellular processes that result in the translocation of proteins across or integration of them into membranes as "intracellular protein topogenesis" (Blobel, 1980). He proposed that the information for these processes is contained in discrete segments of each polypeptide chain, called "topogenic sequences". Depending on intracellular targeting pathways of proteins, the types of topogenic sequences are classified as signal sequences, stop-transfer sequences, insertion sequences and sorting sequences. These segments are cleaved, or uncleaved, from each protein during or after protein synthesis.

Blobel proposed that the signal sequences initiate the translocation of proteins across membranes. He suggests that the information in the signal sequences is recognized by protein translocators which have signal sequence-specific recognition domains in distinct membranes. The interaction between signal sequences and
protein translocators initiates the unidirectional translocation of proteins into specific membranes. Various competent membranes are proposed for translocation and each membrane is suggested to have only a single form of translocator that specifically recognizes only one type of signal sequence. Therefore, the number of different signal sequences could be the same as the number of different translocators. The signal sequences may be cleaved by signal peptidase, or remain uncleaved during translocation. Many proteins have been found to follow one of two modes of translocation. One mode is the cotranslational translocation which occurs during translation of messenger RNA into protein. As the signal sequence is translated, a signal receptor particle on a receiver membrane recognizes the signal, thus resulting in the translocation of proteins as translation is completed (Walter et al., 1984). Otherwise, translation is arrested. Translocation is strictly coupled to the translation which occurs on membrane-bound polysomes in which ribosomes and membranes are associated via junctions between signal sequences and signal receptor particles. The second mode is posttranslational translocation, which occurs after translation is completed. The signal sequence may be recognized by
different receptors only after translation. No receiver membrane may be involved during translation, and therefore, such proteins are synthesized on free polysomes. Proteins which are imported into mitochondria (Schatz and Butow, 1983), nuclei (De Robertis, 1983), or peroxisomes (Goldman and Blobel, 1978) are known to follow the posttranslational mode. A common feature for the two modes of translocation is that a bivalent ligand recognizes a specific receptor domain of translocators, crosslinks to it, and causes the formation of a functional complex. In the cotranslational mode, the signal sequence and a site on the large ribosomal subunit interact, in contrast to two distinct domains in the signal sequence as a bivalent ligand in posttranslational translocation.

The stop-transfer sequences, as proposed by Blobel (1980), interrupt the translocation initiated by the signal sequence, whereas the insertion sequences direct the unilateral integration of proteins into membranes without mediation of translocators. The information in the stop-transfer sequence and the insertion sequence, alone or combined, may result in the asymmetric integration of proteins into membranes. The integration mode of protein into membranes varies according to the orientation of protein with respect to
the membrane and the environment. Therefore, integrated membrane proteins are suggested to be classified as monotopic, bitopic and polytopic. In the monotopic proteins, hydrophilic domains are exposed only on one side of the membrane bilayer, and the hydrophobic domains are anchored to the hydrophobic core of the membrane. Two or multiple hydrophilic domains of the bitopic and polytopic proteins, respectively, are exposed to both sides of the membrane. Therefore, the bitopic protein spans the membrane once and the polytopic protein even more than once. The insertion sequences may direct the simple unilateral integration of proteins and result in monotopic proteins without the need of a translocator. The stop-transfer sequences may be decoded to form bitopic or polytopic proteins by disassembly of the functional translocator complexes into subunits. With these two topogenic sequences, either alone or in combination, the asymmetric mode of protein integration can be predicted.

Finally, Blobel(1980) suggests that the sorting sequences contain the information for posttranslational travel and result in nonrandom distribution of proteins among organelles. The sorting sequences may be required for proteins to anchor to the rough ER as well as for proteins that are transported, and they may be
recognized by specific proteins. In the bilateral integrated membrane proteins, the sorting sequence may interact with a few distinct peripheral membrane proteins. However, the sorting sequence of soluble proteins may interact with a bilateral membrane protein that acts as a carrier protein which shuttles back and forth between a donor and a receiver compartment.

With the concept of topogenic sequences, Blobel (1980) suggests that certain pleiotropic proteins may have similar structures and functions but different topologies. Pleiotropic proteins can result from gene duplication, loss, or acquisition of topogenic sequences, via transposition. It is suggested that such processes are important to achieve dichotomy in protein translocation, to export or bind protein to the plasma membrane, to diversify the organellar distribution of proteins, or to anchor proteins in the intracellular membrane.

Phylogeny of membrane structures

According to Blobel (1980), the membranes of eukaryotic cells may evolve from two different sources. One may be the cell's own primordial plasma membrane. Aggregation of certain membrane functions in the plasma
membrane followed by nonrandom removal of these functions by invagination and fission generates a new intracellular compartment, the ectoplasmic compartment. The continuous ectoplasmic compartment results in the formation of a nuclear envelope continuous with the ER. The endoplasmic compartment is divided further into nucleoplasm and cytoplasm. Other membranes such as lysosomal, peroxisomal, and Golgi complex membranes also may have developed through invagination from the plasma membrane or may be an outgrowth of the ER. The other source is proposed to be the plasma membrane of a foreign symbiotic cell, according to the theory of endosymbiosis. By symbiotic capture of another cell, the foreign plasma membrane generates a different compartment (xenoplasmic) within the ectoplasmic compartment such as mitochondrial matrix and chloroplast stroma. Therefore, only the inner mitochondrial and chloroplast membranes may be derived from foreign plasma membranes (xenoplasmic), whereas their outer membranes may be from the cell's own plasma membrane (orthoplasmic) like all other cellular membranes.

Translocation of Uricase into Peroxisomes

Blobel (1980) speculates that there is a relationship between the translocation mechanism for
proteins and the biogenesis of the membrane structures. As an example, the biogenesis of peroxisomal membrane can be predicted by investigating the translocation mechanisms for peroxisomal proteins. Uricase is an enzyme which is synthesized on ribosomes in the cytoplasm, but is located inside the peroxisomal membranes. Therefore, uricase has to be translocated across the single layer of the peroxisomal membrane. In the biogenesis of peroxisomes, two different models have been proposed. The classical model suggests that the newly synthesized peroxisomal proteins translocate into the ER from which peroxisomes are formed by budding. In an alternative model, peroxisomal proteins are synthesized on free ribosomes and enter the peroxisomal reticulum, which represents connections between peroxisomes. The peroxisomal reticulum exists as a transient structure derived from preexisting peroxisomes, and finally results in the formation of a new peroxisome by fission.

Goldman and Blobel (1978) demonstrated that in rat liver two peroxisomal enzymes, uricase and catalase, were synthesized on free ribosomes rather than on membrane-bound ribosomes. They also demonstrated that when uricase and catalase were synthesized in vitro, they did not segregate into microsomal membranes, unlike
those proteins known to be localized via cotranslational translocation. Although they did not rule out the possibility of the presence of signal peptidase in the cell-free systems, uricase and catalase were not synthesized as larger precursors in vitro; these two enzymes were proposed to have an uncleaved signal sequence. These results allow Goldman and Blobel to suggest the events that occur during the biogenesis of peroxisomes. After cotranslational insertion of peroxisomal membrane proteins including receptors which are specific for signal sequences of peroxisomal membranes into rough ER, these proteins may become segregated and sorted by patching and capping from other integral membrane proteins via sorting sequences. Then newly synthesized peroxisomal proteins are postulated to be directly translocated by the posttranslational mechanism. For translocation, specific signal sequences in internal peroxisomal proteins interact with peroxisomal signal receptors, and resulting in the formation of a transient passageway in the newly assembled peroxisomal membranes. Cleavage of the putative peroxisomal signal sequences may occur during or shortly after passage and might be linked to the disassembly of the passageway.

The model by Goldman and Blobel (1978) described
above suggests that newly synthesized peroxisomal content proteins are found in nascent peroxisomes that are budding from the ER. These nascent peroxisomes may be the major sites for import of proteins destined to reside inside the peroxisomes. Mature peroxisomes may have lost the capacity for import, because of either receptor inactivation or of spatial limitations. Therefore, the synthesis of internal peroxisomal proteins and integral membrane proteins of this organelle may proceed at different intracellular sites, i.e. on free and membrane-bound ribosomes, respectively.

Lazarow et al. (1982) demonstrated the posttranslational translocation of uricase and catalase in vivo in rat liver. In the subcellular distribution of newly synthesized proteins as a function of time, these two peroxisomal proteins were observed to enter peroxisomes at different rates in vivo, and proteolytic cleavage was not involved. They also showed that the peroxisomal catalase was synthesized on free polysomes and that catalase synthesized in wheat germ cell-free systems supplemented with formyl-35S-methionine-tRNA was indistinguishable from the subunit of mature peroxisomal catalase synthesized in vivo. The absence of proteolytic cleavage of signal sequences of catalase was also confirmed by two and three-dimensional peptide
mapping of partially digested catalase from three different sources, i.e. the wheat germ cell-free translation product, the precursor made in vivo but not yet packaged into peroxisomes, and mature catalase. In all three cases, the pattern of catalase polypeptides produced was identical. The results rule out the possibility of cleavage of a signal sequence by signal peptidase during translation in cell-free systems and imply that the information directing catalase to the peroxisome resides within its structure. Lazarow et al (1982) also demonstrated differences in the pattern of proteins from mitochondria, rough and smooth microsomes, and peroxisomes. Based on these results, they concluded that newly synthesized peroxisomal proteins are inserted directly into preexisting peroxisomal membranes rather than by patching and capping from ER membranes.

The available experimental evidence is insufficient to support either of the two models or to explain the detailed mechanism for the biogenesis of peroxisome. Some experimental results are consistent with both models. The peroxisomal content proteins, such as catalase and uricase, are synthesized on free ribosomes and are not mediated by ER. Therefore, their translocation is posttranslational rather than cotranslational and the information to direct their
translocation resides within each polypeptide as a permanent segment, not as a transient feature. Both models also propose two different forms of peroxisomes which recognize the newly synthesized peroxisomal proteins, although their origins are different. The form suggested by Goldman and Blobel is a nascent peroxisome budding from ER by patching and capping after the integration of peroxisomal membrane proteins. The other form is a peroxisomal reticulum newly formed by fission from preexisting peroxisomes. In both models, the translocation of peroxisomal content proteins is postulated to occur by a posttranslation mechanism. There is no experimental evidence to support the presence of putative "receiver" membrane structures except electron microscopic observation which is not sufficient to show the origin of their structures. Current studies have not yet provided sufficient information about the biogenesis of peroxisomes. For example, the membrane structures which recognize the newly synthesized proteins posttranslationally have not been yet identified. Future research should be directed toward the identification of competent membranes which can translocate in vitro translated peroxisomal proteins. Furthermore, our present knowledge is limited only to peroxisomal content proteins. The translocation
mechanism for peroxisomal integral membrane proteins has not been investigated, and will be very important for identifying the competent membrane structures, and to determine their origin.

Purpose of the study

This research has focused upon the enzyme, uricase, which is one of the enzymes related to nitrogen metabolism in the purine catabolic pathway. Uricase was purified and characterized, and the regulation of its synthesis in *Neurospora crassa* was studied. Important steps included obtaining antibodies specific to uricase and their use for affinity chromatography and for immunoprecipitation of the enzyme protein.

In order to understand the control of uricase gene expression at the molecular level, the mechanism of uricase induction was studied. The pattern of uricase synthesis upon induction was studied with *in vivo* labeling of proteins followed by specific immunoprecipitation of uricase with antibody. Moreover the synthesis of uricase mRNA upon induction was monitored by its *in vitro* translation. Possible posttranslational processing and translocation of *in vitro*-translated uricase was studied with microsomal
membranes.

Finally, to investigate the detailed regulation mode of uricase gene expression, the uricase gene was cloned from a lambda gt11 cDNA bank of *Neurospora crassa* by an immunological screening method. The uricase gene fragment was subcloned into pUC8 plasmid and the new plasmids were developed which can be used as a probe to observe the genetic regulation of uricase gene expression in *Neurospora*. 
MATERIALS AND METHODS

Materials

Sephadex G-150 and Sephadex AE-50 were purchased from Pharmacia. Complete and incomplete Freund's adjuvant were from Difco Laboratories. Carrier free $^{35}$S-SO$_4$ and rabbit reticulocyte in vitro translation system with $^{35}$S-methionine and dog pancreatic microsomal membrane were from New England Nuclear. Affigel-Blue, Affigel-10 and protein assay kit were obtained from Bio-Rad Laboratories and oligo(dT) cellulose type 3 from Collaborative Research Inc. A Lambda gt11 cDNA bank of Neurospora crassa, E. coli strain Y1090 and Y1089 were provided by Dr. Rajbhandry, MIT, and ProtoBlot immunoscreening kit was purchased from Promega Biotec. Restriction enzymes and agarose were obtained from International Biotechnologies Inc., and Nusieve agarose was from FMC Inc.

Growth of Neurospora crassa

Neurospora crassa wild type strain 74-OR23-1A (FGSC #987) was conidiated on Vogel's media with 1.5% agar for 7 days and the conidia were harvested by filtration
with cheese cloth. Conidia suspensions of the strain were inoculated to an absorbance of 0.5 at 420 nm into 500 ml of Vogel's minimal media in 2 liter flask and incubated for 16 hrs at 30°C with shaking (150 rpm) in a Psychrotherm incubator shaker (New Brunswick Scientific Co. Inc.). The mycelia were harvested with a Whatman No. 1 filter paper in a Buchner funnel with aspiration. For the induction of uricase, one half of the harvested mycelia was transferred to induction medium in which the nitrogen source was substituted for by 2 mM uric acid. As a control, the other half of mycelia was transferred to fresh Vogel's minimal media. The mycelia were incubated in the minimal and induction media for various times, and then were harvested, lyophilized and used for the isolation of uricase and RNA.

Assay of uricase activity

Two ml of 0.2 mM uric acid in 100 mM Tris-HCl, pH 8.6, was incubated with 0.1 ml of a properly diluted enzyme solution in a quartz cuvette for 10 min at room temperature. The decrease in absorbance of the reaction mixture at 290 nm was measured with a Gilford Spectrophotometer (Model 250) and monitored with a Gilford Recorder (6051). One unit of uricase activity
was defined as the amount that caused a decrease of absorbance of 1.0 at 290 nm unit decrease per min.; specific activity was expressed as the units of uricase activity per mg of protein.

Assay of Protein

The amount of protein was measured with the Bio-Rad protein assay kit according to the manufacturer's procedure using bovine gamma globulin as a standard.

Isolation of Uricase

The procedure for the isolation of uricase (Wang and Marzluf, 1980) was significantly modified as follows. 20 g lyophilized mycelia were ground in a mortar with 10 g of glass beads and homogenized with 200 ml of 0.1 M Tris-HCl (pH 7.2) at 10,000 rpm for 2 min with a VirTis Homogenizer. A crude extract was prepared by centrifugation for 10 min at 15,000 g with a Sorvall HB-4 rotor. Uricase was precipitated with ammonium sulfate in the range from 50 to 60% saturation. The precipitate was dissolved in 10 ml of extraction buffer and dialyzed overnight against 10 mM Tris-HCl buffer (pH 7.2). The dialysate was adjusted to 0.1 M NaCl, 50 mM Tris-HCl, pH 7.2, and loaded onto a Sephadex AE50 column.
previously equilibrated with the same buffer. After washing the column thoroughly with the starting buffer, uricase was eluted with a linear salt gradient from 0.1 M to 0.3 M NaCl in 50 mM Tris-HCl buffer (pH 7.2) at a flow rate of about 20 ml/hr, and 3 ml fractions were collected. The active fractions were pooled and concentrated to 5 ml with an Amicon PM 10 membrane. The concentrated sample was then passed through a Sephadex G-150 column (bed volume 150 ml) at a flow rate of 10 ml/hr, and 3 ml fractions were collected. Those fractions containing uricase activity were pooled and the protein concentration was adjusted to 100 µg/ml. The purity of each preparation was analyzed by activity and protein assay and by 7.5 % polyacrylamide gel electrophoresis (PAGE).

Preparation of Antibody

Anti-uricase antibody was obtained from a New Zealand rabbit (6 month old) by injecting purified uricase. 100 µg of purified uricase was injected into rabbit intramuscularly with the same volume of complete Freund’s Adjuvant. After one month, another 100 µg of uricase with incomplete Freund's adjuvant was injected subcutaneously to boost the antibody production. A week later, 30 ml of blood samples were obtained bleeding
from the ear vein of the immunized rabbit in weekly intervals for 1 month. The blood was clotted for 1 hr at room temperature and allowed to stand overnight at 4°C. After the clot was removed by centrifugation for 10 min at 10,000 g with a Sorvall HB-4 rotor, the supernatant was heated at 65°C for 30 min. For the isolation of immunoglobulin G (IgG) fraction, 7 ml of immunized serum was dialyzed against elution buffer (20 mM Tris-HCl, pH 8.0, 28 mM NaCl, 0.02 % NaN3) and the dialysate was passed through 100 ml bed volume of Affigel-Blue equilibrated with elution buffer. The column was washed with elution buffer, and after the void volume, the next 10 ml of eluate was collected and used as immunoglobulin G fraction. For the isolation of specific anti-uricase antibody, the immunoglobulin G fraction was further purified with uricase-bound Affigel-10. 2 ml of immunized serum or the immunoglobulin G fraction was applied to a 2 ml bed volume column of uricase-bound Affigel-10 and washed thoroughly with 100 mM Tris-HCl buffer (pH 7.2). The column was next washed with 5 ml of 1 M MgCl2 and then the anti-uricase antibody was eluted with 5.0 M MgCl2 at a flow rate of 1.0 ml/min, collecting 1.0 ml fractions. The first two fractions eluted with 5 M MgCl2 were pooled and dialyzed rapidly against 10 mM Tris-HCl
buffer (pH 7.2). This preparation of anti-uricase antibody was adjusted to 100 mM Tris-HCl (pH 7.2), 10 mM DTT and kept frozen at -20°C until use.

Preparation of Uricase- and IgG-bound Affigel-10

For the rapid isolation of uricase and also anti-uricase antibody by a simple procedure, IgG-bound and uricase-bound Affigel-10 were prepared according to the manufacturer's procedure. 2 mg of the immunoglobulin G fraction or 500 μg of purified uricase in 10 ml solution was dialyzed against 10 mM Hepes buffer (pH 8.0) overnight at 4°C. The protein solution was adjusted to 100 mM Hepes buffer (pH 8.0) and added to 2 ml bed volume of the activated gel which had been washed thoroughly with water and 10 mM Hepes buffer. The mixture was reacted for 4 hrs at 4°C with gentle shaking and all remaining active esters were blocked by incubating for one additional hour with 1 ml of 1 M ethanolamine-HCl (pH 8.0). After the reaction, the gel slurry was transferred to a column and washed thoroughly with water. Before use, the column was washed thoroughly with 1 M MgCl₂ and loading buffer.
Isolation of Uricase by Immunoaffinity Chromatography

The IgG antibody column was used to isolate uricase. A crude extract (about 50 unit of uricase activity) was loaded onto 2 ml bed volume column of IgG-bound Affigel-1U. The column was washed thoroughly with 100 mM Tris-HCl buffer (pH 7.2) and 2 bed volumes of 1 M MgCl₂ at a flow rate of 1.0 ml/min. Uricase was eluted with 5.0 M MgCl₂ and 1 ml fractions were collected. The active fractions were pooled and dialyzed against 10 mM Tris-HCl buffer (pH 7.2) overnight at 4°C. The dialysate was concentrated with an Amicon PM 10 membrane, adjusted to 100 mM Tris-HCl, pH 7.2, 10 mM DTT and frozen at -20°C.

In vivo Labeling of Protein

A wild type conidia suspension was inoculated to an absorbance of 0.5 at 420 nm into 500 ml of Vogel's minimal medium and germinated for 12 hrs at 30°C. After harvest, samples representing 1/10 of the mycelia were transferred to flasks containing 50 ml of Vogel's minimal or induction media, each containing 5 uCi/ml of carrier free ³⁵S-SO₄ (specific activity 40 Ci/mg). The mycelia were harvested at various time intervals and lyophilized. The labeled dried mycelia (200 mg) were ground with glass beads in a mortar and then homogenized
with 5 ml of 100 mM Tris-HCl buffer (pH 7.2) at 10,000 rpm for 1 min with a VirTis homogenizer. The crude extract was prepared by centrifugation at 10,000 rpm for 10 min with a Sorvall HB-4 rotor. Fine debris in the crude extract was removed by ultracentrifugation at 30,000 rpm for 30 min in a Beckman SW41 rotor. The solution was used for TCA precipitable count and immunoprecipitation of uricase.

Immunoprecipitation of Uricase

For immunoprecipitation, *Staphylococcus aureus* cells containing protein-A at the cell surface were grown; the cell suspension was prepared according to the method of Kessler (1976). To 50 to 100 ul of a sample solution containing labeled uricase, 200 ul of a 10 % *Staphylococcus aureus* cell suspension was added and incubated in an Eppendorf tube for 15 min at room temperature. After centrifugation in an Eppendorf microcentrifuge for 2 min, the supernatant was incubated with 50 ul of antibody solution (100 ug/ml) for 30 min at room temperature. The solution was further incubated with 200 ul of the *Staphylococcus* cell suspension for 30 min and centrifuged for 2 min. The cell pellet was washed 3 times with 1 ml of washing buffer (50 mM Tris-
HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl$_2$, 0.02% NaN$_3$, 1.0% Triton, 1.0% sodium deoxycholate, 5 mg/ml BSA). The cell pellet was then resuspended with 200 ul of washing buffer, layered onto top of 800 ul of the washing buffer containing 1 M sucrose and centrifuged for 3 min. The pellet was finally washed twice again with the washing buffer and the uricase was eluted with 50 ul of 2% sodium dodecyl sulfate (SUS). Half of the eluted solution was counted in a Beckman Liquid Scintillation counter, and the other half was analyzed with SDS-PAGE.

Isolation of poly(A) RNA

Poly(A) RNA was isolated from Neurospora by an SDS/phenol/chloroform method followed by oligo(dT) cellulose column chromatography. One gram of lyophylized mycelia was homogenized with 10 ml of 100 mM sodium acetate buffer (pH 5.5) containing 0.5% SUS, 5 ml of phenol and 5 ml of chloroform in a VirTis homogenizer for 1 min at 10,000 rpm. The aqueous phase was separated by centrifugation for 20 min at 10,000 g in a corex tube with Sorvall HB-4 rotor at room temperature. The aqueous phase was again extracted with the same volume of phenol/chloroform mixture (1:1) and finally washed with an equal volume of chloroform. The RNA was precipitated with 2 volumes of cold 95% ethanol at least for 1 hr at
-20°C. The precipitate was dissolved in 5 ml of water and the RNA was reprecipitated with 2 volumes of 3.0 M sodium acetate buffer (pH 5.5) for 30 min on ice. After centrifugation for 10 min at 10,000 rpm in a Sorvall HB-4 rotor, the pellet was washed twice with 3.0 M sodium acetate buffer. The RNA pellet was dissolved in 5 ml of water, and the solution was heated at 65°C for 2 min. and cooled rapidly on ice. Then the RNA solution was warmed to room temperature by adding an equal volume of 2X binding buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM EDTA). The RNA solution passed through 1.0 ml bed volume of oligo(dT) cellulose column, and the column was washed thoroughly with binding buffer. Poly(A) RNA was eluted with 2 ml of elution buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA) and 0.5 ml fractions containing RNA were collected. Poly(A) RNA was precipitated at -20°C overnight by adding 2 volumes of cold 95% ethanol in the presence of 0.3 M sodium acetate. The precipitated poly(A) RNA was recovered by centrifugation in an Eppendorf centrifuge for 2 min and washed with cold 70% ethanol. The poly(A) RNA pellet was dried for 10 min in a vacuum dessicator and dissolved in water at concentration of 1 ug/ul. This poly(A) RNA solution was kept frozen at -80°C until use.
In Vitro Translation of Poly(A) RNA

*Neurospora* poly(A) RNA was translated in vitro in a rabbit reticulocyte translation system (NEN) with $^{35}$S-methionine (specific activity 1,000 Ci/m mole) according to the procedure suggested by the manufacturer. 2 ug of poly(A) RNA was incubated for 1 hr at 37°C in a 25 ul reaction mixture containing 50 uCi $^{35}$S-methionine, 1.0 mM MgCl$_2$, 80 mM potassium acetate, 10 ul lysate and 5.5 ul of "translation cocktail". For cotranslational processing, the reaction mixture was incubated for 2 hrs at 27°C in the presence of 2 ul of 50 A280 units of dog pancreatic microsomal membrane. For posttranslational processing, the reaction was stopped after incubation for 2 hrs at 27°C by adding 3 ul of cycloheximide (20 ug/ml) and the reaction mixture was incubated an additional one hour in the presence of dog pancreatic membranes. To study the translocation of protein into membrane vescicles, 5 ul of a trypsin and chymotrypsin mixture (200 ug of each/ml) was added to the translation reaction mixture after processing.

Assay of Protein Synthesis In Vitro

The amount of protein synthesized in vitro was analyzed by measuring the amount of radioactivity
of RNAs (10 ug/ul) was added to the reaction mixture and incubated at 30°C for 10 min. to hydrolyze aminoacyl-tRNAs. 2 ul of the translation reaction mixture was transferred to a glass test tube containing 100 ul of 50 mM Tris-HCl buffer, pH 7.5, and 5 mg/ml of BSA; 1 ml of cold 10% TCA was added and the mixture was cooled on an ice bath for 30 min. The protein precipitate was collected on a Whatman GF/A glass fiber filter, washed with 20 ml of 10% TCA, and finally washed with 10 ml of cold 95% ethanol. The filter was dried under a heat lamp for 10 min and counted in a scintillation counter.

Electrophoresis and Fluorography of Protein

Protein samples were also analyzed by polyacrylamide gel electrophoresis (PAGE), with or without SDS, according to Laemmli (1970). For the detection of protein patterns during the isolation of uricase, samples were run on 7.5% polyacrylamide gels for 5 hrs at 20 mA. The gels were stained with Coomassie Blue. For the assay of labeled radioactive proteins, 20 to 50 ul samples were run on 12.5% SDS-polyacrylamide gels for 5 hrs at 20 mA. The gels were fixed with 10% trichloroacetic acid containing 30% methanol and 10% acetic acid for 1 hr and then washed overnight with 10%
methanol and 10% acetic acid. The gel was soaked in Enlightning (NEN) for 1 hr and dried for 2 hr on a slab gel drier. The dried gel was exposed to Kodak XAR-5 X-ray film for 48 hrs at -70°C.

Screening of lambda gt11 recombinant phage

In order to clone the Neurospora uricase gene, a Neurospora lambda cDNA library in the lambda gt11 expression vector (Young and Davis, 1983 a,b) was used; the desired recombinant phages were detected immunologically with anti-uricase antibody using a ProtoBlot immunoscreening kit (Promega Biotec). E. coli Y1090 cells were grown overnight in 10 ml of LB medium (LB broth supplemented with 10 mM MgSO₄ and 0.2% maltose) containing 100 μg/ml of ampicillin. The culture was centrifuged at 4000 g for 10 min and the cells were resuspended with 10 mM MgSO₄ to an absorbance of 2.5 at 600 nm and stored at 4°C. This stock culture was used as a lawn for the growth of phage. For the primary screening of recombinant phages, 10⁵ phage from the cDNA recombinant library were diluted with TM buffer (10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂) and used to infect 1.0 ml of an E.coli Y1090 stock culture at room temperature for 20 min. 20 ml of LB soft agar was added to the culture mixture and poured onto an LB plate (
The plate was incubated at 42°C for 4 hrs when the plaques became visible. Induction of B-galactoside fusion protein synthesis and transfer of proteins to a nitrocellulose paper was accomplished by placing a nitrocellulose paper which was saturated with 10 mM isopropyl-B-thiogalactopyranoside (IPTG) onto the plate. After incubation for 3 hrs at 37°C, the paper was marked with a needle and then peeled gently off the plate. The nitrocellulose paper was rinsed briefly with TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) to remove cell debris. Any nonspecific protein binding sites were blocked in TBST buffer containing 1% BSA for 30 min with gentle shaking at room temperature. After washed in TBST buffer for 10 min, the nitrocellulose paper was incubated for 30 min in TBST buffer containing affinity-purified antibody (100 fold diluted) which was previously treated with E. coli lysate. The paper was washed in TBST buffer three times (5 min each), and reacted with 10,000 fold diluted goat anti-rabbit IgG-alkaline phosphate-conjugate in TBST buffer for 30 min at room temperature and washed in TBST buffer as above. The paper was blot dried on a 3M filter paper and transferred to the color development solution (50 ml of 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂, 330 ul of nitro blue tetrazolium (50 mg/ml), and
165 μl of 5-bromo-4-chloro-3-indoly1 phosphate (50 mg/ml)). After the color reaction took place for 1 hr at room temperature, it was stopped by replacing the substrate solution with stop solution (20 mM Tris-HCl, pH 8.0, 5 mM EDTA). To purify the desired recombinant phage, an agar plug was removed with a Pasteur pipette from the region of the plate corresponding to the purple positive color signal on the nitrocellulose paper. The agar plug was transferred to 1 ml of TM buffer containing 1 drop of chloroform incubated for at least 1 hr at room temperature with occasional vortexing, and kept at 4°C overnight. The phage were replated on a lawn of E. coli Y1090 in a 90 mm plate and the screening procedure was repeated until all the plaques on the plate produced a purple positive signal.

Confirmation of uricase gene by Western Blot

The cloned recombinant phage which produced a positive signal in the screening procedure was confirmed by an immunological method with Western blot (Johnson et. al., 1985). Standard uricase (1 μg per lane) was run on a 7.5% polyacrylamide gel and then was transferred to a nitrocellulose paper by blotting at 100 V for 3 hrs. Any nonspecific protein binding sites were saturated by incubation in TBST buffer containing 1%
BSA for 30 min with gentle shaking at room temperature as described in the screening procedure. The paper was cut into small pieces and reacted with various antibody preparations described below and visualized with goat anti-lgG-alkaline phosphatase-conjugate as used in the screening procedure. To prepare antibody specific to the cloned recombinant phage, the phage was plated on E. coli strain Y1090 to yield about 3000 plaques per 90 mm plate. After incubation for 4 hrs at 42°C, a nitrocellulose paper which was saturated with 10 mM IPTG was layered onto the plate and left overnight at 37°C. The paper was removed, washed in TBST buffer for 10 min and incubated for 30 min in TBST buffer containing affinity-purified antibody. After washing the paper in TBST buffer three times (5 min each), any bound antibody (specific to the cloned phage) was eluted by adding 3.5 ml of 0.2 M glycine-HCl (pH 2.5), and shaking 2 min in a 50 ml plastic tube. The paper was removed and the antibody solution was neutralized with 1.5 ml of 1.0 M potassium phosphate buffer (pH 9.0). For protein blotting, the neutralized solution was diluted with 6 ml water and 4 ml of TBST containing 1 % bovine serum albumin.
Preparation of Lambda gt11 Recombinant Lysogen

An *E. coli* lysogen which harbors the lambda DNA was prepared by infection of the cloned lambda gt11 recombinant into *E. coli* (Young and Davis, 1983b). *E. coli* Y1089 cells were infected with cloned lambda gt11 recombinant at multiplicity of infection (MOI) of 10 for 30 min at room temperature. A loopful of these cells was streaked on a LB plate containing ampicillin (100 ug/ml) and incubated overnight at 30°C. Individual colonies were then replica-plated onto two plates. One plate was incubated at 30°C and the other at 42°C overnight. Any colony which grew only at 30°C was streaked onto fresh plates and retested for growth at both temperatures. A single colony which grew only at 30°C was used for the preparation of recombinant phage DNA and of cell lysate.

Preparation of Lysate from Induced recombinant Lysogen

A 10 ml culture of *E. coli* Y1089 lysogen was grown at 30°C to a cell density of 2 x 10⁸ and induced by heat treatment at 45°C for 20 min. IPTG was added to 5 mM to induce expression of the B-galactosidase-fusion protein. The culture was incubated at 37°C for 1 hr with
vigorously shaking, then the cells were pelleted, and resuspended in 1 ml of gel sample buffer (50 mM Tris-HCl, pH 6.8, 1.5 % SDS, 1.5 % SDS, 50 mM DTT, 40 % glycerol). The suspension was mixed well by passing several times through a 21 gauge needle. The solution was heated at 70°C for 2 min and insoluble material was removed by centrifugation for 3 min in an Eppendorf centrifuge. The protein pattern was analyzed by 7.5 % SDS-PAGE.

Isolation of Recombinant Phage DNA

Recombinant phage DNA was prepared in large scale from the lysogen culture by heat induction of the phage followed by CsCl density gradient ultracentrifugation and isolation of the DNA by the phenol/chloroform extraction method (Maniatis et al., 1982). An overnight culture of E. coli Y1089 lysogen was inoculated into 1 liter of LB media containing ampicillin (100 ug/ml) and incubated at 30°C with vigorous shaking until the A600 reached 0.5. The culture was induced for 20 min at 45°C with constant shaking, and incubated at 37°C for an additional 3 hrs with vigorous shaking. The bacterial cells were recovered by centrifugation at 4000 g for 10 min at 4°C and resuspended in 10 ml of 10 mM Tris-HCl
buffer, pH 7.4, 50 mM NaCl and 5 mM MgCl$_2$. 10 drops of chloroform were added to the suspension and the solution was vortexed well and incubated at room temperature for 30 min. Pancreatic DNase and RNase were added to a final concentration of 1.0 ug/ml to degrade DNA and RNA liberated from the lysed cells. Cell debris was removed by centrifugation at 15,000 g for 10 min, and the supernatant was recovered. An equal volume of chloroform was added to the supernatant and the mixture was vortexed for 30 seconds. The aqueous phase was separated by centrifugation at 1600 g for 10 min at 4°C. The volume of aqueous phase was adjusted to 11 ml and 0.75 g/ml of solid CsCl was added to the phage solution. The mixture was centrifuged for 24 hrs at 40,000 rpm at 4°C in a Beckman Type 50Ti rotor. The bluish phage band was recovered with a needle from the density gradient and was dialyzed at room temperature for 2 hrs against 50 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM MgCl$_2$ with one change of dialyzing buffer. EDTA, pronase and SDS were added to a final concentration of 20 mM, 50 ug/ml, and 0.5 %, respectively, and the mixture was mixed gently and incubated at 37°C for 1 hr. An equal volume of phenol solution was added and the solution was mixed by inverting the tube several times. The aqueous phase was recovered by centrifugation at 1600 g for 10 min at room
temperature, extracted again with a mixture of phenol and chloroform (1:1), and finally extracted with an equal volume of chloroform. The aqueous phase was recovered and the phage DNA was precipitated with 2 volume of cold ethanol in the presence of 2.5 M ammonium acetate at -20°C overnight. The DNA precipitate was recovered by centrifugation in an Eppendorf centrifuge and washed with 70% ethanol. The phage DNA was dried for 10 min in a vacuum dessicator and dissolved in TE buffer at a final concentration of 1 ug/ul.

Subcloning of *Neurospora* DNA Insert into pUC8 Plasmid

The *Neurospora* DNA fragment in the cloned lambda gt11 recombinant was subcloned into a bacterial plasmid. The pUC8 plasmid was used as the recipient vector and *E. coli* JM 83 was used as a host strain for transformation (Vieira and Messing, 1981). 50 ug of phage DNA was treated with 100 unit of EcoRI restriction enzyme for 3 hrs at 37°C. The digested DNA was run on 4% NuSieve agarose gel at 25 V overnight. A gel slice which contained the *Neurospora* DNA insert was cut out and the DNA was isolated from it by electroelution for 45 min at 100 V. The DNA insert was eluted into 3.0 M sodium acetate buffer (pH 5.0) was washed with water-saturated
butanol twice, precipitated with 2 volumes of cold 95% ethanol and washed with 70% ethanol. The DNA precipitate was dissolved in TE buffer at a final concentration of 10 ng/ul. 800 ng of pUC8 plasmid which was linearized with Eco. RI restriction enzyme was mixed with 200 ng of the Neurospora DNA insert. The mixture was heated at 65°C for 5 min and cooled on an ice bath. One unit of T4 DNA ligase was added in a final volume of 100 ul, and the ligation mixture was incubated at 4°C for 16 hrs. After incubation, 10 ul samples of the reaction mixture with and without the 16 hr incubation were analyzed on a 1% agarose gel. After the ligation reaction, 10 ul of the mixture was added to 100 ul of E.coli JM83 cell suspension which was prepared by the calcium chloride method (Cohen et al, 1972), and incubated on ice for 30 min. As controls, no DNA was added to one batch of cell suspension, and only pUC8 plasmid was added to another. The cell suspension was heated at 42°C for 2 min and 1.0 ml of 2YT medium (Miller, 1972) was added. After incubation for 1 hr at 37°C without shaking, 50 ul of the cell suspension was plated onto 2YT agar medium containing ampicillin (100 ug/ml) and X-gal (4 ml of 2% X-gal in N,N-dimethyl formamide/ liter of medium). The plate was incubated at 37°C overnight. White colonies, which contained an
insert in pUC8 plasmid at Eco. R I site, were streaked on fresh plates and were isolated again. Colonies were individually inoculated into 5 ml of medium and grown overnight. Plasmids were prepared with 1.5 ml of an overnight culture by the rapid alkaline lysis method and run on 1% agarose gel to confirm the presence of the insert (Maniatis et al. 1982).

Isolation of Bacterial Plasmid

The plasmid which has an insert in pUC8 at the EcoR I site was amplified in E.coli JM83 in the presence of chloramphenicol and isolated by the alkaline lysis method (Maniatis et al.,1982). An overnight culture of the E. coli transformant was inoculated into 500 ml of 2YT medium containing ampicillin (100 ug/ul). The culture was incubated at 37°C with vigorous shaking until it reached an A600 of 0.5. 2.5 ml of chloramphenicol (34 mg/ml in ethanol) was added to the culture and incubation was continued for a further 16 hrs. The cells were harvested and washed with 100 ml of ice cold STE buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.8, 1 mM EDTA). The bacterial pellet was resuspended in 5 ml of lysozyme solution (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 5 mg/ml lysozyme) in a 30 ml corex tube and
allowed to stand for 20 min at room temperature. 10 ml of 1% SDS solution in 0.2 N NaOH was added and the contents were mixed gently by inverting the tube several times and incubated on ice for 10 min. 15 ml of ice cold 5 M potassium acetate (pH 4.8) was added and the contents were mixed gently by inverting the tube. The mixture was centrifuged at 10,000 rpm for 20 min at 4°C in a Sorvall HB-4 rotor. DNA in the supernatant was precipitated by adding 0.6 volume of isopropanol. After 30 min at room temperature, DNA was recovered by centrifugation at 10,000 rpm for 20 min at room temperature. The pellet was washed with 70% ethanol at room temperature and dissolved in 9 ml of TE buffer. 1.0 ml of ethidium bromide solution (10 mg/ml) and 10 g of solid CsCl₂ was added to the DNA solution, and plasmid DNA was further purified by ultracentrifugation at 45,000 rpm for 36 hrs at 20°C. The plasmid DNA band was visualized under a UV light and recovered with a 21 gauge needle and the ethidium bromide was extracted with isopropanol three times. The aqueous DNA solution was dialyzed against TE buffer overnight and the plasmid DNA was precipitated with 2 volumes of cold 95% ethanol in the presence of 2.5 M ammonium acetate. The plasmid DNA was washed with 70% ethanol and dissolved in TE buffer to a concentration of 1ug/ul.
RESULTS

Induction of uricase in *Neurospora crassa*:

The production of uricase in *Neurospora crassa* wild type strain (74A-OK23-1A) in induction medium was compared with that produced in Vogel's minimal medium to observe the induction profile of uricase. Conidia were germinated in minimal medium for 12 hrs, when the mycelia were harvested and transferred into fresh minimal and induction media. The synthesis of uricase during growth in both conditions was compared in terms of specific enzyme activity. As shown in Fig.2, uricase specific activity began to increase after 2 hrs of induction and it increased up to 4 fold after 8 hrs of induction, whereas it remained constant in minimal medium. In cells grown in minimal medium, uricase is present at a basal level, which is presumably required to maintain a metabolic steady state. Repression of uricase synthesis is known to be the result of nitrogen catabolite repression, caused by primary nitrogen sources such as ammonia. In the absence of or at low level of primary nitrogen sources, however, repression is released and the rate of uricase synthesis is thought to increase leading to accumulation of substantial amounts of uricase.
Figure 2. Profile of uricase activity of *Neurospora crassa* during cultivation in minimal and induction conditions. Uricase specific activity is expressed as the units of uricase activity per mg of protein.

--- o o --- : induction medium

---------- : Vogel's minimal medium
URICASE SPECIFIC ACTIVITY
($\Delta A_{290} \times 10^{-3}$)

HOUR

0 10 20 30 40

0 10 20 30

2 4 6 8

8
Isolation of Uricase:

In order to study its induction mechanism, uricase was isolated by conventional methods and by immunoaffinity chromatography. At each step in the purification procedure, the degree of purification was expressed as specific activity and protein profiles were monitored by absorbance at 280 nm and with 7.5% polyacrylamide gel electrophoresis. Figure 3 shows the activity profile at each step during the isolation of uricase. As shown in Fig. 3A, uricase activity was present in proteins precipitated at 45% of saturation of ammonium sulfate, and the activity in the precipitate was constant above the 60% saturation level. Therefore, uricase was precipitated from crude extract with ammonium sulfate from 45% to 65% saturation. Further separation of uricase was performed with Sephadex AE-50 anion exchange followed by Sephadex G-150 gel filtration; their separation patterns are shown in Fig. 3B and 3C, respectively. The protein profile at each purification step was analyzed with 7.5% PAGE as shown in Fig. 4. The preparation after gel filtration showed one major protein band. In situ activity staining for uricase revealed that the enzyme had the same mobility as the major protein band (Fig. 4e). This result
Figure 3. Protein and uricase activity elution profiles in the purification of uricase from Neurospora crassa.

--o--o-- : absorbance at 280 nm
--o--o-- : uricase activity
--o--o-- : NaCl gradient
a) ammonium sulfate fractionation
b) Sephadex AE-50 Anion exchange chromatography
c) Sephadex G-150 gel filtration
ABSORBANCE AT 280nm

AMMONIUM SULFATE (% SATURATION)

FRACTION NUMBER

URICASE ACTIVITY (ΔA290/ml)

A

B

Sephadex AE-50

C

Sephadex G-150

NaCl (M)

0.4

0.3

0.2

0.1

0.5

1.0

2.0

0.2

0.1

2.0
Figure 4. Protein patterns of active fractions in the purification procedures on 7.5% polyacrylamide gel electrophoresis. Proteins were stained with Coomassie Blue except lane (e) which was visualized with the in situ activity staining method.

a) Crude extract
b) Ammonium sulfate fractionation (50-60% saturation)
c) DEAE-Sephadex AE 50 Anion exchange chromatography
d) Sephadex G-150 gel filtration
e) In situ activity staining
indicates that the major protein isolated by the procedure is uricase. The purified uricase was used for the preparation of anti-uricase antibody, as described in Materials and Methods.

Isolation of uricase with immunoaffinity chromatography:

After obtaining antiserum against uricase from a rabbit, uricase was subsequently purified by immunoaffinity chromatography. From the antiserum, the immunoglobulin G fraction containing anti-uricase antibody was isolated with Affigel Blue. An Ig G-bound Affigel-10 column was then prepared as described in Materials and Methods in order to isolate uricase in a simple, rapid method. Crude extract containing uricase activity was passed through the antibody column which was then washed thoroughly with starting buffer, followed by 1M MgCl2 to remove impurities bound nonspecifically to the column. Uricase was then eluted with 5M MgCl2 and the preparation was analyzed by 7.5% PAGE as shown in Fig. 5. Only one major protein band was visible by Coomasie Blue staining, and it was shown to have the same mobility as uricase prepared by the conventional procedure. Uricase isolated by this procedure showed the same level of purity as that prepared with the conventional method as shown in Table
Figure 5. Protein pattern of uricase preparations by immunoaffinity chromatography on 7.5% PAGE. Proteins were stained with Coomassie Blue.

a) Crude extract
b) Uricase purified by antibody-bound Affigel-10
c) Uricase purified by the conventional procedure
1. The degree of purification was about 2,200 fold and the specific activity was 33.3 unit/mg of protein in the conventional procedures. Uricase prepared by immunoaffinity chromatography showed about 2,000 fold purification and a specific activity of 30.8 unit/mg protein. These values are very similar, and the use of the antibody column was found to be very efficient and simple for the purification of uricase from *Neurospora crassa*.

**De novo synthesis of uricase**

In order to investigate the regulation of uricase induction, the rates of uricase synthesis were compared between cells in induced and uninduced conditions. Wild type mycelia obtained by growth on minimal medium were transferred to induction medium and to minimal medium. Then the mycelia were grown for various times in the presence of carrier free $^{35}$S-SO$_4$. Crude extracts were prepared and the total radioactivity in them was measured; induced and uninduced crude extracts containing the same amount of TCA precipitable radioactivity (500,000 cpm) were immunoprecipitated. Then, the samples were run on SDS-PAGE gels and fluorographed. In Fig. 6, lane C and I showed the total proteins labeled in minimal and induction conditions,
Table 1. Purification of Uricase.

<table>
<thead>
<tr>
<th>step</th>
<th>activity (U/ml)</th>
<th>protein (mg/ml)</th>
<th>specific activity (U/mg)</th>
<th>yield (U)</th>
<th>purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>0.34</td>
<td>22.0</td>
<td>0.015</td>
<td>149.6</td>
<td>100.0</td>
</tr>
<tr>
<td>(NH4)2SO4 fraction</td>
<td>1.40</td>
<td>16.8</td>
<td>0.083</td>
<td>98.0</td>
<td>65.5</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>1.23</td>
<td>0.11</td>
<td>11.2</td>
<td>36.9</td>
<td>24.7</td>
</tr>
<tr>
<td>Sephadex G-150</td>
<td>6.00</td>
<td>0.18</td>
<td>33.3</td>
<td>24.0</td>
<td>16.7</td>
</tr>
<tr>
<td>Antibody-Affigel-10</td>
<td>4.00</td>
<td>0.13</td>
<td>30.8</td>
<td>6.0</td>
<td>40.0</td>
</tr>
</tbody>
</table>

63
respectively. Lanes C-1, C-2, and C-3 and lanes I-1, I-2, and I-3 show the immunoprecipitated uricase in cells harvested after 2, 4, and 6 hrs in minimal and induction media, respectively. The immunoprecipitated uricase bands from uninduced cells and induced cells had the same mobility in gel electrophoresis as standard uricase. These bands for cells on minimal medium remained constant in minimal media, whereas the bands for the induced cells showed increased intensity with time. The intensity of the bands for induced cells showed similar increases as observed for enzyme activity. The result suggests that the increase in uricase activity does not result from an activation of a pre-existing precursor of uricase, but results from the increase in the amount of uricase synthesized during a given period. Therefore, it can be concluded that the induction of uricase activity in induced cells reflects de novo synthesis of uricase.

In vitro translation of poly(A) RNA

In order to study the induction mechanism for uricase, it was of interest to determine the level of uricase mRNA related to the de novo synthesis of uricase in vivo. Poly(A) RNAs isolated from uninduced and induced cells were translated in vitro in a rabbit
Figure 6. Immunoprecipitation of uricase labelled in vivo with 35-S04. The same amount of radioactive protein extracted from induced and from uninduced cells at time intervals were immunoprecipitated, run on 12.5% SDS-PAGE and autoradiographed. The arrow indicates the location of uricase on the gel.

C) Total Proteins from Control Cell

Immunoprecipitation of uricase from control cells grown for 1) 2 hrs  2) 4 hrs  3) 6 hrs

I) Total Proteins from Induced Cell

Immunoprecipitation of uricase from induced cells grown for 1) 2 hrs  2) 4 hrs  3) 6 hrs

S) Standard Uricase
reticulocyte cell-free translation system with $^{35}$S-methionine. The cells were harvested after induction for various times and poly(A) RNAs were isolated. After translation, TCA precipitable radioactive protein was measured and samples with the same amounts of radioactivity (1,000,000 cpm) were immunoprecipitated with antibody. These samples were run on SDS-PAGE and fluorographed. In Figure 7, lane C and I showed the total spectrum of proteins translated with poly(A) RNAs isolated from uninduced and induced cells, respectively. Lanes C-1 to 3 showed the immunoprecipitated protein translated with poly(A) RNAs isolated from uninduced cells grown for 2, 4, and 6 hrs, whereas lane I-1 to 3 correspond to cells induced for 2, 4, and 6 hrs, respectively. The level of uricase translated from poly(A) RNA from uninduced cells remained constant and it had the same mobility as standard uricase. The immunoprecipitated uricase translated from poly(A) RNA from induced cells also showed the same mobility as standard uricase. However, the level of uricase translated from the RNA from induced cells did not show an increase in intensity of uricase band compared to those observed in uninduced cells as a function of time. This result showed that the level of uricase translated with poly(A) RNA from induced cells does not reflect
Figure 7. *In vitro* translation of poly(A) RNA from *Neurospora*. Poly(A) RNAs from control (C) and induced cells (I) after 2 hrs (lane 1), 4 hrs (lane 2) and 6 hrs (lane 3) induction were translated in a rabbit reticulocyte system, run on 12.5% SDS-PAGE, and autoradiographed. Lane C and 1 show the total translation products. Lanes 1, 2 and 3 show immunoprecipitated uricase (arrow). As a control, no poly(A) RNA was added in the translation system (lane B).
the increase in enzyme activity observed in in vivo labeling experiment in induced cells. However, uricase translated in vitro from poly(A) RNA obtained from noninduced and induced cells showed the same mobility in PAGE as the enzyme synthesized in vivo (see Fig.6.)

Processing of uricase translated in vitro

In order to find the possible translational arrest and processing which occurs during in vitro translation of secreted proteins, poly(A) RNA was translated in vitro in the presence of dog pancreatic microsomal membranes (Fig.8). As a control human placental lactogen mRNA was translated with and without microsomal membranes which are known to possess signal peptidase as well as signal recognition particle receptor. This receptor relieves the translational arrest, which occurs for certain secreted proteins. Lanes a and b show the pattern of proteins synthesized in vitro without exogenous RNA in the absence and presence of microsomal membranes, respectively. Lanes c and d show the translation of human placental lactogen mRNA in the absence and presence of dog pancreatic microsomal membranes. The processing of lactogen did not occur in the absence of microsomal membranes, and showed one major protein band was synthesized from the lactogen RNA
Figure 8. Cotranslational processing of in vitro synthesized Uricase. Messenger RNAs were translated in a cell-free rabbit reticulocyte system in the absence (lane a, c, e) and in the presence of dog pancreatic microsomal membranes (lane b, d, f). No mRNA (lane a, b), placental lactogen mRNA (lane c, d) and Neurospora poly(A) RNA were used as templates for in vitro translation. In lane (e) and (f), in vitro synthesized uricase was immunoprecipitated, run on 12.5% SDS-PAGE and autoradiographed.
In contrast, in the presence of microsomal membranes (lane d), the protein synthesized from lactogen mRNA was largely cleaved to give a second distinct band. The lactogen precursor was cleaved by the signal peptidase activity present in the microsomal membranes, resulting in the production of mature lactogen. Uricase was translated with RNA from uninduced (lane e, f) and induced cells (lane g, h) in the absence and in the presence of microsomal membranes during the translation. As shown by comparing lane e with f, and g with h, neither the mobility of the immunoprecipitated uricase nor its level changed in the presence of microsomal membranes. This result indicated that a proteolytic cleavage of a signal sequence in uricase did not occur in the presence of microsomal membranes. However, the presence of an internal signal sequence which directs the movement of uricase across the peroxisomal membrane can be predicted. This result also ruled out the possibility of a translational arrest caused by the binding of a signal sequence with signal recognition particle. This experiment indicated that a cotranslational processing of uricase did not affect the translational efficiency of uricase poly(A) RNA in the rabbit reticulocyte lysate cell-free translation system. It is possible that a different uricase mRNA is
transcribed in induced cells, and that this new mRNA cannot be efficiently translated in the reticulocyte system.

Posttranslational processing of uricase in vitro:

As shown previously, cotranslational processing of uricase translated in vitro did not occur. There was no change in the size or amounts of uricase synthesized in vitro in the presence of microsomal membranes. Another possibility is that uricase translated in vitro is processed posttranslationally. To observe the possible posttranslational processing of uricase translated in vitro, microsomal membranes were added to the mixture after translation with poly(A) RNA's from induced cells was halted by adding cycloheximide. The result was compared with the cotranslational processing in wild type and induced cells. Fig. 9 shows the total protein profile (T) and the immunoprecipitated uricase (I) translated from poly(A) RNA from uninduced wild type cells in the presence (lane 1) and absence (lane 2) of microsomal membranes and with microsomal membranes added after the reaction (lane 3) (posttranslational processing). Lanes 4, 5 and 6 show the total protein (T) and immunoprecipitated uricase (I) translated with poly(A) RNA from induced cells in the presence and
Figure 9. Posttranslational processing of *in vitro* translated uricase. Total *in vitro* translation product with mRNAs from control (1,2,3) and induced (4,5,6) cells as a template(T). The total translated proteins were immunoprecipitated with anti-uricase antibody(I). No dog pancreatic microsomal membranes were added (1,4), microsomal membranes were added during (2,5) and after (3,6) *in vitro* translation.
absence of microsomal membranes and with post-
translational processing, respectively. Comparing lane
1-3 with 1-1 and 1-2, the immunoprecipitated uricase
translated from uninduced poly(A) RNA has the same
mobility as standard uricase (shown by arrow). After
translation from induced poly(A) RNA, there was also no
change in the mobility of immunoprecipitated uricase as
shown in lanes 1-4, 1-5 and 1-6. The intensity of the
bands varied due to the difference in the total
incorporation of labeled methionine as observed in lane
T-1 to T-6. The results of this experiment indicate that
the posttranslational processing of uricase could not be
detected by any visible size differences. The presence
of microsomal membranes, which can modify certain
proteins co- and post-translationally did not give any
effect on uricase translated in vitro. Therefore, it can
be concluded that the protein translated in vitro in the
absence of microsomal membranes is identical to the
uricase subunit identified in vivo. Although the
possible presence of a signal sequence (sorting
sequence) is suggested by the peroxisomal location of
uricase, cleavage and loss of signal peptide was
clearly not observed in this experiment.
Cloning of *Neurospora* uricase gene

To study the regulation mode of uricase induction, the uricase gene was cloned from a *Neurospora* cDNA bank in lambda gt11 recombinant phage. Lambda gt11 is an expression vector which can express a cloned sequence as a fused protein with B-galactosidase under the control of the lac promoter. The fused protein of desired cDNA clones has uricase sequences, and can be detected (on a blotted filter) by an immunological method. In a primary screening of lambda gt11 cDNA bank, 20 positive plaques, numbered 1 to 20, were isolated from 500,000 recombinant phage (Fig. 10A). The positive plaques were picked and screened again. Among the original 20 positive plaques of recombinant phage, 9 showed a positive signal in the successive screening procedure as shown in Fig. 10B as an example. These positive plaques were picked and further screened until all the plaques gave positive signals as shown in Fig. 10C. Four of 9 isolated recombinant phage were further examined to confirm that they indeed contained uricase cDNA sequences.

Confirmation of Uricase cDNA Clones

The isolated recombinant phage which gave a positive signal in the screening procedure were further examined
Figure 10. Screening for Uricase Gene in a *Neurospora* cDNA bank. The uricase gene was cloned from a *Neurospora* cDNA bank in lambda gt11 by an immunoblotting method. 20 positive plaques were isolated in the primary screening (A), from which 4 uricase-positive recombinant phages were isolated by successive screening procedure (B) until all plaques gave a positive signal (C). Recombinant phage #2, 11, 17, and 18 were isolated.
to demonstrate the presence of uricase cDNA sequences. This was essential to eliminate possible false signals caused by cross-reacting proteins or the presence of antibodies other than anti-uricase in the immune serum. Four different positive recombinant phage (No. 2, 11, 17, 18) were grown on a lawn of E. coli Y1090 and their fusion proteins were expressed by induction with IPTG. Proteins from lysed cells, including the fusion protein, were bound to nitrocellulose paper and reacted with the anti-uricase antibody solution. After washing thoroughly the bound antibody on each filter was eluted. The eluted antibody was reacted with standard uricase on a Western blot and visualized with the same method as in screening procedure. As shown in Fig.11, without antibody (lane a) and with antibody eluted from the filter containing proteins from lambda gt11 control phage (lane f), the uricase band on a Western blot does not show any positive signal. However, with antibody eluted from the filter containing the putative fusion protein from No.2 (lane b) and from No. 18 (lane e) recombinant phage, the uricase band on the Western blot shows a positive signal. On the other hand, uricase was not visualized with the antibody eluted from filter containing proteins from No.11 or No.17 recombinant phage. These results showed that antibody which bound
Figure 11. Confirmation of Cloned Gene by Western Blot.

Cloned recombinant phage were grown on a lawn of \textit{E. coli} Y1090 at 42 C. After 2 hrs, nitrocellulose filters saturated with 10 mM IPTG were layered onto each plate and incubated overnight. The filters were reacted with antibody, washed and then any bound antibody was eluted with glycine-HCl buffer (pH 2.5). The eluted antibody was used to react standard uricase in a Western blot.

a) no antibody
b) antibody eluted from phage recombinant No.2
c) No.11  d) No.17  e) No.18  f) lambda gt11
to No.2 and 18 recombinant phage lysate could recognize native uricase. Thus, it appears that a fusion protein is expressed by these recombinants which interacts with anti-uricase antibody. The most direct explanation of these results is that the cloned cDNA in recombinant phage No.2 and 11 comprises at least a part of the uricase gene.

Expression of Fusion Proteins in *E. coli* Lysogen

To compare the pattern of proteins synthesized in vivo in lysogens which harbor recombinant phage, *E. coli* Y1089 lysogens were prepared by infecting this strain with No. 2, and No.11 recombinant phage and with lambda gt11 vector. Each lysogen was grown and expression of the fusion gene was induced. The lysogens were then lysed and each lysate was run on SDS-PAGE. As shown in Fig. 12, when the lysogens were induced with IPTG, No.2 (lane a) and No.18 lysogens (lane b) each produced a distinct protein which was not produced in the absence of the inducer (lane c and d). Furthermore, the size of the induced protein from the two lysogens which harbored recombinant phage was larger than that of B-galactosidase induced in the control lambda gt11 lysogen (lane e). These results showed that the recombinant phage express the cloned cDNA as a fusion protein, which
Figure 12. Fused proteins from *E. coli* Lysogen Lysates. *E. coli* lysogens were prepared by infecting cloned phage recombinants into *E. coli* Y1089. The lysogens were grown and potential fused proteins were induced with IPTG. After harvesting the cells, *E. coli* lysates were run on 10 % SDS-PAGE. The arrow on the left indicates the distinct band when the lysogen is induced. The arrow on the right indicates the position of authentic β-galactosidase.

a) lysate from No.2 induced lysogen
b) lysate from NO.18 induced lysogen
c) lysate from No.2 uninduced lysogen
d) lysate from No.18 uninduced lysogen
e) lysate from induced lambda gt11 lysogen
is larger than native β-galactosidase as produced in the lambda gt11 lysogen.

Subcloning of the *Neurospora* DNA Insert

To amplify the *Neurospora* cDNA inserts and to facilitate their manipulation, the inserts were isolated and subcloned into the pUC8 vector. The phage DNA was isolated, digested with EcoR I restriction enzyme and run on 4% Nusieve agarose gel electrophoresis. Figure 13 shows that the recombinant phage No.2 (lane a) and No.18 (lane b) had DNA inserts at the Eco R1 site. As expected, the lambda gt11 phage DNA did not have a DNA insert at its Eco.R1 site (lane c). The size of the DNA insert was about 300 bp in each of the lambda recombinants. These DNA fragments were eluted from the gel and inserted into the Eco RI site of pUC8 plasmid. After ligation of pUC8 plasmid linearized with Eco.R1 and the DNA insert, the reaction mixture was used for the transformation of *E. coli* JM83. Transformants which grew as white colonies in LB medium containing ampicillin and X-gal were isolated; two single colonies for each DNA insert were grown. The new plasmids, pNU23, pNU24, which have DNA inserts from No.2 recombinant phage, and pNU183, and pNU184, from No.18 recombinant DNA were isolated. These plasmids were
Figure 13. Inserts from the cloned recombinant phage DNA DNA was isolated from the recombinant phage, and digested with Eco.RI restriction enzyme. The DNAs were run on 4% NuSieve agarose overnight. The arrow indicates the insert in the recombinant phage DNAs. The left lane shows the size marker DNA.

a) recombinant phage No.2
b) recombinant phage No.18
c) lambda gt11
digested with EcoR I and Pst I restriction enzymes, and run on 1.2 % agarose gel electrophoresis. As shown in Fig. 14, when restricted with Eco.RI, each of these plasmids yielded linear pUC8 DNA and a second band of the same size of the DNA inserts in the recombinant phage DNA (lane b, c, e, g and i), whereas pUC8 plasmid did not show any insert (lane a). When these plasmids were digested with Pst I restriction enzyme, all of the new plasmids showed a single DNA fragment (lane d, f, h, j) clearly larger than pUC8 plasmid (lane b). One of the plasmids, pNU183, was larger than the other plasmids (lane h). This plasmid was found to have a dimer of cDNA inserts with partial digestion with Eco.RI restriction enzyme. The new plasmids have inserts derived from the cloned cDNA in each recombinant phage DNA and these represent a successful subcloning of the uricase cDNA.
Figure 14. Digestion of plasmids with Eco RI and Pst I restriction enzymes. DNA inserts from recombinant phage were subcloned into pUC8 plasmid. The new plasmids with inserts, pNU23 (lane c,d), pNU24 (lane e,f), pNU183 (lane g,h) and pNU184 (lane i,j) were digested with EcoRI (lane a, c, e, g, i) and Pst I restriction enzyme (lane b, d, f, h, j) and compared with pUC8 (a,b). The DNA was run on a 1.2% agarose gel overnight.
DISCUSSION

Uricase, an enzyme of purine metabolism, is synthesized and maintained at a basal level even when primary nitrogen sources as ammonia are available. However, under conditions where primary nitrogen sources such as ammonia and glutamine are not available, uric acid can be utilized as the sole nitrogen source, and enzymes required for the utilization of purines are induced. The induction of uricase required the presence of uric acid as an inducer as well as the absence of primary nitrogen sources which are known to exert catabolite repression. A functional nit-2 gene, the major nitrogen regulatory gene, is also required to derepress the expression of the purine catabolic genes. As an induction parameter, specific uricase activities were compared in cells grown in minimal and induction media. The specific activity began to increase after induction for 2 hrs and increased about 4 fold after induction for 8 hrs. The nature of the 2 hrs time lag after induction before the specific activity of uricase began to increase is not clear. However, it is suggested that this lag represents the time required for various combined events such as the recognition of the induction signal, initiation of new transcription, processing and
transport of mRNA, uricase biosynthesis, and its translocation and accumulation in the peroxisome.

In order to understand the regulation of uricase expression at the molecular level, uricase was isolated by conventional and immunoaffinity chromatography methods. In the conventional method, ammonium sulfate fractionation, anion exchange chromatography, and gel filtration techniques were used. By this method, uricase was purified about 2000-fold with 16% recovery. This preparation was used to raise antibodies against uricase in rabbits. After isolating the immunoglobulin G fraction from the immune serum with Affigel-Blue, it was bound to Affigel-10. This antibody-bound matrix was used for the subsequent isolation of uricase. Immunoaffinity column chromatography was very simple and effective in the purification of uricase, and the column was very stable for at least 6 months at 4°C. Uricase isolated by immunoaffinity chromatography showed one band in PAGE and the degree of purification in terms of specific activity was similar to that obtained by the conventional method. Moreover, the recovery of uricase activity was about 40%, which is much higher than that achieved in the conventional method.

To investigate the relation between uricase activity and its synthesis, proteins were labeled in
vivo, crude extracts prepared, and uricase was immunoprecipitated. The results indicated that uricase synthesized under induction conditions is precipitated by the anti-uricase antibody and has the same mobility in SDS-PAGE as the enzyme present in uninduced cells. It was also clear that the increase in uricase activity following induction is due to a parallel increase in the de novo synthesis of uricase. This result ruled out the possibility that the increase in uricase activity was due to activation of a precursor already present in the cells. Therefore, it can be concluded that uricase produced in induction conditions is identical to uricase produced at a basal level in repressed, uninduced conditions, and that the increase of uricase activity upon induction results from de novo synthesis of uricase.

In previous work, when lomofungin which blocks RNA synthesis, was added to cells up to 1.5 hrs after induction, uricase activity did not increase. However, cells treated with lomofungin 2 hrs after induction showed an increase in uricase activity. Therefore, the induction of uricase in Neurospora crassa was proposed to be regulated at the transcriptional level, although the detailed mechanism was not clear (Wang and Marzluf, 1979). In order to study the mode of regulation in the
induction of uricase, the possible relationship between uricase activity increase and the uricase mRNA level was examined by in vitro translation of poly(A) RNAs obtained from cells incubated under uninduced and induced conditions. The level of uricase translated in vitro with poly(A) RNA from induced cells did not increase as observed during in vivo synthesis of uricase. The result indicated that the level of uricase mRNA, as determined by its translation in the rabbit reticulocyte cell-free system, did not increase after induction, although uricase activity does increase several fold in vivo after induction. In in vitro translation of some proteins which are translocated across a membrane barrier, two factors are known to affect translation in cell-free systems (Walter et al., 1984). A translational arrest may occur due to the absence of signal recognition particles or due to the absence of a suitable receiver membrane, or both. During the synthesis of precursor proteins containing a topogenic sequence, their synthesis are known to be arrested when the signal leader sequences are translated in the absence of proper signal recognition particles and receiver membranes. Only in the presence of specific interactions between the leader, the signal recognition particles and a membrane, does the synthesis of the rest
of the amino acid sequence continue. The second factor is the possible presence of free signal peptidase which could cleave the signal leader sequence during translation in the cell-free system. If the signal leader sequence were removed by the signal peptidase, the larger precursor molecule could not be detected in the translation system. In order to examine these possible translational problems, poly(A) RNAs were translated in vitro in the presence of dog pancreatic microsomal membranes which are known to serve as receiver membranes for secreted proteins, and possess a signal peptidase activity which can cleave the signal leader sequence. No differences were detected in the mobility of uricase synthesized in vitro from poly(A) RNA from induced or noninduced cells in the presence of microsomal membranes during or after translation. The proper processing of lactogen served as a positive control that demonstrated that the microsomal membranes were active. Moreover, the translation of lactogen in the absence of microsomal membranes as a larger precursor ruled out the possibility of the presence of signal peptidase in the cell-free translation system. Translocation of uricase into peroxisome could not be examined in this experiment because of difficulty in the preparation of active receiver membranes from
peroxisomes. However, it can be concluded that the cleavage of a signal sequence is not required for the translocation of uricase. The fact that no difference was observed in the level of translation of poly(A) RNA with or without microsomal membranes indicates that no translational arrest occurred because of signal recognition particles, although a possible interaction of uricase with *Neurospora* peroxisomal specific signal recognition particles in the receiver membrane is not still excluded. However, these results rule out the possibility of synthesis of uricase as a larger precursor protein, and demonstrate the absence of co- or post-translational processing of this enzyme. These results are in agreement with the results from the *in vitro* translation of uricase in rat liver (Goldman and Blobel, 1978).

One possible interpretation of the results is that induction leads to the synthesis of a different form of uricase mRNA that encodes the same uricase as found in noninduced cells, but that this new mRNA has a reduced efficiency for translation, at least in the rabbit reticulocyte system. The requirement for the nit-2 regulatory protein which is known to be a positive regulator and for uric acid as an inducer suggests a different mechanism for the controlled synthesis of
uricase. In *Aspergillus nidulans*, the *uavY* gene, identified by genetic studies, mediates the induction of enzymes in purine degradation (Scanzocchio et al., 1981). Although that type of pathway specific regulatory gene has not been identified in Neurospora, the transcription of uricase mRNA may in fact require the product of a purine degradation regulatory gene even during the basal level of expression. And under the condition where the *nit-2* regulatory gene protein is active, possibly in the absence of glutamine, it may bind at a recognition site adjacent to the uricase gene and direct RNA polymerase to initiate transcription from a new promoter. The transcript may have a different regulatory region which permits efficient translation in induced Neurospora cells but which has the same coding sequence as does the mRNA produced during the repressed condition. This kind of regulation is observed in the synthesis of repressor in lambda bacteriophage (Jones et al., 1979). After infection of *E. coli* by lambda phage, lambda repressor protein is synthesized rapidly and competes with other lambda-coded proteins, especially cro protein, the outcome determining the fate of lambda phage in *E. coli*. At the early time, repressor protein is translated from a mRNA transcribed from promoter PE, in the presence of cII and cIII protein.
The transcript contains a Shine-Delgarno sequence which is required for the efficient translation of mRNA. However, in the synthesis of repressor to maintain the lysogenic state, repressor protein is translated with a mRNA transcribed from a different promoter Pr, and which lacks a Shine-Delgarno sequence. During lysogeny, the synthesis of repressor occurs much slower than it does immediately after infection. The regulation of repressor synthesis is controlled by the different transcripts with different translation efficiencies which are initiated from two promoters, although both translation products, the lambda repressor, are identical.

In an effort to study the regulation of uricase gene expression, the uricase gene was cloned from a lambda gt11 cDNA bank of Neurospora by an immunological method. The experiment to confirm the cloned gene by in vitro translation of hybrid selected mRNA failed due to the difficulties in the isolation of any translatable mRNA after hybridization. Therefore, each cloned recombinant phage was confirmed to contain a uricase cDNA sequence using an immunological method. Purified antibody was selected with the fusion protein product from the recombinant phage and shown to specifically react with native uricase. The lysogen which contained the cloned recombinant phage, expressed the foreign gene as a fused
protein with \textit{B}-galactosidase under the control of \textit{lac} operon. The \textit{Neurospora} DNA inserts from the cloned recombinant phage were isolated, and subcloned into pUC8 plasmid, and a series of new plasmids were prepared. The uricase cDNA is the only structural gene of the nitrogen control circuit which has been cloned. Thus the work reported here represents a major step in elucidating not only the control of the uricase gene but should reveal definitive information about the entire nitrogen regulatory system in \textit{Neurospora}. Recently, a \textit{nit-2} amber nonsense mutant, suppressible by Ssu-1, has been obtained. This mutant strongly implies that the \textit{nit-2} gene indeed specifies a regulatory protein (Marzluf et al., 1985; Perrine and Marzluf, 1986). Moreover, with the cloned \textit{nit-2} gene which is now available, it will be possible to address very specific questions about the interaction of the \textit{nit-2} protein with the uricase gene. The uricase cDNA clone will be extremely valuable to use in probing a genomic library in order to isolate the complete uricase structural gene, along with its 5' and 3' flanking DNA sequences. The isolated structural gene will allow a precise determination of control regions which serve as recognition sites for binding of the \textit{nit-2} protein and perhaps a second protein that mediates uric acid induction of the purine catabolic genes.
Numerous mutants are available for several genes which encode purine metabolic enzymes, e.g. xanthine dehydrogenase, allantoinase and allantoicase. However, no uricase mutants are available nor is it even known what chromosome the uricase structural gene is situated upon. The uricase cloned cDNA will allow a reverse genetic approach to obtain uricase gene mutants, by directed gene disruption via transformation (Paietta and Marzluf, 1985). In this gene disruption, the exact function of the uricase gene can be determined. New plasmids will be used as a probe to establish the mode of regulation of the uricase gene expression in Neurospora crassa.
In an effort to understand the mode of regulation of uricase gene expression in *Neurospora crassa*, the enzyme, uricase, was purified and used to obtain a specific antibody. The antibody was purified and employed for immunoprecipitation of uricase synthesized *in vivo* and *in vitro*. Possible posttranslation processing of uricase was examined, but was not found to occur. Finally, uricase cDNA clones were isolated and their identity confirmed by immunological methods. The cDNA sequence was subcloned to develop a series of new plamids. The results of this study lead to the following conclusions:

1) The increase in uricase activity upon induction results from *de novo* synthesis of uricase.

2) Uricase produced in control and in induction conditions is immunoprecipitated by same antibody preparation and has the same mobility in SDS-PAGE, suggesting that the same form of uricase is synthesized under both conditions.

3) *In vitro* synthesized uricase was not processed in the presence of dog pancreatic microsomal membranes and has the same size as that synthesized *in vivo*. This suggests that a putative topogenic sequence of uricase
is not cleaved during translocation.

4) The level of functional uricase mRNA did not show an expected increase (assayed by in vitro translation) in cells following induction as observed in the in vivo studies. This result suggests the possibility that during induction another uricase mRNA is synthesized and that it may not be efficiently recognized by the heterologous cell-free translation system.

5) A portion of the uricase gene was cloned from a lambda gt11 cDNA Neurospora bank and its identity confirmed with a Western blot - immunological method.

6) The cloned recombinant phage DNAs have about a 300 bp cDNA insert. These cDNA inserts have been subcloned into plasmid pUC8.
LIST OF REFERENCES


