UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO
Facultad de Química



# REGULACION DEL NIVEL DE ARN-MENSAJERO ESPECIFICO PARA LA GLUTAMINO SINTETASA EN CONIDIAS DE NEUROSPORA crassa.

TESIS que para obtener el título de Doctor en Ciencias Químicas, área Bioquímica, presenta:



WILHELM HANSBERG TORRES



México, D.F. 1981

# I N T R O D U C C I O N

La glutamino sintetasa (EC 6.3.1.2.) es una enzima clave del metabolismo nitrogenado. El tema ha sido revisado recientemente en el libro titulado: Glutamine: Metabolism, Enzymology, and Regulation (Mora, J. and Palacios, R., eds.) Academic Press, New York. La regulación de la glutamino sintetasa (GS) ha sido estudiada en varios organismos, tanto procariontes (Stadtman and Chock, 1978; Wohlhueter et al., 1973; Foor et al., 1975) como en eucariontes sencillos (Pateman, 1969; Sims et al., 1974; Kapoor and Bray, 1968) y organismos superiores (O'Neal an Joy, -- 1975; Rhodes et al., 1976; Tate et al., 1972; Kulka et al., 1972, Milman et al., 1975; Moscona et al., 1972; Soh and Sarkar, 1978). Para el catabolismo de compuestos nitrogenados en bacterias se ha propuesto un modelo de regulación en el cual la GS juega un papel central (Margasanik, 1977, Pahel and Tyler, 1979; Kustu et al., 1979).

En los últimos cinco o seis años un grupo de investigadores del país hemos emprendido el estudio de la glutamino sintetasa en Neurospora crassa. Este estudio ha abarcado múltiples aspectos: el genético (Dávila et al., 1978), la purificación y estructura de la GS (Palacios, 1976), su caracterización inmunológica (Palacios et al., 1977; Sánchez et al., 1979) su estructura monomérica (Dávila et al., 1980, Sánchez et al., 1980), la

traducción del ARN mensajero específico (Palacios et al., 1977; Sánchez et al., 1978; Hansberg et al., 1979; Sánchez et al., 1980), la estructura oligomérica relacionada a la función (Vichido et al., 1978;, Mora et al; 1980), su síntesis y degradación (Quinto et al., 1977), y su regulación metabólica (Limón et al,, 1977; Espín et al., 1979).

Nuestro grupo de investigación ha estado principalmente involucrado en estudiar la participación de esta enzima durante los procesos morfogenéticos del ciclo de vida vegetativo de Neurospora crassa y hemos colaborado en la medición de los niveles del ácido ribonucléico mensajero (ARNm) específico de GS en dos distintas estructuras morfológicas, la conidia en condiciones de no crecimiento y en el micelio vegetativo en crecimiento exponencial. En ambos tipos celulares, la fuente de nitrógeno regula la actividad de la GS a nivel de su síntesis específica (Quinto et al., 1977; Espín et al., 1979). Debido a ello hemos determinado los niveles del ARNm de la GS en la conidias (Hansberg et al., 1979) y en el micelio vegetativo (Sánchez et al., 1978), incubados en distintas fuentes de nitrógeno.

Para el caso de las conidias, se encontró que Neurospora crassa contiene una cantidad importante de ARN mensajeros preempacados en las conidias que se evidencían al ser traducidos en un gran número de proteínas en dos distintos sistemas ---

de síntesis de proteínas "libres de células". En la población de ARNm de conidias sin incubar no se detectó, por inmunoprecipitación específica, actividad de ARNm para la GS. En cambio, cuando las conidias se incubaron durante varias horas en distintos medios, se detectó actividad del ARNm para GS, principalmente en la fracción de ARNm poli(A)$^+$. La cantidad de GS sintetizada con ARNm de conidias incubadas en distintos medios correlaciona con la actividad específica de la GS en las células, que a la vez depende de la fuente de nitrógeno presente en el medio de incubación. Debido a estos resultados proponemos que las conidias de Neurospora crassa regulan su actividad de GS a nivel de la transcripción del ARNm específico.

En el micelio vegetativo en crecimiento exponencial, la síntesis de GS parece también estar regulada por los niveles del ARNm específico: el ARNm de cultivos crecidos en glutamato como única fuente de nitrógeno sintetizó mayor cantidad de GS en el sistema de síntesis protéica "in vitro" que el ARNm de cultivos crecidos en glutamina como única fuente de nitrógeno. La diferencia fué de diez veces cuando se utilizó para la traducción ARN polisomal y de cinco veces cuando se utilizó ARN total o enriquecido en poli(A).

En conclusión, la regulación de la fuente de nitrógeno sobre la síntesis de la GS en Neurospora crassa parece estar dada por los niveles del ARNm específico para dicha enzima.

# B I B L I O G R A F I A


Dávila, G., Sánchez, F., Palacios, R., and Mora,J. (1978) J. Bacteriol. 134, 693-698.

Dávila, G., Lara, M., Guzmán, J., and Mora, J. (1980) Biochem. Biophys. Res. Comm. 92, 134-140.

Espín, G., Palacios, R., and Mora, J. (1979) J. Gen. Microbiol. 115, 59-68.

Foor, F., Jansen, J.A., Magasanik, K.B. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 4844-4848.

Hansberg, W., Espín, G., Palacios, R., and Sánchez, F., (1979) Develop. Biol. 73, 68-75.

Kapoor, M., and Bray, D. (1968) Biochemistsy 7, 3583-3589.

Kulka, R.G., Tomkins, G.M., and Crook, R.B. (1972) J. Cell Biol. 54, 175-178.

Kustu, S., Burton, D., García, E., McCarter, L., and McFarland, N. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4576-4580

Limón Lason, J., Lara, M., Resendiz, B., and Mora, J. (1977) Biochem. Biophys. Res. Comm. 78, 1234-1240.

Magasanik, B. (1977) Trends Biochem. Sci. 2, 9-12.

Mora, Y., Chávez, O., and Mora, J., (1980) J. Gen. Microbiol. 118, 455-463.

Moscona, M., Frenkel, N., and Moscona, A.A. (1972) Develop. Biol. 28, 229-241.

Milman, G., Portnoff, L.S., and Tiermeier, D.C. (1975) J. Biol. Chem. 250, 1393-1399.

O'Neal, D., and Joy, K.W. (1975) Plant Physiol. 55, 968-974.

Pahel, G., and Tyler, B. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4544-4558.

Palacios, R. (1976) J. Biol. Chem. 251, 4787-4791.

Palacios, R., Campomanes, M., and Quinto, C., (1977) J. Biol. Che. 252, 3028-3034.

Pateman, J.A. (1969) Biochem. J. 115, 769-775

Quinto, C., Mora, J., and Palacios, R. (1977) J. Biol. Chem. 252, 8724-8727.

Rhodes, D., Rendon, G.A., and Stewart, G.R. (1976) Planta 129, 203-210.

Sánchez F., Campomanes, M., Quinto, C., Hansberg, W., Mora, J. and Palacios, R. (1978) J. Bacteriol. 136, 880-885.

Sánchez F., Dávila, G., Mora J. and Palacios, R. (1979) J. Bacteriol. 139, 537-543.

Sánchez F., Calva, E., Campomanes, M., Blanco, L., Guzmán, J., Saborío, J.L., and Palacios (1980) J.Biol. Chem. 225, 2231-2234.

Sims, A.P., Toone, J., and Box, V. (1974)) J. Gen Microbiol. 84, 149-162.

Soh, B.M., and Sarkar, P.K. (1978) Develop. Biol. 64, 316-328.

Stadtman, E.R. and Chock, P.B. (1978) in Current Topics in Cellular Regulation (Horecker, B.L., and Stadtman, E.R. - eds.) Vol. 13, pp. 53-95, Academic Press, New York.

Tate, S.S., Leu, F.Y., and Meister, A. (1972) J. Bio. Chem. 247, 5312-5321.

Vichido, I., Mora, Y., Quinto, C., Palacios R., and Mora, J. (1978) J. Gen. Microbiol. 106, 251-259.

Wohlhueter, R.M., Schutt, H., Holzer, N. (1973) in The Enzymes of Glutamine Metabolism (Prusiner, S. and Stadtman, E.R., eds.) pp. 45-64, Academic Press, New York.

DEVELOPMENTAL BIOLOGY 73, 68–75 (1979)

# Regulation of Glutamine Synthetase Messenger Ribonucleic Acid in Conidia of *Neurospora Crassa*

WILHELM HANSBERG,[1] GUADALUPE ESPÍN,[2] RAFAEL PALACIOS, AND FEDERICO SÁNCHEZ[3]

*Departamento de Biología Molecular, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México 20, D.F., México*

*Received January 25, 1979; accepted in revised form May 9, 1979*

A large quantity of prepackaged mRNA is present in conidia of *Neurospora crassa* as evidenced by its capacity to direct the synthesis of proteins in two heterologous cell-free systems. With this mRNA no *in vitro* synthesis of glutamine synthetase could be detected by specific immunoprecipitation. Upon incubation of the conidia under nongrowing conditions, the specific mRNA for glutamine synthetase was evidenced in the polyadenylated RNA and to a lesser degree in the nonpolyadenylated RNA. The amount of specific mRNA correlated well with the specific activity of glutamine synthetase in the conidia, which was dependent on the nitrogen source in the incubation medium. Based on these results we propose that conidia of *N. crassa* regulate its glutamine synthetase activity at the transcriptional level.

## INTRODUCTION

Preexisting mRNA in conidia of *Neurospora crassa* is suggested by the increase in polysomes during the first 10 min of conidial hydration (Mirkes, 1974) and is also thought to be responsible for RNA competition in hybridization experiments (Bhagwat and Mahadevan, 1970) and for germination in the absence of RNA synthesis (Inoue and Ishikawa, 1970). We now present direct evidence for the existence of active mRNA stored in the conidia: Polyadenylated RNA from unincubated spores is capable of directing the synthesis of a variety of proteins in two heterologous cells free systems.

*N. crassa* conidia have an active nitrogen metabolism when incubated under nongrowing conditions as evidenced by the synthesis and accumulation of glutamine and arginine (Espín and Mora, 1978) and glutamine synthetase (GS) *de novo* synthesis which is dependent on the nitrogen source supplied in the medium (Espín *et al.*, 1979). The question then arises as to how the conidia of *N. crassa* regulate GS synthesis: Is it by adjusting the level of its specific mRNA, or is specific mRNA prepackaged and translated according to enzyme demand? Our results indicate that GS mRNA, seemingly absent in the unincubated conidia, appears during incubation of the spores in amounts depending on the nitrogen source and in direct proportion to the GS specific activity.

## MATERIALS AND METHODS

*Organisms and culture conditions.* Wild-type 74A and proline auxotroph *prol-3A* strains came from the Fungal Genetics Stock Center at Humboldt State University Foundation, Arcata, California. They were grown on slants with minimal medium N (MM) of Vogel (1954) supplemented with 1.5% (w/v) sucrose during 3 days in the dark at 29°C followed by 2 days in the light at 25°C. For the *prol-3A* slants, 100 μg/ml

[1] To whom correspondence should be addressed.
[2] Present address: Agricultural Research Council, Unit of Nitrogen Fixation, The University of Sussex, Brighton, Sussex, BN 19 RQ England.
[3] University of California, San Francisco, Department of Biochemistry and Biophysics, San Francisco, California 94143.



0012-1606/79/110068-08$02.00/0

proline was added to the medium.

Conidia were harvested with distilled water, filtered through glass wool, and incubated 1 hr in MM-sucrose without a nitrogen source or with 5 m*M* glutamine or 1 m*M* cysteine. After incubation, spores were filtered through Whatman No. 41 paper and dehydrated with acetone. When no incubation was carried out, conidia were collected with ice-cold water and immediately filtered and dehydrated.

*RNA extractions.* Poly(A)$^+$ RNA was extracted according to a method developed in our laboratory which, instead of eliminating proteins with solvents, uses protein denaturation with sodium dodecyl sulfate (SDS), high salt, and mercaptoethanol; extracts were passed directly through oligo(dT)cellulose columns. A comparison of this method with two other reported methods will be published elsewhere. Briefly, 1 g of conidial acetone powders was ground with dry ice, resuspended, and homogenized in 10 ml cold buffer containing 10 m*M* Tris–HCl, pH 7.4, 10 m*M* $MgCl_2$, 500 m*M* NaCl, 1 *M* 2-mercaptoethanol, 0.5% SDS, 0.5% sodium deoxycholate, and 0.005% dextran sulfate. The homogenates were incubated 10 min at 30°C and centrifuged 5 min at 1500*g*. Each pellet was washed twice with T Na-SDS (10 m*M* Tris–HCl, pH 7.4, 500 m*M* NaCl, 0.5% SDS), and the three supernatants were pooled and stored overnight at −20°C. After thawing, the suspension was centrifuged 10 min at 12,000*g* and the supernatant was passed through a column of oligo(dT)cellulose (Type 7, P-L Biochemicals, Inc.), previously equilibrated with TNa-SDS buffer at room temperature. The column was washed exhaustively with TNa-SDS solution until no absorbance at 230 nm in the flowthrough could be detected. The poly(A)$^+$ RNA was eluted with T-SDS buffer (10 m*M* Tris–HCl, pH 7.4, 0.5% SDS), adjusted to 150 m*M* sodium acetate, and precipitated overnight at −20°C with 2 vol of ethanol.

The poly(A)$^-$ RNA was extracted from the supernatant that did not adsorb to the oligo(dT)cellulose column. It was precipitated overnight at −20°C with 2 vol of ethanol, resuspended in 20 ml of a buffer containing 100 m*M* Tris, pH 9, 100 m*M* NaCl, 10 m*M* EDTA, and 1% SDS, and extracted three times with an equal volume of phenol:chloroform:isoamylalcohol (50:50:1) and once with 1 vol of chloroform. The aqueous phase was adjusted to 150 m*M* sodium acetate and precipitated at −20°C with 2 vol of ethanol.

Poly(A)$^+$ and poly(A)$^-$ RNAs were washed three times with 2 and 10 ml, respectively, of 3 *M* sodium acetate and precipitated again with ethanol. The RNAs were collected by 30-min centrifugation at 15,000*g*, washed with cold 70% ethanol solution, and dried. The RNAs were dissolved in distilled water at concentrations of 1 μg/μl for the poly(A)$^+$ and 20 μg/μl for the poly(A)$^-$ RNA.

*Determination of glutamine synthetase activity.* Glutamine synthetase was extracted according to the method described by Vichido *et al.* (1978). Briefly, conidial acetone powders were ground with dry ice and homogenized with 10 vol of extraction buffer (5 m*M* $K_2HPO_4$, 50 m*M* $K_2SO_4$, 0.5 m*M* EDTA, pH 7.2). After centrifugation for 20 min at 12,000 *g*, the extraction procedure was repeated on the pellet. Transferase activity of GS was measured in the combined supernatants and in the pellet following the method described by Ferguson and Sims (1971). Units of enzyme activity are expressed as micromolecules of γ-glutamyl hydroxamate produced per minute at 30°C. Specific activity was obtained by calculating the total enzyme activity of the culture and dividing it by the total protein content of the culture. Protein was determined by the method of Lowry *et al* (1951).

*Protein synthesis in cell-free systems.* Wheat germ extract for the cell-free system was prepared according to Roberts and Paterson (1973). Incubation mixtures (100 μl)

contained: 20 m*M* Hepes–KOH, pH 7.6, 48 m*M* KCl, 20 m*M* potassium acetate, 2 m*M* magnesium acetate, 2 m*M* dithiothreitol, 2 m*M* 2-mercaptoethanol, 0.4 m*M* spermidine, 1 m*M* ATP, 0.2 m*M* GTP, 11 m*M* creatine phosphate, 4 μg creatine phosphokinase, 0.25 m*M* amino acid mix lacking methionine, 20–70-μCi of L-[$^{35}$S]methionine (100–400 Ci/mmole, New England Nuclear), 20 μl of wheat germ extract, and 1–15 μg of poly(A)$^+$ RNA. Incubation was carried out for 2 hr at 30°C and the reaction was stopped by cooling at 4°C and adding 0.2 vol of a solution containing 10 m*M* Tris–HCl, pH 7.2, 100 m*M* methionine, 720 m*M* NaCl, 5% sodium desoxycholate, and 5% Triton X-100. Aliquots of 5 μl were applied to Whatman GF/C filters and precipitated with 10% trichloroacetic acid (TCA). The filters were boiled, washed exhaustively with 5% TCA, dried, and counted in PPO-toluene (4 g/liter) in a liquid scintillation spectrometer.

The rabbit reticulocyte cell-free system was used essentially as described by Palmiter (1973) with minor modifications (Palacios *et al.*, 1977). When it was pretreated with micrococcal calcium-dependent nuclease (P-L Biochemicals, Inc.), the method of Pelham and Jackson (1976) was followed.

*Immunoprecipitation of glutamine synthetase.* Glutamine synthetase was purified by a procedure based on affinity chromatography on anthranilate-bound Sepharose (Palacios, 1976). Goat γ-globin monospecific for GS was obtained as described (Sánchez *et al.*, 1978). GS was immunoprecipitated after incubation of the cell-free system by a direct procedure using 5 μg of purified GS as a carrier and 3 mg of specific antibody (Palacios *et al.*, 1977). The immunoprecipitates were kept at −20°C until dissolved for electrophoresis.

*Electrophoresis and fluorography.* Either immunoprecipitates or 5 μl of the incubated cell-free mixtures was applied on a 1.5-mm-thick, 11% polyacrylamide–SDS gel and electrophoresed according to Laemmli (1970). Gels were stained with 0.1% Coomassie blue in 25% isopropanol–10% acetic acid and destained in 5% isopropanol–5% acetic acid. Gels were then impregnated with PPO, dried, and fluorographed as described by Bonner and Laskey (1974). GS bands of the same gels were sometimes cut off, then autoclaved 30 min in vials containing 0.2 ml of 30% $H_2O_2$, and the incorporated radioactivity was counted in 10 ml Bray solution in a liquid scintillation spectrometer.

## RESULTS

All incubations of the conidia were carried out under nongrowing conditions, using either the proline auxotroph *prol-3A* without proline or the wild-type 74A strain in MM-sucrose devoid of nitrogen. Under these conditions no increase in biomass was detected and GS synthesis was regulated by the nitrogen source present in the medium (Espín *et al.*, 1979).

*Poly(A)$^+$ RNA from unincubated conidia.* Poly(A)$^+$ RNA was extracted from unincubated conidia of 74A and *prol-3A* strains. The extraction method described in this paper allowed us to obtain around 100 μg of poly(A)$^+$ RNA per gram of conidial acetone powder. This RNA was assayed for its capacity to direct protein synthesis in two heterologous cell-free systems. Incorporation of L-[$^{35}$S]methionine into proteins using the wheat germ cell-free system challenged with poly(A)$^+$ RNA extracted from conidia of strain 74A is shown in Fig. 1A. There was a linear increase in the label incorporated into total protein with increasing amounts of poly(A)$^+$ RNA until a saturation point around 0.2 mg/ml was reached. Subsequent experiments were performed at nonsaturating RNA concentrations. The label was incorporated into a large number of proteins of different molecular weight as evidenced by SDS–polyacrylamide gel electrophoresis and fluorography (Fig. 1B). The fluorographic pattern resembled the one obtained with polysomal

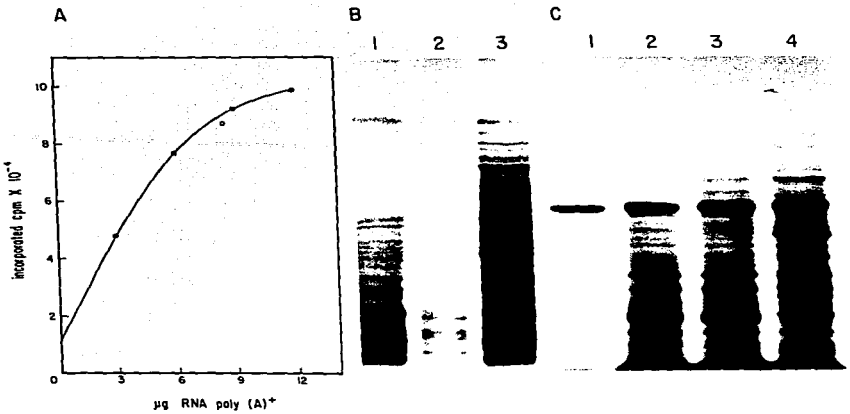


FIG. 1. Protein synthesis in the wheat germ cell-free system. (A) Incorporation of L-[$^{35}$S]methionine into total protein with increasing amounts of poly(A)$^+$ RNA from unincubated conidia (●) or from conidia incubated 1 hr in a nitrogen-free medium (○). (B) Fluorography of total labeled proteins synthesized in the wheat germ system and separated in a SDS–polyacrylamide slab gel. Proteins synthesized with poly(A)$^+$ RNA from unincubated conidia (1), without adding RNA (2), with polysomal RNA from growing mycelium (3). (C) Fluorography of total labeled proteins synthesized in the reticulocyte system (pretreated with calcium-dependent nuclease) and electrophoresed in a SDS-polyacrylamide slab gel. Without added RNA (1), with poly(A)$^+$ RNA from unincubated conidia (2), with poly(A)$^+$ RNA from a mixture of unincubated conidia and exponentially growing mycelium (3), with poly(A)$^+$ RNA from exponentially growing hyphae (4).

RNA from exponentially growing hyphae, although some qualitative and quantitative differences were detected (Fig. 1B). Similar results were obtained with the reticulocyte cell-free system pretreated with calcium-dependent nuclease and challenged with poly(A)$^+$ RNA from unincubated conidia, a mixture of unincubated conidia and exponentially growing mycelium, and exponentially growing hyphae (Fig. 1C).

*Glutamine synthetase activity.* GS specific activity of 74A and *prol-3A* conidia was very low but increased upon incubation in MM-sucrose without a nitrogen source and remained relatively low in the *prol-3A* strain when glutamine was added to the medium (Fig. 2). During incubation there was no measurable increase in biomass, although formation of a small germ tube was observed. Upon addition of cysteine to the medium, which is known to inhibit germ tube formation (Schmit and Brody, 1976), GS activity increased at the same rate as without cysteine (Fig. 2).

*Detection of GS mRNA.* In order to detect whether GS was synthesized *in vitro*, using poly(A)$^+$ RNA from unincubated conidia, immunoprecipitations with monospecific antibodies directed against purified GS were carried out. Figure 3A shows the immunoprecipitates from both the reticulocyte and the wheat germ cell-free systems, separated by electrophoresis in a SDS–polyacrylamide slab gel. Immunoprecipitation, which has been shown to be 70–80% efficient in the reticulocyte cell-free system

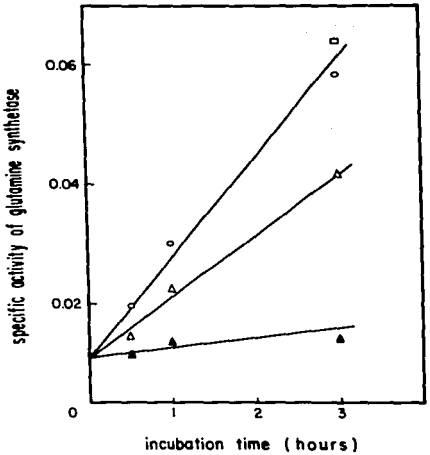


FIG. 2. Specific activity of GS during incubation of conidia. Wild-type 74A strain in MM-sucrose without nitrogen source (○——○). *Prol-3A* strain in the same medium (△——△) or supplemented with glutamine (▲——▲). Strain 74A in nitrogen-free medium with cysteine (□——□).

(Sánchez *et al.*, 1978), was comparable in both systems as judged by the amount of immunoglobulins and carrier GS that entered into the gel. As seen in the fluorography of this gel (Fig. 3B), radioactive GS was obtained with the polysomal RNA extracted from vegetative hyphae, but no detectable amount of active mRNA for GS seemed to be present in the poly(A)$^+$ RNA derived from unincubated conidia. An experiment was performed in which equal amounts of hyphae and unincubated conidia were mixed, then poly(A)$^+$ RNA was extracted as usual and assayed in the reticulocyte cell-free system pretreated with calcium-dependent nuclease (Fig. 1C). Synthesis of GS with this RNA corresponded to about half the amount of mycelium poly(A)$^+$ RNA added to the assay (Fig. 4A). This result discarded the possibility that the absence of *in vitro* GS synthesis with RNA from unincubated conidia wsas due to an inhibitory effect or to RNA degradation during conidial RNA extraction.

When the poly(A)$^+$ RNA was extracted from spores after 1 hr of incubation in a nitrogen-free medium, GS synthesis was readily detected in the cell-free system. To test if the amount of mRNA for GS was different in the conidia incubated in a nitrogen-free medium, as compared with those incubated in the presence of glutamine, poly(A)$^+$ RNA from *prol-3A* spores was isolated and assayed in a reticulocyte lysate cell-free system pretreated with calcium-dependent nuclease. Poly(A)$^+$ RNA from unincubated conidia seemed not to incorporate label into GS protein, while both RNAs from incubated conidia had label associated with GS (Fig. 4B). RNA extracted from conidia incubated in a nitrogen-free medium incorporated 2.4 times more radioactivity into GS than RNA derived from spores incubated in a glutamine-containing medium (Fig. 4B). Poly(A)$^-$ RNA from incubated conidia also showed some radioactivity associated with GS, although to a much lesser extent than poly(A)$^+$ RNA (data not shown).

## DISCUSSION

Poly(A)$^+$ RNA in the unincubated conidia efficiently directed the synthesis of a great number of proteins in the wheat germ cell-free system (Figs. 1A and B) and in the rabbit reticulocyte cell-free system pretreated with calcium-dependent nuclease (Fig. 1C). Since conidia are dormant cells, this RNA represents stored mRNA. The analysis of the proteins which are coded by these prepackaged messengers could give us information on the mechanism of conidiogenesis and the germination process. Conidia have been thought of as metabolically arrested cells formed because of water and nutrient shortage at the tip of the conidiophores, germination being the rever-

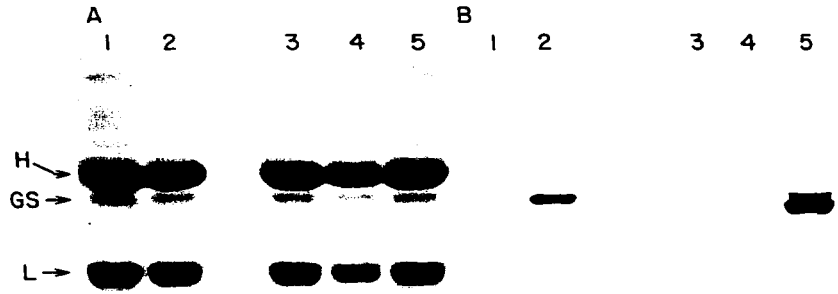


FIG. 3. Immunoprecipitation of *N. crassa* glutamine synthetase synthesized in the reticulocyte and wheat germ cell-free systems. (A) Immunoprecipitation separated by electrophoresis in a 11% SDS–polyacrylamide slab gel. Reticulocyte cell-free system challenged with 10 μg of poly(A)$^+$ RNA from unincubated conidia (1) or with 40 μg of polysomal RNA from exponentially growing vegetative hyphae (2). Wheat germ cell-free system: without RNA (3), with 10 μg of poly(A)$^+$ RNA from unincubated conidia (4), and with 40 μg polysomal RNA from exponentially growing mycelium (5). Total protein counts subjected to immunoprecipitation were $2 \times 10^6$ for each sample of wheat germ incubation mixture. (B) Fluorography of the same gel.

sal of the whole process (Mirkes, 1974). If this is so, one would expect to find in the conidia the mRNAs for proteins synthesized in the conidiophores. Messenger for different enzymes would certainly be included. On the other hand, specific mRNAs could be actively stored during conidiogenesis, and this may confer some advantages to the conidia at the moment of germination. We personally favor this last possibility, since spores, like plant seeds, pollen grains, and unfertilized eggs, contain large amounts of stored products which are used during a later developmental stage. Conidia of *N. crassa* consume the large pool of glutamic acid (2.5% of the conidial dry weight) within the first hour of germination (Schmit and Brody, 1975). Stored mRNA could be part of a "survival package" required by the conidia to rapidly activate their metabolism and adjust it to growing conditions.

Glutamine synthetase is a key enzyme in nitrogen metabolism (Stadtman, 1973). The regulation of eukaryotic GS has been studied in different biological systems, and controls at the levels of enzyme synthesis (Moscona *et al.*, 1971; Sims and Ferguson, 1974), enzyme degradation (Arad *et al.*, 1976; Sims and Ferguson, 1974), and amount of translatable GS mRNA (Sarkar and Griffith, 1976; Soh and Sarkar, 1978) have been demonstrated.

In the exponentially growing mycelium of *N. crassa*, GS activity and concentration are regulated by the nitrogen source in the medium (Vichido *et al.*, 1978). Regulation of GS by the nitrogen source is exerted at the level of enzyme synthesis (Quinto *et al.*, 1977) by adjusting the amount of active GS mRNA (Sánchez *et al.*, 1978).

As first found by Espín *et al.* (1979), the nitrogen source in the medium regulates GS synthesis in the conidia incubated un-

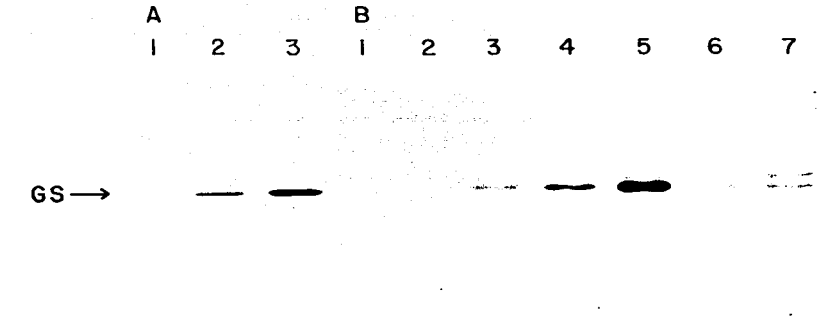


FIG. 4. Fluorography of the immunoprecipitated GS synthesized in the reticulocyte cell-free system pretreated with calcium-dependent nuclease and electrophoresed in a SDS–polyacrylamide slab gel. (A) Mixing experiment with: poly(A)$^+$ RNA from unincubated conidia (1), poly(A)$^+$ RNA from a mixture of unincubated conidia and exponentially growing mycelium (2), and poly(A)$^+$ RNA from exponentially growing hyphae (3). Total protein counts subjected to immunoprecipitation were $0.5 \times 10^6$ for each sample. (B) GS synthesis during incubation of the conidia with different nitrogen sources: without RNA (1), with poly(A)$^+$ RNA from unincubated conidia ($0.2 \times 10^6$ counts incorporated into total protein) (2), with poly(A)$^+$ RNA from conidia incubated for 1 hr in a nitrogen-free medium (3, 4, and 5) ($0.5 \times 10^6$, $1 \times 10^6$, and $2 \times 10^6$ counts, respectively), and with poly(A)$^+$ RNA from conidia incubated for 1 hr in a medium with glutamine as the sole nitrogen source (6 and 7) ($1 \times 10^6$ and $0.5 \times 10^6$ counts, respectively).

der nongrowing conditions. Enzyme activity appears to be regulated even in the absence of germ tube formation, since the addition of cysteine to the medium did not affect the GS activity (Fig. 2), while it prevented the emergence of the germ tube (Schmit and Brody, 1976). Right from the beginning of incubation there was a great increase in GS activity in the absence of a nitrogen source, while the addition of glutamine prevented this increase (Fig. 2). The GS-specific mRNA seemed to be absent in the unincubated conidia (Figs. 3 and 4). RNA extracted from a mixture of exponentially growing mycelium and unincubated conidia gave an intermediate GS synthesis value (Fig. 4A), excluding RNA degradation during conidial RNA extraction or an inhibitor effect in the translation assay.

The GS-specific mRNA appeared during germination in parallel to the enzyme-specific activity. After 1 hr of incubation, conidia had two to three times more GS mRNA in a medium devoid of nitrogen than in a medium with glutamine as the sole nitrogen source (Fig. 4B). We conclude from these experiments that conidia of *N. crassa* regulate GS-specific activity by adjusting the amount of active mRNA for this enzyme. This regulation is probably exerted at the transcriptional level, although regulation during the processing of the GS-specific mRNA could be an alternative. This last possibility could best be tested by detection of GS-specific RNA sequences with a specific DNA probe.

The authors are indebted to Dr. Jaime Mora for encouragement and criticism throughout this work. We also would like to thank Dr. Fernando Bastarrachea and Dr. Carlos Larralde for critically reading the

manuscript. This research was partially supported by CONACYT (Consejo Nacional de Ciencia y Tecnología, México).

## REFERENCES


ARAD, G., FREIKOPF, A., and KULKA, R. G. (1976). Glutamine stimulated modification and degradation of glutamine synthetase in hepatoma tissue culture cells. *Cell* **8**, 95–101.

BHAGWAT, A., S., and MAHADEVAN, P. R. (1970). Conserved mRNA from the conidia of *Neurospora crassa*. *Mol. Gen. Genetics* **109**, 142–151.

BONNER, W. M., and LASKEY, R. A. (1974). A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**, 83–88.

ESPÍN, G., and MORA, J. (1978). Effect of the deprivation of amino acids on conidia of *Neurospora crassa*. *J. Gen. Microbiol.* **104**, 233–240.

ESPÍN, G., PALACIOS, R., and MORA, J. (1979). Glutamine metabolism in conidia from *Neurospora crassa*. *J. Gen. Microbiol.* (in press).

FERGUSON, A. R., and SIMS, A. P. (1971). Inactivation *in vivo* of glutamine synthetase and NAD-specific glutamate dehydrogenase: Its role in the regulation of glutamine synthesis in yeasts. *J. Gen. Microbiol.* **69**, 423–427.

INOUE, H., and ISHIKAWA, T. (1970). Macromolecule synthesis and germination of conidia in temperature-sensitive mutants of *Neurospora crassa*. *Jap. J. Genet.* **45**, 357–369.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.

MIRKES, P. E. (1974). Polysomes, ribonucleic acid, and protein synthesis during germination of *Neurospora crassa* conidia. *J. Bacteriol.* **117**, 196–202.

MOSCONA, M., FRENKEL, N., and MOSCONA, A. A. (1972). Regulatory mechanisms in the induction of glutamine synthetase in embryonic retina: Immunochemical studies. *Develop. Biol.* **28**, 229–241.

PALACIOS, R. (1976). *Neurospora crassa* glutamine synthetase: Purification by affinity chromatography and characterization of subunit structure. *J. Biol. Chem.* **251**, 4787–4791.

PALACIOS, R., CAMPOMANES, M., and QUINTO, C. (1977). *Neurospora crassa* glutamine synthetase. Translation of specific messenger ribonucleic acid in a cell-free system derived from rabbit reticulocytes. *J. Biol. Chem.* **252**, 3028–3034.

PALMITER, R. D. (1973). Regulation of protein synthesis in chick oviduct. Role of testosterone. *J. Biol. Chem.* **248**, 2095–2106.

PELHAM, H. R. B., and JACKSON, R. J. (1976). An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* **67**, 247–256.

QUINTO, C., MORA, J., and PALACIOS, R. (1977). *Neurospora crassa* glutamine synthetase. Role of enzyme synthesis and degradation on the regulation of enzyme concentration during exponential growth. *J. Biol. Chem.* **252**, 8724–8727.

ROBERTS, B. E., and PATERSON, B. M. (1973). Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. *Proc. Nat. Acad. Sci. USA* **70**, 2330–2334.

SÁNCHEZ, F., CAMPOMANES, M., QUINTO, C., HANSBERG, W., MORA, J., and PALACIOS, R. (1978). Nitrogen source regulates glutamine synthetase mRNA levels in *Neurospora crassa*. *J. Bacteriol.* **136**, 880–885.

SARKAR, P. K., and GRIFFITH, B. (1976). Messenger RNA for glutamine synthetase: Its partial purification, translation in a cell-free system and its regulation by hydrocortisone. *Biochem. Biophys. Commun.* **68**, 675–680.

SIMS, A. P., and FERGUSON, A. R. (1974). The regulation of glutamine metabolism in *Candida utilis*: Studies with $^{15}NH_3$ to measure *in vivo* rates of glutamine synthesis. *J. Gen. Microbiol.* **80**, 159–171.

SOH, B. M., and SARKAR, P. K. (1978). Control of glutamine synthetase messenger RNA by hydrocortisone in the embryonic chick retina. *Develop. Biol.* **64**, 316–328.

SCHMIT, J. C., and BRODY, S. (1975). *Neurospora crassa* conidial germination: Role of endogenous amino acid pools. *J. Bacteriol.* **124**, 232–242.

SCHMIT, J. C., and BRODY, S. (1976). Biochemical genetics of *Neurospora crassa* conidial germination. *Bacteriol. Rev.* **40**, 1–41.

STADTMAN, E. R. (1973). A note on the significance of glutamine in intermediary metabolism. *In* "The Enzymes of Glutamine Metabolism" (S. Prusiner and E. R. Stadtman, eds.), pp. 1–6. Academic Press, New York.

VICHIDO, I., MORA, Y., QUINTO, C., PALACIOS, R., and MORA, J. (1978). Nitrogen regulation of glutamine synthetase in *Neurospora crassa*. *J. Gen. Microbiol.* **106**, 251–259.

VOGEL, H. J., and BONNER, D. M. (1954). On the glutamate-proline-ornithine inter-relations in *Neurospora crassa*. *Proc. Nat. Acad. Sci. USA* **40**, 688–694.

JOURNAL OF BACTERIOLOGY, Dec. 1978, p. 880-885
0021-9193/78/0136-0880$02.00/0

Vol. 136, No. 3
Printed in U.S.A.

# Nitrogen Source Regulates Glutamine Synthetase mRNA Levels in *Neurospora crassa*

FEDERICO SÁNCHEZ, MIGUEL CAMPOMANES, CARMEN QUINTO, WILHELM HANSBERG, JAIME MORA, AND RAFAEL PALACIOS*

*Departmento de Biología Molecular, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico, D.F.*

Received for publication 9 June 1978

*Neurospora crassa* glutamine synthetase mRNA was measured by its capacity to direct the synthesis of the specific protein in a cell-free system derived from rabbit reticulocytes. *N. crassa* cultures grown on glutamate as the sole nitrogen source had higher mRNA activities than did those grown on glutamine. The differences were about 10-fold when polysomal RNA was used for translation and about 5-fold when either total cellular RNA or polyadenylic acid-enriched cellular RNA was used. These data indicate that in exponentially growing *N. crassa*, the nitrogen source regulates glutamine synthetase by adjusting specific mRNA levels.

Glutamine synthetase (EC 6.3.1.2) is a key enzyme in nitrogen metabolism. Its regulation has been extensively studied in different organisms, including procaryotes (1, 4, 20), simple eucaryotes (13, 16), and higher organisms (6, 8, 9, 17, 18). We have studied *Neurospora crassa* glutamine synthetase and found that both the nitrogen and the carbon sources participate in its regulation (7; I. Vichido, Y. Mora, C. Quinto, R. Palacios, and J. Mora, J. Gen. Microbiol., in press). In both exponentially growing mycelia (14) and nongrowing conidia (G. Espín, R. Palacios, and J. Mora, submitted for publication), the regulation of glutamine synthetase by the nitrogen source is expressed at the level of specific enzyme synthesis. *N. crassa* glutamine synthetase has been purified and partially characterized (10), and its specific mRNA has been translated in vitro (11). The present study deals with the quantification of glutamine synthetase mRNA levels of cultures grown on either glutamate or glutamine as the sole nitrogen source. The data indicate that the nitrogen source regulates glutamine synthetase synthesis by adjusting specific mRNA levels.

## MATERIALS AND METHODS

**Strains and growth conditions.** *N. crassa* wild-type strain 74 A was used throughout this study. A mycelium was grown from an inoculum of conidia on Vogel minimal medium N (19) supplemented with 1.5% sucrose and containing 5 mM glutamate or 5 mM glutamine as the sole nitrogen source as previously described (14). Eight hours after the inoculation of conidia, the mycelium was filtered through Whatman no. 41 paper and stored at −70°C until used for polysome or RNA extraction.

**Preparation and characterization of anti-glutamine synthetase antibodies.** Glutamine synthetase was purified by a procedure based on affinity chromatography on anthranilate-bound Sepharose (10). To obtain specific antibodies, goats received intramuscular injections of 1 mg of the purified protein in complete Freund adjuvant, followed by two boosters of the same dose at 15-day intervals. One week later, goats were bled from the carotid artery. Sera obtained from the different bleedings were pooled, and the total gamma globulin fraction was prepared by three consecutive precipitations with 40% saturation ammonium sulfate. The final precipitate was dissolved in 10 mM sodium phosphate (pH 7.2) containing 150 mM NaCl, dialyzed against the same buffer, and frozen at −70°C. This goat antibody fraction appeared to be monospecific for glutamine synthetase by different experimental criteria: double immunodiffusion, immunoelectrophoresis, precipitation of in vivo-labeled glutamine synthetase, and precipitation of glutamine synthetase activity. Conditions of immunoprecipitation were similar to those previously reported (11, 14).

**Preparation of in vivo-labeled glutamine synthetase.** An *N. crassa* culture grown 8 h on glutamate as the sole nitrogen source was labeled for 2 h with 1 μCi of [$^3$H]methionine (New England Nuclear, 15 Ci/mmol) per ml. After the labeling, the mycelium was collected and glutamine synthetase was purified as previously described (10). The purified preparation had a specific activity of $10^6$ cpm/mg of protein.

**Preparation of *N. crassa* polysomes.** Polysomes were prepared as described by Gray and Cashmore for plant leaf tissue (5) with some modifications. Glassware was autoclaved, and all procedures were performed at 4°C. Mycelia were homogenized in 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8.5) containing 0.4 M KCl, 20 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 0.25 M sucrose, and 500 μg of heparin per ml. The homogenate was centrifuged 15 min at 10,000 rpm in a Sorvall H-B4 rotor; the supernatant

was made 0.5% in both Nonidet P-40 and sodium deoxycholate and layered over discontinuous sucrose gradients containing 3 ml of 2.5 M, 3 ml of 1.0 M, and 1 ml of 0.5 M sucrose prepared in 50 mM Tris (pH 8.5) containing 0.1 M KCl, 20 mM $MgCl_2$, 14 mM 2-mercaptoethanol, and 100 μg of heparin per ml. Gradients were centrifuged at 27,000 rpm for 8 h in the Sorvall HB-4 rotor. After centrifugation, the supernatant and the 0.5 and 1.0 M sucrose layers were discarded and the pellet was suspended in the 2.5 M sucrose cushion. The sample was then filtered through a Sephadex G-25 column equilibrated in the same buffer. The polysome preparation was frozen at −70°C for up to 1 month.

**Preparation of *N. crassa* RNA.** To prepare total cellular RNA, mycelia were homogenized at room temperature with 2 volumes of 0.1 M Tris-hydrochloride (pH 9.0 at 25°C), 0.1 M NaCl, 10 mM ethylenediaminetetraacetate, 1% sodium dodecyl sulfate, and 2 volumes of phenol–chloroform–isoamyl alcohol (50:50:1). The aqueous phase was separated by centrifugation for 10 min at 10,000 rpm in the Beckman SW27 rotor and extracted twice more with 1 volume of phenol–chloroform–isoamyl alcohol (50:50:1). The aqueous phase was made 0.15 M in NaCl and precipitated overnight with 2 volumes of ethanol. The pellet after centrifugation was washed three times with 2.0 M $LiCl_2$ containing 10 mM ethylenediaminetetraacetate (12), dissolved in water, and precipitated twice more with ethanol. The final pellet was blown with a stream of nitrogen gas to remove traces of ethanol and suspended in a small volume of water.

To prepare polysomal RNA, 1 volume of the polysome preparation was mixed with 2 volumes of 0.1 M Tris-hydrochloride (pH 9.0 at 25°C), 0.1 M NaCl, 10 mM ethylenediaminetetraacetate, and 1% sodium dodecyl sulfate and treated as described for the preparation of total cellular RNA.

To prepare polyadenylic acid-enriched cellular RNA, mycelia were homogenized at room temperature in 3 volumes of 10 mM Tris-hydrochloride (pH 7.6) containing 0.5 M NaCl, 10 mM magnesium acetate, 1.0 M 2-mercaptoethanol, 0.5% sodium dodecyl sulfate, 0.5% sodium deoxycholate, and 0.005% dextran sulfate. The homogenate was incubated 10 min at 37°C and centrifuged 10 min at 10,000 rpm in the Sorvall HB-4 rotor. The supernatant was then chromatographed in a column of oligodeoxythymidylic acid–cellulose equilibrated in 10 mM Tris-hydrochloride (pH 7.6) containing 0.5 M NaCl and 0.5% sodium dodecyl sulfate. After washing with the same buffer until no absorbance at 260 nm was obtained, RNA was eluted with 10 mM Tris-hydrochloride containing 0.5% sodium dodecyl sulfate. The fractions containing absorbance at 260 nm were pooled, made 0.15 M in NaCl, and precipitated with 2 volumes of ethanol at −20°C. The pellet obtained after centrifugation was washed with $LiCl_2$ and processed as described above. All RNA preparations were stored at −70°C.

**Incubation of reticulocyte lysate and quantification of in vitro-synthesized glutamine synthetase.** Reticulocyte lysate was incubated as previously described (11), using [$^{35}$S]methionine (New England Nuclear, 400 Ci/mmol), at a concentration of 200 μCi/ml, as the labeled amino acid. After 90 min of incubation, the reaction was stopped and 100-μl portions were used to immunoprecipitate glutamine synthetase by a direct procedure (11), using 5 μg of carrier purified glutamine synthetase and 3 mg of specific antibody. In some experiments, a trace amount of purified glutamine synthetase labeled in vivo with [$^{3}$H]methionine was added before immunoprecipitation. Immunoprecipitates were separated by centrifugation through discontinuous sucrose gradients (11) and subjected to acrylamide slab gel electrophoresis followed by staining with Coomassie brilliant blue and fluorography (3). To quantify in vitro-synthesized glutamine synthetase, the specific protein band was cut and digested with $H_2O_2$. Bray solution was then added (10 ml), and the samples were counted for $^{3}$H/$^{35}$S double label in a Packard Tri-Carb model 3390 liquid scintillation spectrometer. The spillover of $^{35}$S into the $^{3}$H channel was 8%.

## RESULTS

The experimental approach followed to determine glutamine synthetase mRNA levels depended on the quantitative isolation of the in vitro-synthesized enzyme protein as well as on obtaining nondegraded polysomes and RNA from *N. crassa*. The isolation of the protein was accomplished by specific immunoprecipitation. The reticulocyte lysate system that incorporates [$^{35}$S]methionine as the labeled amino acid was incubated in the presence or in the absence of *N. crassa* polysomal RNA (Fig. 1). After incubation, nonlabeled purified glutamine synthetase was added, and each reaction mixture was divided into two aliquots, one of them receiving, in addition, a trace amount of purified glutamine synthetase labeled in vivo with [$^{3}$H]methionine. The preparations were immunoprecipitated with specific antibody, and the immunoprecipitates were subjected to acrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate, stained with Coomassie brilliant blue, and treated for fluorography. After fluorography, gels were cut and each slice was counted for double label ($^{3}$H/$^{35}$S). As judged by the stained pattern, the immunoprecipitates contained three major protein bands, corresponding to the heavy chain of gamma globulin, glutamine synthetase, and the light chain of gamma globulin. On the other hand, the fluorographic and radioactivity patterns indicated that the only labeled protein in the immunoprecipitates migrated with the mobility of glutamine synthetase. When the protein-synthesizing system was incubated in the absence of *N. crassa* RNA, no $^{35}$S-protein was found in the immunoprecipitates; when the system was incubated in the presence of *N. crassa* RNA and glutamine synthetase labeled in vivo with $^{3}$H was added before immunoprecipitation, both the $^{3}$H and the $^{35}$S labels migrated in the gel as a single peak. Al-

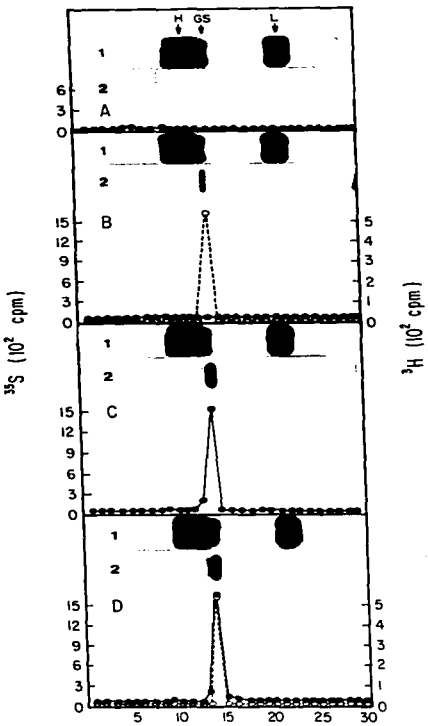


FIG. 1. *Electrophoresis of immunoprecipitated glutamine synthetase synthesized in vivo and in vitro. The reticulocyte lysate system was incubated in the absence (A, B) or in the presence (C, D) of polysomal RNA (320 μg/ml) extracted from a culture grown on glutamate as the sole nitrogen source. Immunoprecipitation (see text) of the in vitro-synthesized protein was carried out after addition of 5 μg of purified N. crassa glutamine synthetase as carrier and in the absence (A, C) or in the presence (B, D) of a trace amount (700 cpm) of glutamine synthetase labeled in vivo with [$^3$H]methionine. Immunoprecipitates were subjected to electrophoresis on acrylamide slab gels in the presence of sodium dodecyl sulfate, stained with Coomassie brilliant blue, and treated for fluorography. After 3 days of exposure, the gels were cut in 3-mm slices and counted for $^3$H/$^{35}$S double label. Panels show the stained gel (1), the gel after fluorography (2), $^{35}$S counts per minute (●), and $^3$H counts per minute (○). The arrows in (A) indicate the positions of the heavy chain of gamma globulin (H), glutamine synthetase (GS), and the light chain of gamma globulin (L).*

though the immunoprecipitates did not show the presence of contaminating proteins bands, in all experiments performed in the present study the immunoprecipitates were run in acrylamide gels and subjected to fluorography before quantitation of the radioactivity present in the glutamine synthetase band. This approach provides direct evidence on the specificity of the immunoprecipitation in each individual point.

The following experiments were performed to quantitate the levels of glutamine synthetase mRNA in *N. crassa* mycelia grown on glutamate or glutamine as the sole nitrogen source. We have previously shown that when *N. crassa* is grown on glutamate, the glutamine synthetase specific activity is about 10-fold higher than that found when it is grown on glutamine and that this difference correlates positively with the relative rates of enzyme synthesis found under both conditions (14).

The reticulocyte lysate system was incubated in the presence of different concentrations of polysomal RNA from cultures grown on glutamate or glutamine as the nitrogen source (Fig. 2). Immunoprecipitation of the in vitro-synthesized protein was performed in the presence of purified glutamine synthetase labeled in vivo with [$^3$H]methionine. The amount of $^3$H in the specific immunoprecipitates was fairly constant and accounted for about 80% of the input radioactivity (Fig. 2A and B). On the other hand, $^{35}$S radioactivity increased in the immunoprecipitates as a function of RNA concentration. To accurately quantitate the amount of in vitro-synthesized glutamine synthetase, either the net amount of $^{35}$S or the $^{35}$S/$^3$H ratio found in the glutamine synthetase band was used. Both procedures coincided and were linear with respect to RNA concentration (Fig. 2C). The slope of the line was about 10-fold higher for the RNA extracted from the culture grown on glutamate than that for the RNA from the culture grown on glutamine. When mycelia from both conditions were mixed before the RNA was extracted, the amount of glutamine synthetase-translatable mRNA gave intermediate results (data not shown), indicating that there was no preferential degradation of RNA in either of the conditions

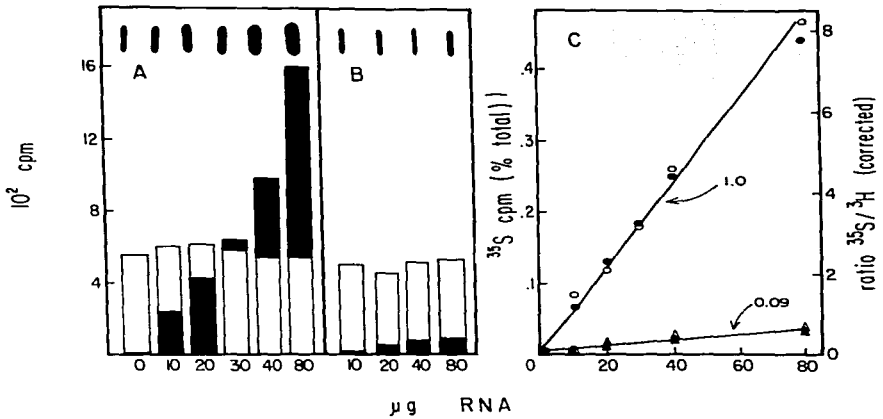


FIG. 2. *Glutamine synthetase mRNA activity of N. crassa polysomal RNA. The reticulocyte lysate system was incubated in the presence of different concentrations of polysomal RNA (see text) extracted from parallel cultures of N. crassa grown on glutamate or glutamine as the nitrogen source. The immunoprecipitation reaction was carried out in the presence of a trace amount (700 cpm) of glutamine synthetase labeled in vivo with [$^3$H]methionine. The immunoprecipitates were subjected to acrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate, stained, and treated for fluorography. After fluorography, the stained glutamine synthetase protein band was cut, digested, and counted. A (culture grown on glutamate) and B (culture grown on glutamine) show the net amounts of $^3$H (open bars) and $^{35}$S (shadowed bars) radioactivities in the glutamine synthetase bands. Autoradiography of the glutamine synthetase bands is also shown. C shows the $^{35}$S counts per minute immunoprecipitated expressed as total protein synthesized (closed symbols) or the $^{35}$S/$^3$H ratio in the immunoprecipitate corrected by the total protein synthesized in each reaction (open symbols) for cultures grown on glutamate (circles) or glutamine (triangles). The indicated slopes of the lines were normalized to that of the culture grown on glutamate, set arbitrarily at 1.0. RNA concentration corresponds to a 125-µl amount of the reticulocyte lysate reaction.*

due to technical manipulation. RNA recovery from mycelia grown on glutamine was 70% or more of that grown on glutamate.

Figures 3 and 4 present experiments similar to that of Fig. 2 but performed with total cellular RNA or with polyadenylic acid-enriched cellular RNA, respectively. In each case, RNA extracted from the culture grown on glutamate presented higher activity than that extracted from the culture grown on glutamine. With both RNA preparations, the differences in mRNA activities between both cultures were about fivefold.

## DISCUSSION

The regulation of eucaryotic glutamine synthetase has been studied in different biological systems, and controls at both the level of enzyme synthesis (8, 16) and the level of enzyme degradation (2, 16) have been demonstrated. Sarkar and Griffith (15) have measured the levels of translatable glutamine synthetase mRNA from chick retina, and their data indicate that steroid hormones increase the concentration of specific mRNA in polysomes.

We have studied the regulation of *N. crassa* glutamine synthetase by growing the fungus in the presence of the substrates or the product of the enzyme and have found that the nature of the nitrogen source during exponential growth regulates enzyme synthesis while exerting no effect on enzyme degradation (14).

In the present study, the in vitro translation of *N. crassa* RNA isolated by three different procedures (Fig. 2 through 4) indicates that the nitrogen source used for growth regulates the

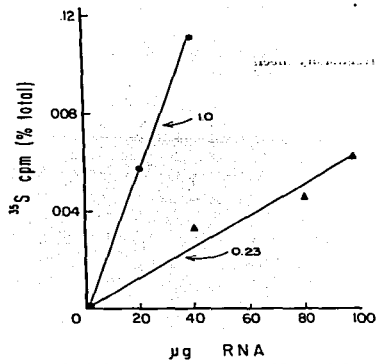


FIG. 3. *Glutamine synthetase mRNA activity of N. crassa total cellular RNA. The reticulocyte lysate system was incubated in the presence of different concentrations of total cellular RNA (see text) extracted from parallel cultures of N. crassa grown on glutamate or glutamine as the sole nitrogen source. The immunoprecipitation reactions were carried out in the absence of in vivo-labeled glutamine synthetase. The immunoprecipitates were processed as in Fig. 2. Symbols are as in Fig. 2C.*

levels of translatable glutamine synthetase mRNA. To our knowledge, this is the first direct demonstration of the regulation of specific mRNA levels in *N. crassa*. To find out if regulation is exerted at the level of transcription, mRNA processing, or mRNA degradation, a specific hybridization probe must be obtained. The search for such a probe is now in progress in our laboratory.

When polysomal RNA was used for translation (Fig. 2) a 10-fold difference in active specific mRNA was found between cultures grown on glutamate and on glutamine as sole nitrogen sources. This difference is in accordance with that previously obtained for the relative rates of enzyme synthesis measured in vivo (14). On the other hand, when using total cellular RNA (Fig. 3) or polyadenylic acid-enriched cellular RNA (Fig. 4) for translation, less marked differences (4- to 5-fold) were obtained between cultures grown on glutamate and on glutamine as nitrogen sources. The quantitative differences obtained with the different procedures of RNA isolation are difficult to explain at present. They could be due to differential degradation of specific mRNA due to technical manipulation, to different proportions of mRNA versus other cellular RNAs in the two nitrogen conditions, or to the presence of glutamine synthetase mRNA in ribonucleoprotein particles outside of polysomes.

Polysome profiles from cultures grown on glutamate or glutamine as the nitrogen source do not show significant differences in amount and size of total polysomes (data not shown). Furthermore, the translation of RNA isolated from mixtures of mycelia from both cultures has given intermediate values (data not shown), between those found with RNA, extracted from both cultures. These findings suggest that there is not significant preferential degradation during the preparation of RNA from either of the cultures.

We have postulated that the induction of *N.*

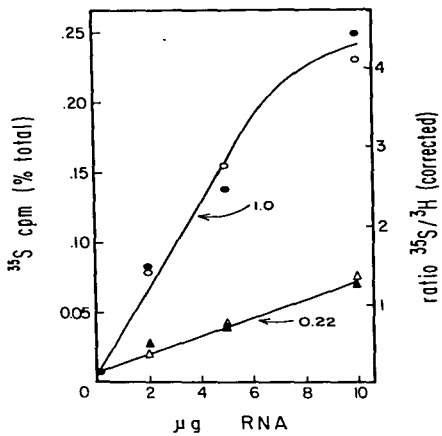


FIG. 4. *Glutamine synthetase mRNA activity of N. crassa polyadenylic acid-enriched cellular RNA. The reticulocyte lysate system was incubated in the presence of different concentrations of polyadenylic acid-enriched cellular RNA (see text) extracted from parallel cultures of N. crassa grown on glutamate or glutamine as the nitrogen source. Immunoprecipitation was carried out in the presence of 700 cpm of glutamine synthetase labeled in vivo with [$^3$H]methionine; the immunoprecipitates were processed as in Fig. 2. Symbols are as in Fig. 2C.*

*crassa* glutamine synthetase when glutamate is used as the nitrogen source is the result of ammonia being limited. Furthermore, we have recently reported that ammonia limitation results in the induction of glutamine synthetase activity, whereas carbon limitation prevents this induction (7). These findings, together with the fact that ammonia per se does not impair the induction of enzyme activity (7), have led us to propose that carbon exerts a positive role and that glutamine exerts a negative one on glutamine synthetase induction (Vichido et al., J. Gen. Microbiol., in press). Experimental approaches similar to those in the present study will be useful to demonstrate the level at which this regulation takes place.

## ACKNOWLEDGMENTS

This work was supported in part by grant PNCB 036 from the Consejo Nacional de Ciencia y Tecnologia, Mexico.

The authors are grateful to Fernando Bastarrachea and Francisco Bolivar for critically reading the manuscript.

## LITERATURE CITED

1. **Adler, S. P., D. Purich, and E. R. Stadtman.** 1975. Cascade control of *Escherichia coli* glutamine synthetase. J. Biol. Chem. **250**:6264–6272.
2. **Arad, G., A. Frelkopf, and R. G. Kulka.** 1976. Glutamine stimulated modification and degradation of glutamine synthet*ase* in hepatoma tissue culture cells. Cell **8**:95–101.
3. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucelic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
4. **Foor, F., K. A. Janssen, and B. Magasanik.** 1975. Regulation of synthesis of glutamine synthetase by adenylated glutamine synthetase. Proc. Natl. Acad. Sci. U.S.A. **72**:4844–4848.
5. **Gray, R. E., and A. B. Cashmore.** 1976. RNA synthesis in plant leaf tissue: the characterization of messenger RNA species lacking and containing polyadenilic acid. J. Mol. Biol. **108**:595–608.
6. **Kulka, R. G., G. M. Tomkins, and R. B. Crook.** 1972. Clonal differences in glutamine synthetase activity of hepatoma cells. J. Cell Biol. **54**:175–178.
7. **Limon-Lasson, J., M. Lara, B. Resendiz, and J. Mora.** 1977. Regulation of glutamine synthetase in feed-batch cultures of *Neurospora crassa*. Biochem. Biophys. Res. Commun. **78**:1234–1240.
8. **Moscona, M., N. Frenkel, and A. A. Moscona.** 1972. Regulatory mechanisms in the induction of glutamine synthetase in embryonic retina: immunochemical studies. Dev. Biol. **28**:229–241.
9. **O'Neal, T. D., and K. W. Joy.** 1975. Pea leaf glutamine synthetase. Plant Physiol. **55**:968–974.
10. **Palacios, R.** 1976. *Neurospora crassa* glutamine synthetase. Purification by affinity chromatography and characterization of subunit structure. J. Biol. Chem. **251**:4787–4791.
11. **Palacios, R., M. Campomanes, and C. Quinto.** 1977. *Neurospora crassa* glutamine synthetase. Translation of specific messenger ribonucleic acid in a cell-free system derived from rabbit reticulocytes. J. Biol. Chem. **252**:3028–3034.
12. **Palmiter, R. D.** 1973. Regulation of protein synthesis in chick oviduct. Role of testosterone. J. Biol. Chem. **248**:2095–2106.
13. **Pateman, J. A.** 1969. Regulation of synthesis of glutamate dehydrogenase and glutamine synthetase in micro-organisms. Biochem. J. **115**:769–775.
14. **Quinto, C., J. Mora, and R. Palacios.** 1977. *Neurospora crassa* glutamine synthetase. Role of enzyme synthesis and degradation on the regulation of enzyme concentration during exponential growth. J. Biol. Chem. **252**:8724–8727.
15. **Sarkar, P. K., and B. Griffith.** 1976. Messenger RNA for glutamine synthetase: its partial purification, translation in a cell-free system and its regulation by hydrocortisone. Biochem. Biophys. Res. Commun. **68**: 675–681.
16. **Sims, A. P., and A. R. Ferguson.** 1974. The regulation of glutamine metabolism in *Candida utilis*: studies with $^{15}NH_3$ to measure *in vivo* rates of glutamine synthesis. J. Gen. Microbiol. **80**:159–171.
17. **Tate, S. S., F. Y. Lew, and A. Meister.** 1972. Rat liver glutamine synthetase. Preparation, properties and mechanism of inhibition by carbamyl phosphate. J. Biol. Chem. **247**:5312–5321.
18. **Tiemeier, D. C., and G. Milman.** 1972. Chinese hamster liver glutamine synthetase: purification, physical and biochemical properties. J. Biol. Chem. **247**:5722–5727.
19. **Vogel, H. J., and D. M. Bonner.** 1954. On the glutamate-proline-ornithine inter-relations in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S.A. **40**:688–694.
20. **Weglenski, P., and B. Tyler.** 1977. Regulation of *glnA* messenger ribonucleic acid synthesis in *Klebsiella aerogenes*. J. Bacteriol. **129**:880–887.