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

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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-

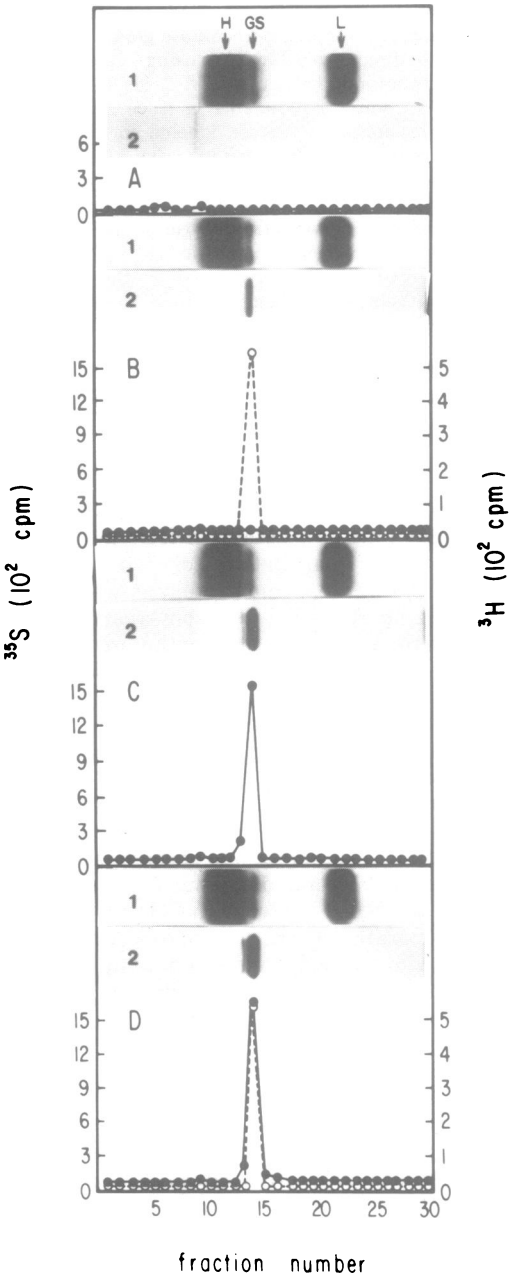

FIG. 1. *Electrophoresis of immunoprecipitated glutamine synthetase synthesized in vivo and in vitro. The reticulocyte lysate system was incubated in the absence (A, B) or in the presence (C, D) of polysomal RNA (320 μg/ml) extracted from a culture grown on glutamate as the sole nitrogen source. Immunoprecipitation (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

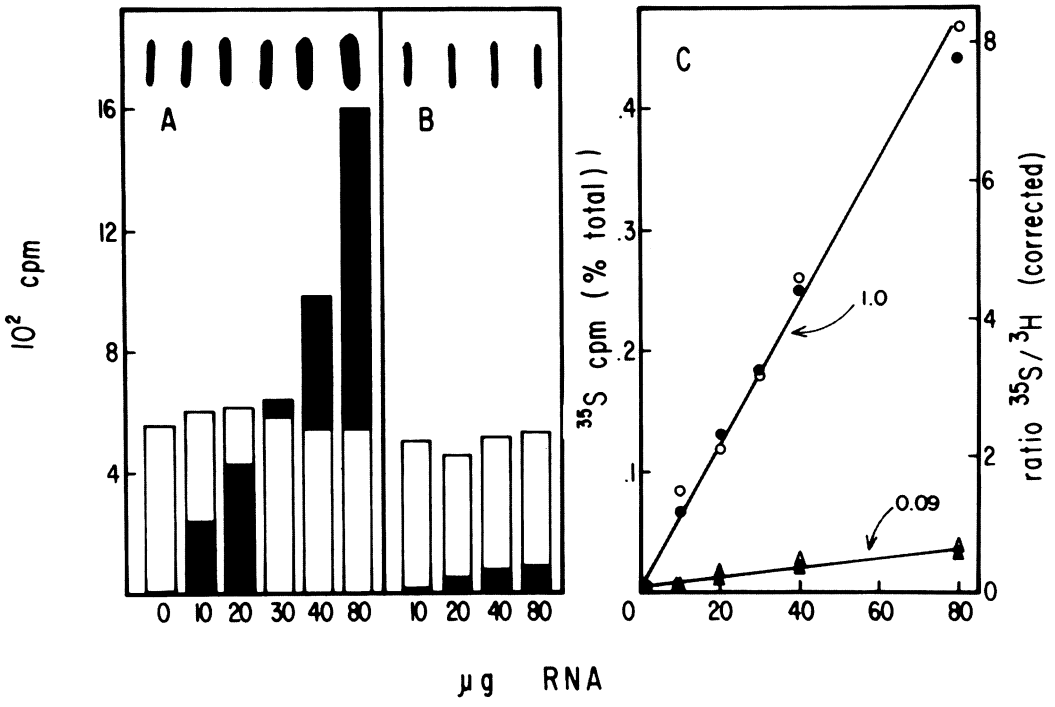

FIG. 2. *Glutamine synthetase mRNA activity of N. crassa polysomal RNA. The reticulocyte lysate system was incubated in the presence of different concentrations of polysomal RNA (see text) extracted from parallel cultures of N. crassa grown on glutamate or glutamine as the nitrogen source. The immunoprecipitation 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 $^{35}S/^3H$ 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

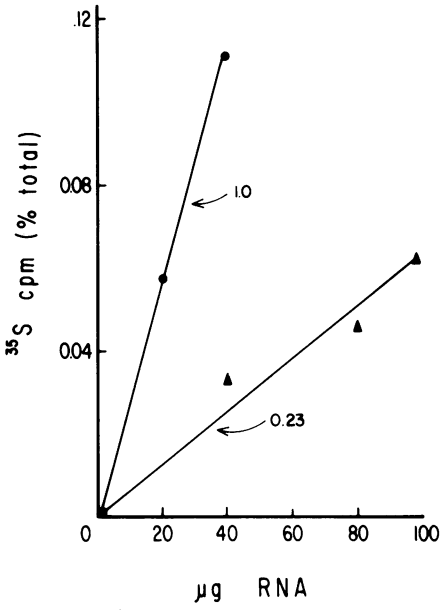

FIG. 3. *Glutamine synthetase mRNA activity of N. crassa total cellular RNA. The reticulocyte lysate system was incubated in the presence of different concentrations of total cellular RNA (see text) extracted from parallel cultures of N. crassa grown on glutamate or glutamine as the sole nitrogen source. 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.*

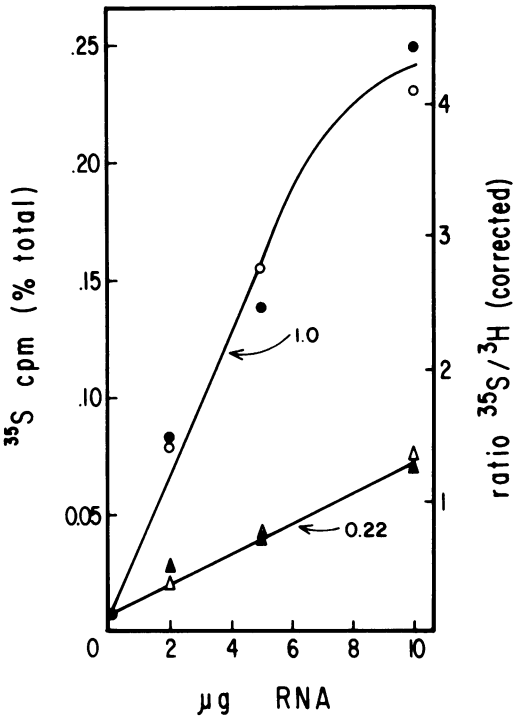

FIG. 4. *Glutamine synthetase mRNA activity of N. crassa polyadenylic acid-enriched cellular RNA. The reticulocyte lysate system was incubated in the presence of different concentrations of polyadenylic acid-enriched cellular RNA (see text) extracted from parallel cultures of N. crassa grown on glutamate 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.

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