Growth Regulation by GTP

REGULATION OF NUCLEOTIDE POOLS IN NEUROSPORA BY NITROGEN AND SULFUR CONTROL SYSTEMS*

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A number of intracellular messengers are involved in cellular regulation including cyclic AMP, cyclic GMP, Ca²⁺, fructose 2,6-bisphosphate, and the guanosine polyphosphates (of the stringent response). Several other regulators also have central nonregulatory functions such as adenine nucleotide energy charge (1), protons or intracellular pH (2), and polyamines (3, 4). It has recently been proposed that GTP should be added to this latter group, with GTP having a general role in regulating (stimulating) anabolic processes involved in growth and cell proliferation (5).

Whereas there is not space here to reiterate evidence reviewed elsewhere (5), the three most important types of evidence supporting this hypothesis are the following. 1) Declines in GTP pools have been demonstrated to have a specific role in triggering the formation of dormant spores in cultures of several prokaryotic species (6–9) and in the yeast, Saccharomyces cerevisiae (10, 11) and in triggering terminal differentiation in mammalian cells in culture (12, 13). 2) GTP stimulates an extremely wide variety of anabolic process in vitro, acting to provide energy for them and to stimulate them by allosteric means and other mechanisms (5). 3) ras proteins are involved in stimulating cell proliferation in animal cells and in yeast, with the proliferative response requiring the GTP-bound ras protein (14–20).

The hypothesis that GTP has a general role in stimulating growth-related processes predicts that GTP levels themselves should be regulated such that GTP pools will be highest in growing, nutritionally sufficient cells and lower in nongrowing, nutritionally deprived cells. However, except for GTP pool studies during sporulation in microorganisms (6–11), no systematic studies of GTP pools have been made that could be used to test this prediction (5, 21). We use here four strains of the fungus Neurospora crassa to determine the pattern of regulation to GTP pools and the mechanism involved in such regulation. The results show that GTP/ATP ratios decline on nitrogen or sulfur deprivation, with that decline being mediated by known regulatory systems. These nitrogen and sulfur regulatory systems control a number of nucleotide pools and therefore have much broader functions than their previously proposed roles in regulating utilization of alternative exogenous nitrogen and sulfur sources.

MATERIALS AND METHODS

Strains—The following strains were used in these studies: St. Lawrence wild-type 74-OR25-1A; cr-1, B123 that had been backcrossed to the St. Lawrence wild-type background eight times (22); cys-3, P22, a (F.G.S.C. 4029) that had been backcrossed to the St. Lawrence wild-type background seven times; and nit-2, nr37, a (F.G.S.C. 982) that had been isolated in and backcrossed to the St. Lawrence wild-type background. The latter two strains were obtained from the Fungal Genetics Stock Center (Kansas City, KS).

Culture Conditions—Standard growth medium was Vogel's medium N (23) containing 2% sucrose. Conidia were obtained from 5–7-day-old cultures grown at 25 °C on agar-containing (2%) medium. All experiments were performed on cultures grown and treated at 25 °C except as noted in the text for the data in Table I and Fig. 1.

Nitrogen-free medium was Vogel's medium N without ammonium nitrate. Sulfur-free medium was Vogel's medium N with MgSO₄ replaced by equimolar MgCl₂. Where L-methionine was used as a sulfur source, the sulfur-free medium was supplemented with 50 μg/ml L-methionine.

Where mycelia were to be placed into new media, they were collected onto a sterile 47-mm Gelman glass-fiber filter, washed with sterile deionized water, placed into new sterile medium, and shaken in a New Brunswick G76 incubator shaker. Small, 10-ml samples were prepared and collected by rapid filtration as described earlier (25). They were quickly placed into 1 ml of cold, 10% trichloroacetic acid, extracted for 15 min at 0 °C, and processed as described earlier (26). In most cases, 50 μl of extract was
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RESULTS

GTP/ATP RATIOs IN NEUROSPORA-Initial experiments were aimed at measuring changes in GTP/ATP ratios in the nucleotide pools of Neurospora. This ratio may reflect the division of the purine nucleotide synthetic pathway into adenine and guanine nucleotides. Such ratio measurements also have the advantage that errors due to fluctuations in sampling or extraction of cultures or of injection volumes for HPLC analysis are eliminated.

As shown in Table I, GTP/ATP ratios in wild-type Neurospora decline upon nitrogen starvation. These data support the view that guanine nucleotide pools drop as growth ceases. When the adenylate cyclase- and cyclic AMP-deficient mutant of Neurospora (31-34), cr-1, is studied, it shows essentially the same pattern of GTP/ATP ratios as does the wild type (Table I). We conclude that GTP/ATP ratios are controlled in a cyclic AMP-independent fashion.

The decline of GTP/ATP ratios on nitrogen starvation raises the question of whether the ratio is controlled by growth, per se, by nitrogen deprivation, or by some other mechanism. In order to probe this question, we looked at pool ratios in a nit-2 mutant of Neurospora. The nit-2 gene is a positive regulatory gene which is responsible for the derepression of a whole series of nitrogen catabolic enzymes on nitrogen deprivation (35-37). nit-2 mutants are unable to respond to nitrogen deprivation with such derepression. As shown in Table I, in a nit-2 mutant, GTP/ATP ratios do not decline on nitrogen starvation. These results support the view that nitrogen starvation produces a drop in GTP/ATP ratios by a mechanism mediated by the wild-type nit-2 gene.

A time course of response to nitrogen nutritional change at 30 °C is shown in Fig. 2. GTP/ATP ratios decline after the cells are nitrogen-deprived. When the nitrogen source ammonium nitrate is added to the culture medium, GTP/ATP ratios drop further before increasing to a high level 30 min after nutrient addition. Thus, after a transient decline, nitrogen source addition restores GTP/ATP ratios.

Nucleotide Pool Regulation in Wild-type N. crassa—Data on several nucleotide ratios in pools of wild-type Neurospora are shown in Table II. These data, which are based on different experiments than those shown in Table I or Fig. 2, show that GTP/ATP ratios decline on either nitrogen or sulfur deprivation, but show little change after 3-5 h of carbon deprivation. Furthermore, both ATP/ADP and GTP/GDP ratios were subsequently counted to determine the number of viable germinated conidia.

Statistical Analysis—Data in Tables II, IV, and V were all analyzed by an analysis of variances. Samples (generally three each) collected from a single culture were averaged together, and means were used as single datum values. The means (of these means) were then compared with the means of undeprived, growing controls using a Scheffe test (30) for statistically significant differences at the 0.05 or 0.01 level. The data in Tables I and III were analyzed by a Student's t test.

\[ \text{GTP/ATP} = \frac{\text{GTP}}{\text{ATP}} \]

Nutritional Control of Nucleotide Pools

\[ \text{GTP/ATP ratios in growing and nitrogen-starved Neurospora cultures} \]

| Condition | Wild type | cr-1 | nit-2 |
|-----------|-----------|------|-------|
| Nutritional control | 0.246 ± 0.013 | 0.244 ± 0.023 | 0.233 ± 0.006 |
| (n = 5) & (n = 5) | (n = 5) | (n = 5) |

\[ \text{p} < 0.01 \text{, when compared with nutritionally sufficient, growing controls.} \]

1 The abbreviations used are: HPLC, high pressure liquid chromatography; G\( _{\text{m}} \), stimulatory regulatory GTP-binding protein of adenylate cyclase.

2 Nutritional controls were grown in nitrogen-deprived 27-28 °C.
Nutritional Control of Nucleotide Pools

Fig. 2. Nucleotide profiles of nutritionally sufficient and 6 h sulfur-deprived eye-3 mutants. 50-μl injections of nutritionally sufficient and 6-h sulfur-deprived mycelium extracts were used to obtain the HPLC profiles. The nutritionally sufficient and sulfur-deprived mycelia had dry weights of 11.7 and 16.0 mg, respectively. In 1 ml of 1 M trichloroacetic acid. Peak identification is discussed in Ref. 41. AdoMet, S-adenosylmethionine.

Regulation—As discussed briefly above, N. crassa has a nitrogen catabolic control system that acts upon nitrogen deprivation to derepress a series of genes coding for enzyme activities involved in nitrogen catabolism. This nitrogen catabolite regulatory system acts via the wild-type nit-2 gene; and therefore, this regulatory system is inactive in nit-2 mutants (35–37). The nit-2 gene product is thought to activate transcription of a set of genes having roles in transport and catabolism of alternative exogenous nitrogen sources (35–37).

When a nit-2 mutant is studied, some aspects of nucleotide pool control differ from those found in wild type, whereas others are unaffected by the mutation. As shown in Table IV, ATP/ADP ratios and GTP/GDP ratios increased in the nit-

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| Table III |
| --- |
| ATP and GTP levels in wild-type N. crassa |

When absolute levels of GTP and ATP are measured, these are also found to decline on nitrogen or sulfur deprivation, with GTP levels being lowered about 25% and lesser declines in ATP levels (Table III). Thus, nutritional (nitrogen or sulfur) deprivation leads to declines in GTP pools either when expressed in terms of amount per dry weight or in levels relative to ATP.

Nitrogen Control and Role of nit-2 Gene in Nucleotide Pool Regulation—As discussed briefly above, N. crassa has a nitrogen catabolic control system that acts upon nitrogen deprivation to derepress a series of genes coding for enzyme activities involved in nitrogen catabolism. This nitrogen catabolite regulatory system acts via the wild-type nit-2 gene; and therefore, this regulatory system is inactive in nit-2 mutants (35–37). The nit-2 gene product is thought to activate transcription of a set of genes having roles in transport and catabolism of alternative exogenous nitrogen sources (35–37).

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Nutritional Control of Nucleotide Pools

TABLE IV
Nucleotide ratios and ATP levels in nit-2 mutant of N. crassa

|                         | ATP/ADP | GTP/GDP | GTP/ATP | UTP/ATP | CTP/ATP |
|-------------------------|---------|---------|---------|---------|---------|
| Nutrient sufficient, growing control (n = 3) | 7.40 ± 0.31 | 6.16 ± 0.62 | 0.241 ± 0.004 | 0.254 ± 0.018 | 0.114 ± 0.009 | 5.55 ± 0.20 |
| 3-h nitrogen-deprived (n = 4) | 13.46 ± 0.72 | 10.54 ± 1.63 | 0.252 ± 0.004 | 0.330 ± 0.010 | 0.118 ± 0.005 | 4.30 ± 0.37 |
| 4.5-h nitrogen-deprived (n = 5) | 14.00 ± 6.00 | 7.86 ± 2.98 | 0.235 ± 0.012 | 0.335 ± 0.024 | 0.119 ± 0.008 | 3.51 ± 0.66 |
| 3-h sulfur-deprived (n = 3) | 14.83 ± 0.80 | 12.10 ± 0.34 | 0.215 ± 0.013 | 0.220 ± 0.008 | 0.101 ± 0.005 | 5.12 ± 0.94 |
| 6-h sulfur-deprived (n = 3) | 14.25 ± 0.90 | 10.10 ± 2.28 | 0.186 ± 0.027 | 0.231 ± 0.020 | 0.115 ± 0.016 | 3.68 ± 0.41 |

* p < 0.05, when compared with nutritionally sufficient, growing controls.

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TABLE V
cys-3 and wild-type Neurospora grown in sulfur-free methionine plus medium

|                         | ATP/ADP | GTP/ATP | UTP/ATP | CTP/ATP | ATP |
|-------------------------|---------|---------|---------|---------|-----|
| Nutrient sufficient, growing control (n = 6) | 10.66 ± 2.87 | 0.215 ± 0.019 | 0.225 ± 0.028 | 0.107 ± 0.018 | 7.38 ± 2.09 |
| 3-h sulfur-deprived cys-3 (n = 4) | 6.38 ± 8.24 | 0.250 ± 0.032 | 0.313 ± 0.065 | 0.118 ± 0.030 | 4.70 ± 2.42 |
| 6-h sulfur-deprived cys-3 (n = 4) | 2.03 ± 0.40 | 0.243 ± 0.019 | 0.224 ± 0.040 | 0.102 ± 0.023 | 0.90 ± 0.45 |
| 3-h nitrogen-deprived cys-3 (n = 6) | 13.41 ± 1.57 | 0.217 ± 0.017 | 0.271 ± 0.066 | 0.130 ± 0.012 | 6.15 ± 2.47 |
| 4.5-h nitrogen-deprived cys-3 (n = 6) | 11.88 ± 1.74 | 0.203 ± 0.008 | 0.253 ± 0.055 | 0.127 ± 0.008 | 5.47 ± 2.01 |

* p < 0.05, when compared with nutritionally sufficient, growing controls.

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TABLE VI
Viability of germinated conidia of wild type and cys-3 after sulfur deprivation

| Sulfur deprivation period | % % of nonstarved control | Wild type | cys-3 |
|--------------------------|---------------------------|----------|------|
| h                        | 0                         | 100      | 100  |
| 5                        | 92 ± 15                   | 94 ± 21  |      |
| 8.5                      | 88 ± 15                   | 76 ± 20  |      |

2 mutant on nitrogen or sulfur deprivation, as they did in the wild type (compare with Table II). However, GTP/ATP ratios, which decline in wild type on nitrogen deprivation (Tables I and II and Fig. 2), fail to decline when nit-2 is nitrogen-deprived (Table IV). When the nit-2 mutant is sulfur-starved, GTP/ATP ratios show a decline similar to that seen in wild type. Thus, the mutant shows a specific defect with respect to nucleotide control by nitrogen deprivation consistent with a specific nitrogen control role of the nit-2 gene. The aberrant control of GTP/ATP ratios in the nit-2 mutant on nitrogen deprivation seen here confirms the same pattern seen in Table I. UTP/ATP ratio response to nitrogen deprivation is also aberrant, with increases in ratio being found in nit-2 compared with modest decreases in wild type (compare Table IV with Table II). The absolute level of ATP per unit weight decreases in the mutant on nutritional deprivation, much like it does in the wild type (compare Table IV with Table III). Sulfur Control and Role of cys-3 Gene in Nucleotide Pool Regulation—N. crassa has a sulfur control system very similar to its nitrogen control system. On sulfur deprivation, the organism derepresses a series of enzymes and transport systems involved in sulfur catabolism (38–40). This derepression is mediated by a positive regulatory gene, cys-3, whose gene product activates the individual controlled genes on sulfur deprivation (38–40). cys-3 then is the sulfur analog of the nitrogen control locus nit-2. cys-3 mutants are missing the derepression seen in wild type on sulfur deprivation (38–40).

The cys-3 mutant strains are unable to utilize the sulfate in minimal medium as a sulfur source and therefore must be grown on an alternative sulfur source such as methionine. When a cys-3 mutant and the wild type are grown on methionine-containing medium, the absolute levels of ATP are elevated above those characteristic of wild type or nit-2 growing on standard Vogel's medium N (compare Table V with Tables III and IV). The elevated ATP pools appear to be due to the methionine medium rather than the genetic strain because elevation also occurs in wild type grown on this medium. In addition, methionine-grown wild type and cys-3 have somewhat lower GTP/ATP ratios than does wild type or nit-2 grown in standard, sulfate-containing medium. When the methionine-grown wild type is deprived of nitrogen or sulfur, GTP/ATP ratios decline in similar fashion to cultures studied earlier (compare Table V with Table II). When cys-3 is nitrogen-deprived, its GTP/ATP ratios resemble those of...
the wild type. However, when cys-3 is sulfur-deprived, several nucleotide changes occur that are dramatically different from those seen in the wild type (Table V and Fig. 2). 1) GTP/ATP ratios, instead of declining, actually increased (although not to a statistically significant extent). 2) The energy charges as measured by ATP/ADP ratios decline drastically. GTP/GDP ratios showed similar declines (data not shown). 3) Absolute levels of ATP also decline (by about 88%). 4) Other nucleoside triphosphate pools also decline drastically, as seen by multiplying absolute ATP levels by nucleoside triphosphate/ATP ratios. Essentially the whole nucleotide profile measured by HPLC declines including acetyl-CoA, four nucleotide sugars, and NAD (Fig. 2). 5) The 6-h sulfur-deprived cys-3 chromatograms (Fig. 2, lower) show two, as yet unidentified peaks which are present in relatively high amounts as compared with wild type or growing, nondeprived cys-3. One of these elutes very early in the region of nucleosides (at about 2 min), and free bases and the other migrates in the region of nucleoside monophosphates (at about 7.5 min). It should be clear then that nucleotide pool regulation in cys-3 on sulfur deprivation is extremely aberrant.

**Debilitation of Sulfur-deprived cys-3**—The low energy charge and the depletion of nucleotide pools of sulfur-deprived cys-3 might be expected to lead to debilitation or even death of the organism. In order to determine whether such debilitation was occurring, two types of experiments were performed. Six-hour sulfur-deprived mycelia of wild type and cys-3 had L-methionine (50 μg/ml) added back to the medium. Dry weight samples were taken immediately before methionine addition and at 7.5 h after methionine addition. The wild type showed 174% increase in dry weight after methionine addition, whereas cys-3 showed only a 14% increase. It should be clear then that cys-3 has a greatly decreased ability to grow after a period of sulfur deprivation.

Does sulfur deprivation actually kill cys-3? This is difficult to answer from studies of the older mycelia used in the experiments reported above because whole sections of mycelium might die while other sections still survive. In order to answer this question, conidia of wild type and cys-3 were germinated for 4 h in shaken sulfur-free medium plus methionine. These were then washed and resuspended in sulfur-free medium and shaken. Samples were taken at various times, diluted, and plated onto methionine-containing (50 μg/ml) sorbose medium plates, and colonies were counted after 24-36 h to determine viability of the germinated conidia. The results are shown in Table VI. The results show relatively little loss of viability in either wild type or cys-3.

What was also seen, however, was that the cys-3 colonies grown from sulfur-deprived germinated conidia were much delayed in their growth when compared with similarly treated wild type. We estimate that the 8.5 h sulfur-deprived cys-3 colonies were delayed roughly 6 h in their growth when compared with 8.5 h sulfur-deprived wild type. In contrast, non-sulfur starved wild type and cys-3 germinated conidia showed similar colony growth. Thus, the ability to resume growth after sulfur deprivation is greatly impaired by the cys-3 mutation.

**DISCUSSION**

GTP/ATP ratios in *N. crassa* are regulated by nutritional state, with nitrogen or sulfur deprivation producing a decline in these nucleotide ratios. The nitrogen starvation response is mediated by the wild-type nit-2 gene and the sulfur starvation response by the wild-type cys-3 gene.

Five different conclusions can be drawn from these results. 1) GTP pools or at least GTP/ATP ratios are important enough to the organism that it uses two known regulatory systems to regulate these ratios. These results then suggest that GTP pools have substantial biological significance to the cell. 2) It is the drop in GTP/ATP ratios that requires the positive regulatory role, rather than the maintenance of the ratio. Both nit-2 and cys-3 are positive regulatory genes involved in the derepression of nitrogen source and sulfur source catabolism on nitrogen and sulfur deprivation, respectively (35-40). Because mutants defective in these genes lack the GTP/ATP ratio declines normally produced by starvation, it follows that the declines require a positive regulatory role. 3) A corollary of conclusion 2 is that the GTP/ATP ratio declines are not a trivial response of the cells "feeling poorly" because they are deprived of a nitrogen or sulfur source. The nit-2 and cys-3 mutants are also nitrogen- or sulfur-deprived when placed into nitrogen- or sulfur-free medium, but each mutant fails to respond to deprivation of only one element with GTP/ATP ratio declines characteristic of the wild type. As discussed below, several other nucleotide changes are produced in nit-2 mutants, as they are in wild type. 4) The pattern of regulation is consistent with a role of GTP in stimulating anabolism and cell proliferation. GTP pools decline in response to nitrogen and sulfur deprivation and may then, in turn, trigger other changes in adjusting to the growth limitation. Conversely, in nutritional sufficiency, the increase in GTP may activate processes involved in subsequent growth.

The results presented (Tables III-V) provide information on the absolute levels of ATP, GTP, CTP, and ATP, as well as nucleotide ratios. The estimated concentration of ATP in growing cultures (averaging 2.49 μmol/ml of cell water) is in good agreement with those reported earlier (42). Absolute levels of all four nucleoside triphosphates expressed in nanomoles/milligram, dry weight, decline on nitrogen, sulfur, or carbon starvation. GTP levels decline by about 25-50% (Table VII). One central question to be discussed below is whether these declines may be of regulatory significance. The nitrogen control nit-2 mutant can be compared in its nucleotide control with that of the wild type. Its growing and sulfur-deprived cultures are essentially identical in properties to those shown in the wild type. Some properties of nitrogen-deprived cultures, ATP/ADP ratios, and absolute levels of ATP do not differ significantly from those of similarly treated wild type. However, whereas GTP/ATP and UTP/ATP ratios decline in wild type on nitrogen deprivation, the nit-2 mutant shows no decline in GTP/ATP ratios and shows substantial, significant increases in UTP/ATP ratios when similarly treated. These results show that the nit-2 gene influences cellular properties other than the previously reported (35-37) utilization of alternative exogenous nitrogen sources. Whereas it is possible to explain the pool regulation properties of the nit-2 mutant by hypothesizing that the wild-type nitrogen control system may degrade part of the GTP and UTP pools to provide a nitrogen source, sulfur source degradation cannot explain many of the properties of the cys-3 mutant.

The cys-3 mutation of the sulfur regulation system (38-40) has an even broader effect than that of nit-2. Whereas a cys-3 mutant is essentially like wild type on nitrogen deprivation, it shows multiple aberrations when it is sulfur-deprived. In addition to aberrations in GTP/ATP and UTP/ATP ratios, sulfur-deprived cys-3 shows dramatic declines in energy charge (as measured by ATP/ADP or GTP/GDP ratios) and
also dramatic declines in total nucleotide pools. During sulfur starvation, ATP pools decline by almost 50% in cys-3, whereas wild type shows a mere modest 29% decline. Whereas our data do not explain how the cys-3 transcriptional regulatory gene influences nucleotide pools, we wish to suggest one possible relationship. Sulfur-containing coenzyme A derivatives have a special role in energy metabolism, and a deficiency in the pools of such derivatives in sulfur-deprived cys-3 may explain the low energy charge that was measured.

It is clear that the cys-3 mutant is defective in the ability to resume growth on sulfur addition to sulfur-deprived cultures. Koch (43) has discussed the fact that microorganisms typically live in rapidly changing nutritional environments (fast or famine) and have probably been selected for rapid adjustment to such changing environments. The wild-type cys-3 gene and possibly other similar regulatory systems may have important roles in adjusting to such changing environments.

We report here that the cr-1 adenylate cyclase and cyclic AMP-deficient mutant of Neurospora has normal GTP/ATP ratios in growing or nitrogen-deprived cultures. We have previously reported that absolute levels of GTP are important in energy metabolism, and a deficiency in living mammalian cells. The stimulation of adenylate cyclase by hormones requires the involvement of a regulatory component, Gs, which is active only in the GTP-bound form (17, 49). Depletion of GTP pools by 50–75% in mammalian cell lines leads to a clear decline in the cellular increase of cyclic AMP levels when treated with hormone (49–51). The surprising thing about these results is that whereas GTP pools as measured here and elsewhere are typically several hundred micromolar, the Ks of the Gs for GTP is about 0.1 µM (51–53). Consequently, if the measured pool of GTP is mostly free, thermodynamically active GTP, even after a 75% decrease in GTP, there should be more than enough to saturate the Gs. Because this does not seem to be the case in vivo, it has been suggested (51, 52) that there are separate pools of GTP, and perhaps most of the GTP in cells may be bound to a large number of GTP-binding proteins including protein elongation and initiation factors and tubulin. If much of the GTP is protein-bound, changes in total GTP pools may be reflected in much larger percentage changes in the free GTP pool.

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