Identification of new regulatory genes controlling synthesis of folate-dependent enzymes in *Aspergillus nidulans*

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Prototrophic revertants of a *meth2* strain of *Aspergillus nidulans* which is impaired in the regulation of synthesis of folate-dependent enzymes were isolated and six of them analysed. In three of the isolates reversion was the result of an intragenic suppressor mutation in the *methH* locus. In the remaining strains suppressor mutations occurred in independent genes. These genes, designated *folA*, *folB* and *folC*, are linked and located in chromosome VI. Mutations in these genes render synthesis of some folate enzymes, particularly folypolyglutamate synthetase, insensitive to methionine-mediated repression.

Keywords: *Aspergillus nidulans*, folate regulatory genes

INTRODUCTION

Folates are essential cellular components serving as one-carbon units in the biosynthesis of methionine, thymidylate, the formyl group of fMet-tRNA in prokaryotes and interconversion of glycine and serine. There are several enzymes involved in folate metabolism, the core part of which is shown in Fig. 1. The regulation of the activity of these enzymes has been studied in various organisms (for reviews see McGuire & Coward, 1984; Blakley, 1984; Rowe, 1984; Santi & Danenberg, 1984; Schirch, 1984; Schirch & Strong, 1989 and references therein). Much less is known about regulation of their synthesis, particularly about regulatory genes which control these processes. Two regulatory genes, *metJ* and *metR*, have been described in *Escherichia coli* and *Salmonella typhimurium*. The former is a negative regulator of a number of methionine biosynthetic genes including *metE*, encoding the vitamin B₁₂-independent methyltransferase (for a review see Saint-Girons *et al.*, 1988). The *metR* gene product mediates the activation of the *metE* gene by homocysteine (Urbanowski & Stauffer, 1989; Byerly *et al.*, 1990) and also regulates expression of the *glyA* gene (Plamann & Stauffer, 1989).

We have found that the synthesis of folate-dependent enzymes in *Aspergillus nidulans* is regulated by intracellular pools of homocysteine and methionine, the

former acting as an inducer and the latter as a co-repressor (Nadolska-Lutyk *et al.*, 1989; Natorff *et al.*, 1993). This regulation is impaired by two, apparently allelic, mutations *meth2* and *metD10* (no recombinants among 4000 progeny in the cross *meth2* × *metD10*; Clutterbuck, 1987). Both mutants are leaky: the growth of *meth2* is stimulated only by methionine, while

Fig. 1. An outline of folate metabolism. 1, Methionine synthase (MS); 2, dihydrofolate reductase (DHFR); 3, serine hydroxymethyltransferase (SHMT); 4, methylenetetrahydrofolate reductase (MTHFR); 5, folypolyglutamate synthetase (FPGS). H₂PteGlu_n, dihydropteroylpolyglutamate; H₄PteGlu_n, tetrahydropteroylpolyglutamate; CH₂H₄PteGlu_n, methylenetetrahydropteroylpolyglutamate; CH₃H₄PteGlu_n, methyltetrahydropteroylpolyglutamate.

Abbreviations: DHFR, dihydrofolate reductase; FPGS, folypolyglutamate synthetase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SHMT, serine hydroxymethyltransferase.

metD10 also grows on betaine. The mutants show a repressed level of folylpolyglutamate synthetase (FPGS) coinciding with a ratio of folate monoglutamate to folate polyglutamates higher than that found in the wild-type (Lewandowska *et al.*, 1993, 1996).

By searching for *metH2* revertants we have isolated six methionine-independent strains, some of which show regulation of folate enzymes insensitive to methionine-mediated repression (Lewandowska *et al.*, 1996). In the present study we have characterized these strains. In three cases reversion occurred intragenically, while in the remaining three strains suppressor mutations occurred in independent but loosely linked loci, designated *folA*, *folB* and *folC*.

METHODS

Strains. The following strains of *A. nidulans* from our collection which carry standard markers (Clutterbuck, 1994) were used: *metH2*, *pyroA4*, *yA1*; *argA1*, *biA1*, *pabaA2* and *pyroA4*, *yA1*. The latter strain was used as a reference wild-type strain for enzyme assays. The *metH2* revertants, R5, R7, R8, R10, R14 and R15, which in primary tests showed altered regulation of two folate-dependent enzymes as compared with the *metH2* strain (Lewandowska *et al.*, 1996), all carried the *pyroA4* and *yA1* markers. The tester strain FGSC 465 carrying the chromosome markers *sulA1*, *acrA1*, *galA1*, *pyroA4*, *facA303*, *lacA1*, *choA1* and *chaA1* was used for allocation of newly described genes in chromosomes.

Media, culture conditions and enzyme assays. The strains were grown in liquid minimal medium (Paszewski & Grabski, 1974) which was supplemented with 5 mM L-methionine as indicated, in a rotary shaker (200 r.p.m.) at 30 °C for 18 h. Mycelia were collected on a glass filter, washed thoroughly with water and blotted on filter paper. Cell-free extracts for enzyme assays were prepared by mixing mycelial pads with equal volumes of powdered glass and grinding in a chilled mortar with 0.1 M potassium phosphate buffer (pH 7.5). The resulting slurry was centrifuged at 10000 g for 15 min at 4 °C and the supernatant used as an enzyme source. Freezing of the extracts did not affect the activity of the enzymes studied.

Folylpolyglutamate synthetase (FPGS) (EC 6.3.2.17) was assayed according to McGuire *et al.* (1980), methionine synthase (MS) (5-methyltetrahydrofolate:L-homocysteine methyltransferase; EC 2.1.1.13) was assayed as described previously (Paszewski *et al.*, 1977), serine hydroxymethyltransferase (SHMT) (EC 2.1.2.1) and methylenetetrahydrofolate reductase (MTRF) (EC 1.5.1.20) were assayed according to Mudd *et al.* (1972), and dihydrofolate reductase (DHFR) (EC 1.5.1.3) was assayed as described by Hanggi & Littlefield (1974). Protein was estimated according to Bradford (1976) with bovine serum albumin (fraction V) as a standard.

Isolation and genetic analysis of *metH2* suppressors. Conidia of the *metH2*, *pyroA4*, *yA1* strain suspended in water (10^8 conidia ml⁻¹) were irradiated by UV light (survival about 1%) and plated on minimal medium. Methionine-independent colonies were isolated and analysed genetically and biochemically. Genetic crosses followed the procedures of Pontecorvo *et al.* (1953). Genes were allocated to their linkage groups by mitotic haploidization (Forbes, 1959) facilitated by the use of benlate (Hastie, 1970).

RESULTS

Isolation of *metH2* suppressor mutations and their genetic analysis

About 50 Met⁺ colonies were obtained after mutagenesis of the *metH2*, *pyroA4*, *yA1* strain. Six of these, R5, R7, R8, R10, R14 and R15, representing different phenotypes (Fig. 2), were chosen at random. They were crossed with the wild-type. In the case of R5, R7 and R14 strains no methionine-requiring segregants were recovered among 125–150 progeny scored. These three strains grow more slowly than the wild-type on minimal medium. Their phenotypes suggest that reversion resulted from intragenic suppressors or mutations in an adjacent gene rather than from back mutations. These suppressor mutations are designated *metH2R5*, *metH2R7* and *metH2R14*. They are all dominant in heterokaryons with the *metH2* strain. Crosses of the R8, R10 and R15 strains with the wild-type gave about 25% Met⁻ segregants, indicating that reversion resulted from a suppressor mutation in an independent gene. From each cross a few Met⁺ segregants were chosen for enzymic examination. Those which showed deregulation of folate-dependent enzymes were crossed with the wild-type strain to select segregants carrying only a suppressor mutation, i.e. not segregating the *metH2* mutation, and segregants carrying both mutations but with different auxotrophic markers. The latter strains were crossed pairwise to find out how many genes represent the suppressor mutations. The results indicated that the three suppressor mutations studied occurred in different but linked genes. These genes, which we designate *folA*, *folB* and *folC*, are mutated in the R8, R10 and R15 strains, respectively.

The genes are arranged in the order *folB*–*folA*–*folC*, with 17.2% linkage between *folB* and *folA*, and 20.4% linkage between *folA* and *folC*.

The linkage values are based on 381 and 581 segregants scored in the crosses *folA8 metH2* × *folB10 metH2* and *folA8 metH2* × *folC15 metH2*, respectively. In the cross *folB10 metH2* × *folC15 metH2* 117 Met⁻ colonies were found among 428 tested, which gives a recombination frequency of 54.6%. The *fol* genes were allocated to the linkage group VI by mitotic haploidization with the use of the tester strain FGSC 465 and, additionally, with a strain carrying the *argA1* marker located in the same chromosome. As the suppressor phenotype is not always easily distinguishable we determined chromosome segregation in haploids carrying both *metH2* and the suppressor mutation. The *folA8* and *folC15* mutations are recessive, while *folB10* is semidominant, as determined in heterokaryons between the original suppressor strains and the *metH2* mutant.

Regulation of folate-dependent enzymes in *metH2* suppressor strains

The results presented in Table 1 indicate several marked differences in enzyme levels between strains carrying extragenic suppressors and the *metH2* and wild-type

Fig. 2. (a) Growth of the *meth2* (*pyroA4*, *yA1*) and wild-type (*pyroA4*, *yA1*) strains; (b) growth of strains carrying *meth2* intragenic suppressors: *meth2R5* (*pyroA4*, *yA1*), *meth2R7* (*pyroA4*, *yA1*), *meth2R14* (*pyroA4*, *yA1*); (c) growth of strains carrying *fol* mutations: *folA8 meth2* (*pyroA4*, *yA1*), *folA8* (*pyroA4*, *yA1*), *folB10 meth2* (*pyroA4*, *yA1*), *folB10* (*pyroA4*, *yA1*), *folC15 meth2* (*pyroA4*, *yA1*) and *folC15* (*yA1*). In each case growth was for 48 h at 37 °C. MM, minimal medium; MM + Met, minimal medium supplemented with 5 mM L-methionine.

strains. These differences are particularly pronounced in the case of FPGS. The mutations *folA8*, *folB10* and *folC15* result in an elevated level of this enzyme and, what is more important, relieve its synthesis, completely or partially, from methionine-mediated repression. Essentially, the same is true in the case of MS and DHFR. MTFR is elevated in all strains carrying *fol* mutations but in the case of *folB10 meth2* and *folB10* strains grown in the presence of methionine a repressed enzyme level is observed. The SHMT levels in the revertants do not appear to differ from that of the wild-type strain except for the *folA8 meth2* and *folC15 meth2* strains where the enzyme appears to be less repressed in the presence of methionine.

The results shown in Table 1 also indicate that the mode of folate enzyme regulation in the *fol* mutants is essentially similar to that observed in the *fol meth2*

strains, with the exception of strains carrying the *folA8* mutation: the *folA8/17* strain shows higher levels of MS, MTFR and DHFR. Interestingly, the two segregants from the cross *folA8 meth2* × wild-type carrying the *folA* mutation, *folA8/9* and *folA8/17*, differ in the level of MS in minimal-grown cells, indicating that genetic background may modulate the *folA* action.

The folate-dependent enzyme levels in the strains carrying intragenic suppressors are shown in Table 2. In all three strains grown in minimal medium the FPGS level is slightly higher than in the wild-type but the enzyme is repressible in the *meth2R5* and *meth2R7* strains but not in the *meth2R14* strain. On the other hand MS is not repressible in the *meth2R7* and *meth2R14* strains. In all three strains grown in minimal medium the MTFR and DHFR levels are elevated as compared to the wild-type but repressed in the presence

Table 1. Activity of folate-dependent enzymes in the wild-type, *meth2* and *fol* strains of *A. nidulans*

Results represent mean values $\pm$ SD from three or more experiments. In a few cases two experiments were performed and in three cases only one. Enzyme activities exceeding the corresponding wild-type values by more than 50 % are shown in bold type. MM, minimal medium; + Met, minimal medium supplemented with 5 mM L-methionine.

Strain	Specific activity [nmol h ⁻¹ (mg protein) ⁻¹] of:									
	FPGS		MS		MTFR		DHFR		SHMT	
	MM	+ Met	MM	+ Met	MM	+ Met	MM	+ Met	MM	+ Met
Wild-type	4.56 $\pm$ 0.26	0.76 $\pm$ 0.06	12.70 $\pm$ 0.14	6.50 $\pm$ 0.14	2.72 $\pm$ 0.10	0.86 $\pm$ 0.05	17.48 $\pm$ 0.97	8.07 $\pm$ 1.40	4266 $\pm$ 417	2236 $\pm$ 70
<i>meth2</i>	1.29 $\pm$ 0.00	0.73 $\pm$ 0.00	10.00 $\pm$ 2.00	5.90 $\pm$ 0.20	3.00 $\pm$ 0.20	1.20 $\pm$ 0.20	20.50 $\pm$ 0.50	9.50 $\pm$ 0.70	4666 $\pm$ 152	2826 $\pm$ 253
<i>folA8</i>	8.76 $\pm$ 3.50	4.71 $\pm$ 0.06	15.45 $\pm$ 1.90	12.85 $\pm$ 0.07	3.23 $\pm$ 0.15	1.03 $\pm$ 0.20	21.56 $\pm$ 0.25	9.06 $\pm$ 1.32	3686 $\pm$ 57	3092 $\pm$ 70
<i>meth2</i>										
<i>folA8/9</i>	9.80	4.35 $\pm$ 0.35	14.00 $\pm$ 0.00	8.40 $\pm$ 0.00	3.60 $\pm$ 0.34	2.00 $\pm$ 0.74	22.90 $\pm$ 0.85	12.80 $\pm$ 0.91	3891 $\pm$ 71	3081 $\pm$ 85
<i>folA8/17</i>	7.75 $\pm$ 2.37	3.55 $\pm$ 1.18	22.70 $\pm$ 0.42	17.00 $\pm$ 6.20	3.80 $\pm$ 0.00	3.26 $\pm$ 0.30	24.46 $\pm$ 0.56	21.40 $\pm$ 4.65	4406 $\pm$ 7	2771 $\pm$ 56
<i>folB10</i>	5.80 $\pm$ 0.75	2.70 $\pm$ 1.80	17.76 $\pm$ 1.09	8.70 $\pm$ 2.30	3.86 $\pm$ 0.45	0.93 $\pm$ 0.32	22.35 $\pm$ 0.68	12.50 $\pm$ 6.10	3725 $\pm$ 97	2371 $\pm$ 338
<i>meth2</i>										
<i>folB10</i>	5.30	1.50	17.10 $\pm$ 1.83	6.45 $\pm$ 0.35	4.95 $\pm$ 0.80	0.76 $\pm$ 0.05	25.46 $\pm$ 1.18	8.10 $\pm$ 1.70	4228 $\pm$ 130	2056 $\pm$ 22
<i>folC15</i>	7.80 $\pm$ 4.20	5.35 $\pm$ 0.07	21.25 $\pm$ 1.60	16.90	5.40 $\pm$ 2.12	3.20 $\pm$ 0.56	28.05 $\pm$ 4.10	19.80 $\pm$ 2.77	4489 $\pm$ 527	3126 $\pm$ 22
<i>meth2</i>										
<i>folC15</i>	12.87 $\pm$ 0.15	6.40 $\pm$ 0.46	19.60 $\pm$ 0.70	17.80 $\pm$ 0.84	6.72 $\pm$ 0.23	3.60 $\pm$ 0.16	31.40 $\pm$ 4.40	20.64 $\pm$ 1.20	4289 $\pm$ 68	3214 $\pm$ 28

Table 2. Activity of folate-dependent enzymes in the wild-type and *meth2R* strains of *Aspergillus nidulans*

Results represent mean values $\pm$ SD from three or more experiments. In a few cases only two experiments were performed. Enzyme activities exceeding the corresponding values for the wild-type strain by more than 50 % are shown in bold type. MM, minimal medium; + Met, minimal medium supplemented with 5 mM L-methionine.

Strain	Specific activity [nmol h ⁻¹ (mg protein) ⁻¹] of:									
	FPGS		MS		MTFR		DHFR		SHMT	
	MM	+ Met	MM	+ Met	MM	+ Met	MM	+ Met	MM	+ Met
Wild-type	4.56 $\pm$ 0.26	0.76 $\pm$ 0.06	12.70 $\pm$ 0.14	6.50 $\pm$ 0.14	2.72 $\pm$ 0.10	0.86 $\pm$ 0.05	17.48 $\pm$ 0.97	8.07 $\pm$ 1.40	4266 $\pm$ 417	2236 $\pm$ 70
<i>meth2</i>	1.29 $\pm$ 0.00	0.73 $\pm$ 0.00	10.00 $\pm$ 2.00	5.90 $\pm$ 0.20	3.00 $\pm$ 0.20	1.20 $\pm$ 0.20	20.50 $\pm$ 0.50	9.50 $\pm$ 0.70	4666 $\pm$ 152	2826 $\pm$ 253
<i>meth2R5</i>	4.80 $\pm$ 0.48	0.89 $\pm$ 0.19	14.46 $\pm$ 0.46	7.50 $\pm$ 0.14	3.60 $\pm$ 0.00	0.80 $\pm$ 0.00	18.65 $\pm$ 0.77	5.75 $\pm$ 0.90	2625 $\pm$ 267	1687 $\pm$ 63
<i>meth2R7</i>	5.38 $\pm$ 0.32	1.16 $\pm$ 0.27	17.46 $\pm$ 1.16	17.10 $\pm$ 0.00	3.70 $\pm$ 0.14	0.65 $\pm$ 0.07	20.75 $\pm$ 0.91	8.40 $\pm$ 0.28	2814 $\pm$ 000	1626 $\pm$ 000
<i>meth2R14</i>	5.46 $\pm$ 0.69	5.46 $\pm$ 0.28	13.80 $\pm$ 1.17	12.75 $\pm$ 0.20	4.00 $\pm$ 0.28	1.50 $\pm$ 0.14	22.35 $\pm$ 0.77	13.10 $\pm$ 1.27	2686 $\pm$ 000	1558 $\pm$ 113

of methionine. However, the repression is markedly weaker in the *meth2R14* strain. Interestingly all three strains exhibit an SHMT level lower than that found in the wild-type and *meth2* strains. This level is even lower when methionine is present in the growth medium.

DISCUSSION

We demonstrated in our earlier studies that the synthesis of a number of folate-dependent enzymes in *A. nidulans* is regulated by a system in which homocysteine acts as an inducer and methionine as a co-repressor (Lewandowska *et al.*, 1993, 1996). This regulatory system is impaired in the *meth2* and *metD10* mutants, which is particularly manifested by a repressed (or non-inducible) level of FPGS.

By isolating *meth2* revertants we selected six strains in which synthesis of folate-dependent enzymes was in-

sensitive, to differing extents, to methionine-mediated repression. In three of these strains reversion was due, most likely, to an intragenic suppressor mutation. Clearly, these mutations differ in their effect on folate enzyme synthesis. For instance FPGS and MS are not repressible in the *meth2R14* strain while in the *meth2R7* strain only the latter enzyme is. It is worth noting that the SHMT level in these strains is lower than that in the wild-type and *meth2* strain. If these variations in the regulatory effects occur at the transcriptional level they might reflect differences in the responsive regulatory elements in the promoters of the structural genes encoding particular enzymes. A similar reasoning may be applied to explain differences in effects of suppressor mutations occurring in three independent genes, designated *folA*, *folB* and *folC*. We assume as the simplest hypothesis that these are regulatory genes which control synthesis of folate enzymes although

there is no direct evidence that their primary effect is regulatory.

Expression of particular structural genes may be regulated by different transcriptional complexes which form at various promoters. The *fol* gene products altered by mutation might affect the properties of these complexes. The different protein sequences resulting from the various alleles of these genes might have specific and unpredictable effects on the modulation of regulation.

To our knowledge the *fol* genes described here, together with the *metH/metD* locus, are the first identified eukaryotic regulatory genes controlling folate metabolism, although the existence of such genes can plausibly be deduced from results obtained with other organisms. For instance a three- to fourfold increase in DHFR and thymidylate synthase transcripts observed in human cisplatin-resistant cells (Scanlon & Kashani-Sabet, 1988) can be attributed to a modulation in activity of a regulatory gene.

It appears that the system governing the regulation of folate-dependent enzymes in *A. nidulans* may be rather complex, especially taking into account the possibility that the list of genes involved may not be complete yet. This conclusion is supported by the fact that mutants representing three different loci were identified among only six mutants tested from 50 originally isolated. It is very likely that among the enzymes studied here DHFR is also regulated by cell-cycle-controlling genes. The complexity of mammalian DHFR gene promoter structure as well as the transcriptional complex involving both nonspecific and specific proteins (for review see Slansky & Farnham, 1996) is compatible with the idea of a multigene regulatory system that might control the expression of a particular structural gene. In this connection it is worth noting that genes encoding SHMT in *Neurospora crassa* (McClung *et al.*, 1992) and *Saccharomyces cerevisiae* (McNeil *et al.*, 1994) are under control of the amino acid cross-pathway regulatory system.

In our previous work (Natorff *et al.*, 1993) we found that the *scon* mutations, which impair the sulphur metabolite repression system, also lead to the derepression of a number of folate-dependent enzymes. It was shown that this derepression is a secondary effect – it results from an increased pool of homocysteine. The direct role of the *scon* gene products is the regulation of sulphur-related genes. It will be interesting, in turn, to check if the *fol* genes are specific for folate metabolism, or also affect, directly or indirectly, sulphur metabolism.

ACKNOWLEDGEMENTS

This work was supported by the Polish State Committee for Scientific Research, grant no. 6P 20300407.

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Received 21 April 1997; revised 17 June 1997; accepted 25 June 1997.