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FOLYLPOLYGLUTAMATE SYNTHESIS IN *NEUROSPORA CRASSA*: TRANSFORMATION OF POLYGLUTAMATE-DEFICIENT MUTANTS

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Key Word Index—*Neurospora crassa* wild type (FGSC 853); Ascomycetes; fungi; *met*-6 and *mac* mutants; transformation to prototrophy; folylpolyglutamates; folylpolyglutamate synthetase.

Abstract—The methionine auxotrophy of *Neurospora crassa met*-6 and *mac* mutants is related to an inability to synthesize long-chained folylpolyglutamates. Both of these lesions affect folylpolyglutamate synthetase activity, but it is not clear whether these mutations occur in different genes or in functional domains of the same gene. To address this question, copies of the *met*-6^+ gene have been introduced into both mutants using plasmid and cosmid vectors. Transformation to prototrophy was achieved in both mutants. The ability of these mutant and transformant strains to synthesize folylpolyglutamates was assessed by HPLC analysis of folate cleavage products. Mycelial extracts of the wild type revealed a folate pool dominated by folylhexaglutamates. These folates were also detected in the transformants but were lacking in both mutants. In the latter strains, the conjugated folates were mainly di- and triglutamates. When incubated for 24 hr with [^{14}C]*p*-aminobenzoate, transformant and wild type cultures synthesized long chain folates, *ca* 60–80% of these being hexaglutamyl derivatives. In contrast, the labelled folates of *mac* and *met*-6 were mainly mono- and diglutamyl derivatives, respectively. Polyglutamate synthesis was also studied *in vitro* by partial purification and characterization of mycelial folylpolyglutamate synthetase protein. Mycelial extracts of the wild type and transformant cultures utilized 5,10-methylenetetrahydrofolate monoglutamate and its diglutamate as substrates in this synthetase reaction. In contrast, extracts of *met*-6 and *mac* mycelia utilized only one of these folate substrates. Gel filtration of folylpolyglutamate synthetase protein indicated apparent M_r values of *ca* 66 000 in all strains. It is suggested that polyglutamate synthesis in *Neurospora* is probably mediated, as in other eukaryotic species, by a single folylpolyglutamate synthetase protein.

INTRODUCTION

Folate coenzymes serve as carriers of one-carbon units in a variety of reactions involved in amino acid and nucleotide metabolism, collectively known as one-carbon metabolism. These reactions are preferentially mediated by folylpolyglutamates, the predominant intracellular form of folate [2–5]. The sequential addition of L-glutamate moieties to monoglutamyl folates (Eqn 1) is catalysed by folylpolyglutamate synthetase [FPGS; tetrahydrofolate: L-glutamate gamma-ligase (ADP forming), EC 6.3.2.17]†. Most folate-dependent enzymes have greater affinities for these polyglutamates and there is evidence that they are important in the cellular retention of folate [6, 7].

The synthetases of prokaryotes [8–11] and mammalian tissues [12–18] have now been largely characterized. Studies of this enzyme have drawn attention to mutations of FPGS genes that result in polyglutamate deficiencies [11, 13, 19–28]. In addition, some of these studies have involved transformation of mutant cell lines. Thus, various wild type FPGS genes restore ability to express this enzyme activity [11, 22, 24–26] and folylpolyglutamates are synthesized *in vivo* [26–28]. In this regard, recent studies show that the level of FPGS expression in mammalian cells affects the chain length of the endogenous polyglutamate pool [28]. In addition, transfection of mutant cell lines by genes of bacterial or human origin dramatically affects the polyglutamate status of the folate pool [28].

In *Neurospora crassa*, polyglutamate synthesis is affected by mutations at the *met*-6 and *mac* loci [29–34]. Consequently, these mutants fail to generate polyglutamates *in vivo* and display methionine auxotrophy. Based on alterations of FPGS activity in these mutants it has been suggested [29, 33] that polyglutamate synthesis may involve more than one synthetase activity. The first activity, detected in the *met*-6 mutant [33], produces folyldiglumate (Eqn 2), while the second activity, found in the *mac* mutant [33], forms longer chained folylpolyglutamates from $H_4PteGlu_2$ (di-γ-glutamate derivative)

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The abbreviations for folate derivatives are those suggested by the IUPAC-IUB Commission, as summarized in ref. [1].

(Eqn 3).

$$H_4PteGlu_n + ATP + \text{L-glutamate} \rightarrow H_4PteGlu_{n+1} + ADP + P_i \quad (1)$$

$$H_4PteGlu + ATP + \text{L-glutamate} \rightarrow H_4PteGlu_2 + ADP + P_i \quad (2)$$

$$H_4PteGlu_2 + 4ATP + 4\text{L-glutamate} \rightarrow H_4PteGlu_6 + 4ADP + 4P_i \quad (3)$$

The genetic relationships between *met*-6 and *mac* have not been fully elucidated. For example, both *mac* and another allele, *met*, complement *met*-6, but *mac* and *met* do not complement each other [35]. On the other hand, in high resolution recombination studies, *met*-6 and *met* behaved as alleles of the same gene, while *mac* appeared to be a very closely linked gene [35]. Thus, the two FPGS activities observed in the mutants [33] might define different genes or separate functional domains of the same gene. It is also not clear whether these FPGS activities are associated with distinct proteins in the wild type.

As an extension of our earlier work [36] this paper reports the cloning of the *Neurospora crassa met*-6^+ gene by functional complementation of the *met*-6 mutant. Transformation of both *met*-6 and *mac* mutants resulted in prototrophy and the expression of an FPGS activity that produced polyglutamates from mono- and diglutamylfolate substrates.

RESULTS AND DISCUSSION

Transformation of polyglutamate-deficient mutants

In order to study the nature of the two FPGS activities (Eqs 2 and 3), both *met*-6 and *mac* cells were transformed to restore the wild type phenotype. Cosmid vectors (a gift from Dr T. Schmidhauser, Southern Illinois University) containing the wild type *met*-6^+ gene and a benomyl resistance gene for selection, were isolated by complementation of *met*-6 spheroplasts [37, 38] to restore polyglutamate synthesis. Both mutants were successfully transformed as evidenced by prototrophy and growth in the presence of benomyl. With the use of various restriction endonucleases to divide the insert, the *met*-6^+ gene was subcloned into recombinant plasmid vectors which were then used for subsequent transformations. A 3.5 kb genomic DNA fragment was isolated which was able to rescue both mutants to prototrophy, indicating that the two loci were in close proximity, Furthermore, Kpn I cleavage of the 3.5 kb fragment into two smaller fragments of 0.7 and 2.7 kb virtually eliminated transformation in both mutants. Since most insertions are ectopic in *Neurospora*, it is unlikely that either of the small fragments would replace the non-functional, mutant region of the affected locus. Therefore, we conclude that the Kpn I cleavage disrupted a single genetic locus. This implies that *met*-6 and *mac* may actually represent alterations to a single gene.

HPLC analysis of endogenous polyglutamates

The folylpolyglutamate chain lengths of the wild type, mutants and transformant strains were examined by quantitative reductive cleavage of the folates to *p*-ABAGlu$_n$ (*p*-aminobenzoate polygutamate derivative, where n = the number of L-glutamate moieties) [39]. In these analyses, extracts were prepared from cultures harvested 24 hr after spore inoculation. The *p*-ABAGlu$_n$ derivatives were purified, separated by HPLC and quantified by their absorbance at 280 nm.

In agreement with our earlier microbiological assays [34], the data in Table 1 suggest that the folates of *N. crassa* wild type are almost exclusively pteroylpolyglutamates. As in other species [2, 4], an individual polyglutamate chain length dominated the folate pool. In *Neurospora* this polyglutamate was a hexaglutamate. In contrast, highly conjugated folates represented less than 10% of the polyglutamates detected in *met*-6 and *mac* (Table 1). In these mutants, the conjugated folates were mainly di- and triglutamates. Our earlier assays [34] showed that monoglutamyl folates were the major derivatives in the *mac* mutant. Unfortunately, *p*-ABAGlu, arising by cleavage of these derivatives, was not clearly resolved by the HPLC procedure to allow accurate quantitation by A_{280} measurement. As a result, the A_{280} data in Table 1 only give the distributions of di- to hexaglutamyl folates. The possible contribution of monoglutamyl derivatives to these folate pools was therefore assessed by [^{14}C]*p*-ABA (*p*-aminobenzoate) feeding experiments (see below). Transformation of *met*-6 and *mac* was associated with the generation of long chain polyglutamates with the major derivative being the hexaglutamate (Table 1). Tetra- and pentaglutamates occurred in significant amounts in both transformants. These analyses are in agreement with transfection and transformation studies of mammalian cells [24–28] where FPGS expression and synthesis and conjugated folates relieved multiple auxotrophic requirements for compounds related to one-carbon metabolism. The loss of methionine auxotrophy in the *Neurospora* transformants and their ability to form long-chained polyglutamates *in vivo* suggests that FPGS expression and folate synthesis may be comparable to that occurring in the wild type. These possibilities were examined in further experiments.

Incorporation of [^{14}C]p-*ABA into folylpolyglutamates*

Folate distributions were also examined after administration of [^{14}C]*p*-ABA. This allowed an assessment of folate biosynthesis during mycelial production. The growth media of the wild type, mutants and transformed strains were supplemented with ring-labelled [^{14}C]*p*-ABA, and the folates extracted after 24 hr. These conditions result in an extensive labelling of the endogenous folate pools of *N. crassa* [34]. In the present work, these folates were cleaved to *p*-ABAGlu$_n$ derivatives [39] and isolated by HPLC. Labelled *p*-ABA was incorporated primarily into hexaglutamyl folate in the wild type (Table 1); only minimal labelling was detected in less highly conjugated derivatives. The labelled folates of both

Table 1. Net distribution of intracellular folates by HPLC analysis of *p*-ABA polyglutamates

Strain and method	Folates recovered		Polyglutamate chain length (%)					
of folate analysis	A_{280}*	[^{14}C]*p*-ABA†	1	2	3	4	5	6
1. Wild type								
A_{280}	60.20		—	n.d.	n.d.	0.7	2.0	97.3
^{14}C		1.95	2.7	2.2	4.6	4.0	12.4	74.0
2. *Met*-6 mutant								
A_{280}	52.54		—	71.1	13.8	11.6	2.6	0.8
^{14}C		0.10	0.8	49.2	22.0	18.6	10.2	n.d.
3. *Mac* mutant								
A_{280}	44.28		—	84.7	6.0	2.5	3.6	3.2
^{14}C		0.14	91.3	8.8	n.d.	n.d.	n.d.	n.d.
4. *Met*-6 transformant								
A_{280}	71.56		—	7.2	7.5	11.6	13.7	60.1
^{14}C		1.13	0.3	n.d.	4.0	5.5	7.3	82.9
5. *Mac* transformant								
A_{280}	100.09		—	3.3	2.6	8.7	13.9	71.6
^{14}C		1.58	4.0	0.1	3.8	7.3	9.8	62.5

Values are based on triplicate analyses.
*Data are expressed as pmol *p*-ABAGlu$_n$ (n = 2–6) detected from extracts per g fresh weight.
†Data are expressed as pmol [^{14}C] *p*-ABA incorporated per g fresh weight.
n.d. = Not detected.
— = Monoglutamate derivative not clearly resolved from background absorbance.

Table 2. Fractionation of *Neurospora* FPGS activity

	Wild type		Mutants				Transformants			
			Met-6		*Mac*		*Met*-6		*Mac*	
Fractionation step	Activity	Yield	Activity	Yield	Activity	Yield	Activity	Yield	Activity	Yield
1. Crude extract	0.17	100	0.17	100	0.07	100	0.21	100	0.14	100
2. Streptomycin SO_4	0.17	99	0.16	109	0.07	106	0.20	104	0.13	101
3. 45–70% $(NH_4)_2SO_4$	1.26	181	0.57	132	0.27	150	0.99	189	0.69	207
4. Sephadex G-200	6.92	76	9.64	75	3.43	63	6.10	58	5.04	74

Mycelia (*ca* 40 g fresh weight) were harvested after 24 hr growth in Vogels minimal medium at 30°. Enzyme activity is expressed in pkat L-glutamate incorporated into 5,10-CH_2-H_4PteGlu per mg protein. The substrate for the *mac* mutant was 5,10-CH_2-H_4PteGlu$_2$. Yield is expressed as a per cent. Increase of activity in step 3 protein was associated with the removal of an FPGS inhibitor (ref. [41]).

mutants lacked the hexaglutamate. When compared to the wild type, the *met*-6 cultures incorporated less total [^{14}C] into folate derivatives, and label was mainly associated with di- and triglutamates (Table 1). A similar distribution of the label occurred in the *mac* mutant (Table 1), but in this case, the monoglutamate derivative was a significant product. These data, and that of earlier work [34, 40], suggest that each mutation resulted in polyglutamate deficiency. The reason for the decreased incorporations of [^{14}C]*p*-ABA by these mutant strains as compared to the wild type is not clear. Previous studies on the effects of media supplements on polyglutamate labelling [40] suggest that exogenous methionine in the media of the *met*-6 and *mac* cultures may account for part of the observed decrease. Furthermore, the absence of long-chained polyglutamates in *met*-6 and *mac* (Table 1) might affect the cellular retention of labelled folates [6, 7] and reduce the amount of [^{14}C] recovered in the harvested mycelia.

Transformation and rescue of the mutant strains re-established the dominance of hexaglutamate as the major labelled folate (Table 1). It is also clear that both transformants had polyglutamate distributions that were similar to those of the wild type. Thus, extracts of both transformants had relatively large percentages of the recovered [^{14}C] in *p*-ABAGlu$_6$. Also, significant amounts of label were associated with tetra- and pentaglutamates in these cultures. Thus, the 3.5 kb insert, used in these transformations, appeared to relieve the polyglutamate deficiencies of both *met*-6 and *mac*.

Fractionation of FPGS activity and general properties

FPGS, which may regulate folylpolyglutamate levels in *Neurospora*, was partially purified and characterized using extracts of the wild type, mutants and transformants. A protein fractionation, employing $(NH_4)_2SO_4$ precipitation and gel filtration, gave *ca* 40-fold partial purification of the FPGS activity in each case (Table 2).

Table 3. FPGS activities and native molecular weights

Strain	Folate Substrate*		Molecular Weight† $\times 10^3$
	$CH_2H_4PteGlu_1$	$CH_2H_4PteGlu_2$	
1. Wild type	1.264	0.193	65.72
2. *Met*-6 mutant	0.501	n.d.	66.35
3. *Mac* mutant	n.d.	0.281	64.65
4. *Met*-6 transformant	0.938	0.198	67.21
5. *Mac* transformant	0.706	0.146	62.76

*Data are expressed as pkat of L-glutamate incorporated into 5,10-CH_2-H_4PteGlu per mg protein. $(NH_4)_2SO_4$ 45–70% fractions were desalted prior to assay.

†As calculated from the linear regression log $(M_r) = -0.8706\ (V_e/V_o) + 3.5002$ from a curve of known molecular weight protein standards. Average of three independent extractions (see Experimental).

n.d. = Not detected.

Furthermore, the specific enzyme activities were comparable after the Sephadex G-200 step. The ability of these proteins (step 3) to incorporate [^{3}H]-glutamate into 5,10-CH_2-H_4PteGlu$_n$ (5,10-methylenetetrahydropteroylmonoglutamate [n = 1 and 2]) is summarized in Table 3. In common with the wild type, FPGS protein isolated from the transformants was able to utilize both of these folate substrates. On the other hand, FPGS protein of *met*-6 and *mac* used only 5,10-CH_2-H_4PteGlu$_1$ or 5,10-CH_2-H_4PteGlu$_n$, respectively (Table 3). Although we did not analyse the chain lengths of the polyglutamates formed in these reaction systems it follows from our earlier work [33, 36] that the wild type FPGS protein would form 5,10-CH_2-H_4PteGlu$_n$ (n = 3–5) from the diglutamate substrate. It may also be assumed, from the data in Table 3, that the *met*-6 and *mac* transformants converted 5,10-CH_2-H_4PteGlu$_2$ into polyglutamates with at least three glutamate residues. We therefore conclude that the DNA insert used in these transformations allowed the expression of FPGS protein whose activity was complementary to that expressed by either mutant.

When samples of step 3 protein (Table 2) were chromatographed in the presence of marker proteins (Table 3) it was clear that wild type and transformant FPGS protein had an apparent M_r of 66 000 ± 4000. This value is in close agreement with our earlier SDS–PAGE analyses of this protein [36] from the wild type. These gel filtration studies of wild type and transformant FPGS protein also provided evidence for only a single peak of FPGS activity that catalysed the production of labelled polyglutamates from 5,10-CH_2H_4PteGlu$_1$ and its diglutamate (data not shown). Thus, we could find no direct support for the possibility that polyglutamate synthesis in these strains was catalysed by two synthetase proteins of distinct molecular size. The apparent M_r values for FPGS protein isolated from the mutants also averaged 66 000 as shown in Table 3. This implied that each mutation, although affecting the nature of the FPGS reaction, had little apparent effect on the overall size of the native protein.

The present studies suggest that the *met*-6 and *mac* mutations probably occur in a single, FPGS-encoding gene. The changes in nucleotide sequence that produce these mutations are now being investigated in our laboratory. Based on similar work on the *folC* gene that encodes FPGS and dihydrofolate synthetase in *E. coli* [42], it is conceivable that *met*-6 and *mac* are produced by point mutations that affect the active site. In this regard, site directed mutagenesis of *folC* has shown that single amino acid substitutions can profoundly affect the affinity of bacterial FPGS for glutamate and various folate substrates [42]. Similar studies of the *Neurospora met*-6$^+$ gene now appear warranted.

EXPERIMENTAL

Chemicals. Reagent grade chemicals were supplied by Sigma Chemical Co., St. Louis and Fisher Scientific, Edmonton. Folylpolyglutamates (n = 1–7) and *p*-aminobenzoylpolyglutamates (n = 1–7) markers were supplied by Dr B. Schircks Laboratories, Jonas, Switzerland. [6*R*, *S*]-tetrahydrofolates (n = 1,2) were generated by catalytic conversion of folic acid [43]. [U-^{3}H]-L-glutamate, supplied by Amersham-Searle, was diluted with L-glutamate to give a sp. act. of 25 μCi μmol^{-1}. Ring labelled [^{14}C]*p*-ABA, purchased from Sigma, was dissolved in H_2O to give a final concn of 100 μCi ml^{-1} with a sp. act. of 6.8 mCi mmol^{-1}. BioGel P2 (200–400 mesh) was purchased from Bio-Rad Laboratories, Richmond, California. M_r marker proteins were supplied by Sigma. DE-52 anion exchange cellulose and Partisil 10 SAX HPLC columns were purchased from Whatman Biosystems Ltd. Protein was measured using the method of Bradford [44] by the Bio-Rad Protein assay kit. In experiments involving [^{3}H] and [^{14}C], Bray's scintillation fluor [45] was used to measure radioactivity in a Beckman LS6000TA liquid scintillation counter.

Strains and culture conditions. The wild type of *N. crassa* (Lindegren A, FGSC 853), and 2 methionine-requiring mutant strains (*met*-6, FGSC 1330; *mac*, FGSC 3609) were maintained and cultured in liquid or solid Vogels medium [46]. The mutant cultures received supplements of 0.2 mM L-methionine. Transformant strains m6-T4: 11, m6-TIA50, mac-T4: 11 and mac-TIA50 re-

ceived supplements of benomyl (0.5 $\mu g\,ml^{-1}$). Control strains m6-pSV50 and mac-pSV50 received supplements of benomyl and methionine. The *N. crassa* cosmid clone pSV50-4.11.C (a gift from Dr T. Schmidhauser, Southern Illinois University containing the *met*-6^+ gene) constructed in the cosmid vector pSV50, was maintained in *E. coli* strain DH1 [47]. Bacterial plasmid pUC19, as well as recombinant clones from this plasmid were maintained in *E. coli* strain JM 83 [48].

Construction of the Met-6^+ *recombinant plasmid.* Small-scale plasmid prepns were performed according to the methods of Birnboim and Doly [49]. Restriction endonuclease digestion, plasmid isolation, CsCl-ethidium bromide gradient centrifugation and *E. coli* transformations were performed according to manufacturers, instructions or as described by Maniatis *et al.* [50]. A 3.5 kg *Eco R1/Bam H1* digested fragment was isolated from 0.8% submarine agarose gels in 0.1 M Tris–acetate pH 8.3, 2 mM EDTA, using either electroelution or a Geneclean protocol from Bio 101 Inc. When isolated by electro-elution the DNA was further purified by 1 extraction with phenol, 1 extraction with $CHCl_3$–isoamyl alcohol (24:1), pptd with EtOH and dried. Purified DNA was resuspended in the H_2O and ligated into plasmid pUC 19, designated pIA50. The 3.5 kb Eco R1/Bam H1 fragment was digested using the enzyme *Kpn* 1, and the fragments were used to transform the *met*-6 mutant spheroplasts. In addition, each fragment was ligated into plasmid pUC 19, designated pIA62 (0.7 kb insert; Eco R1/Kpn 1 fragment) and pIA68 (2.7 kb insert; Kpn 1/Bam H1 fragment), and these were also used in subsequent transformations.

Transformation of mutants to the wild type phenotype. The *Neurospora* transformation procedure was that of ref. [37] with the modifications described in ref. [38]. *Met*-6 and *mac* conidia were germinated 7 hr before spheroplasts were prepd. After transformation, plates were incubated at 30° for 36–48 hr. Individual transformed colonies were isolated from agar plugs and cultured at 30° on slants containing solidified Vogels minimal medium with 0.25 $\mu g\,ml^{-1}$ benomyl.

Folate extraction, cleavage and HPLC analysis. Liquid cultures (3 l) were inoculated with *ca* 3×10^7 conidia and grown with aeration at 30° for 24 hr [33]. In studies of folate synthesis, 500 ml cultures received [^{14}C]*p*-ABA (10 μCi, 68 μmol) followed by growth as above. Mycelia were harvested by vacuum filtration, the pad was washed (200 ml sterile ddH_2O), and folates were extracted. Transformant cultures were routinely supplemented with 0.5 $\mu g\,ml^{-1}$ benomyl. Mycelial samples (*ca* 40 g fr. wt) were finely cut and heated at 100° for 10 min in 25 ml of 20 mM KPi (pH 7.0) containing 50 mM 2-mercaptoethanol. After cooling and hand grinding with *ca* 2 g acid washed sand, the extract was heated to 100° for 10 min, cooled and centrifuged at 15 000 *g* for 10 min. Folates in the supernatant frs were quantitatively cleaved to *p*-ABAGlu$_n$ purified as azo dyes, reconverted to *p*-ABAGlu$_n$, and subjected to HPLC analysis [39]. Quantitative determinations were based on calibration curves that related peak area to nmol *p*-ABAGlu$_n$ injected [51].

Fractionation of cytosolic FPGS activities. Mycelia (100 g fr. wt) were homogenized in 250 ml of extraction buffer 20 mM K_2PO_4 buffer (pH 7.4) containing 50 mM 2-mercaptoethanol, 1 mM PMSF and 2 mM benzamidine. The homogenate was clarified by centrifugation, soluble protein was fractionated with streptomycin sulphate, and FPGS protein was recovered with ammonium sulphate at 45–70% satn [36]. This procedure effectively removed mitochondrial FPGS protein, which ppd at 0–35% satn [33]. The resulting pellet was redissolved in 5–15 ml extraction buffer, and the extract was applied to a 2.6×80 cm column of Sephadex G-200 (Pharmacia LKB Biotechnology Inc.). Protein was eluted at a flow rate of 0.5 ml min^{-1}, and frs of 3 ml were collected and assayed for FPGS activity (see below). Determinations of M_r were based on V_e values of the following marker proteins: beta-amylase (200×10^3), alcohol dehydrogenase (150×10^3), bovine serum albumin (66×10^3), carbonic anhydrase (29×10^3), and cytochrome c (12.4×10^3).

Assay of FPGS activities. FPGS assay was based on the incorporation of [^{3}H] glutamate into folylpolyglutamates [16]. The standard reaction system contained: Tris–HCl pH 9.7, 100 μmol; $MgCl_2$, 5 μmol; ATP, 5 μmol; [*R*, *S*]H_4PteGlu, 0.1 μmol; 2-mercaptoethanol, 100 μmol; L-glutamate, 1.5 μmol containing 2.5 μCi of [^{3}H]glutamate; and extract protein. The reaction tubes were purged with H_2 and incubated at 37° for 2 hr. The reaction was terminated by adding 3 ml of 30 mM 2-mercaptoethanol followed by incubation at 100° for 5 min. After centrifugation to remove denatured protein, [^{3}H]folylpolyglutamate products were isolated by ion exchange chromatography on DE-52 cellulose [14], and assessed for radioactivity, as noted above. FPGS activity was corrected for glutamate incorporation that was not folate dependent.

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