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Isolation and Characterization of Glycerol-3-Phosphate Dehydrogenase-Defective Mutants of *Neurospora crassa*

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Three glycerol-nonutilizing mutants deficient in the mitochondrial glycerol-3-phosphate (G3P) dehydrogenase (EC 1.1.99.5) were isolated from *inl*^{ts} derivatives of *Neurospora crassa* following inositolless death at elevated temperatures on minimal glycerol medium. These mutants failed to grow on glycerol as a sole carbon source, but could grow on acetate, glucose, or mannitol media and were female fertile in genetic crosses, thereby distinguishing them from the previously reported polyol-protoperithecial defective *Neurospora* mutants. In addition, these *glp* mutants exhibited a distinct morphological alteration during vegetative growth on sucrose slants and colonial growth on sorbose-containing semicomplete medium. The *glp-2* locus was assigned a location between *arg-5* and *nuc-2* on chromosome IIR on the basis of two-factor crosses and by duplication coverage by insertional translocation ALS176, but not NM177. All mutations were allelic as judged from the absence of both complementation in forced heterokaryons and genetic recombination among *glp-2* mutations. The reversion frequency of all three mutations was less than 10^{10}, indicating probable deletions in these strains. No G3P dehydrogenase activity could be detected in either cytosolic or mitochondrial extracts from mutant strains grown on glycerol, glucose, or galactose media. These results suggest that the *glp-2* locus may be the structural gene for both the cytosolic and mitochondrial forms of G3P dehydrogenase or for a cytosolic precursor of the mitochondrial G3P dehydrogenase. The defect is specific for the G3P dehydrogenase since normal activities of the mitochondrial cytochrome oxidase and succinate dehydrogenase and the cytosolic glycerol dehydrogenase and dihydroxyacetone phosphate reductase are detected in mutant extracts. During attempted growth of *glp-2* mutants on glycerol media, there was an accumulation of G3P in culture filtrates, a reduction in the mycelial growth rate, and a decreased level of glycerokinase induction.

The utilization of glycerol by *Neurospora crassa* requires at least two inducible enzymes, a cytosolic glycerokinase (ATP:glycerol phosphotransferase, EC 2.7.1.30) and a mitochondrial glycerol-3-phosphate (G3P) dehydrogenase (*sn*-G3P:(acceptor) oxidoreductase, EC 1.1.99.5) (3). The G3P dehydrogenase is located on the mitochondrial inner membrane and apparently requires one or more translational products of the mitochondrial genome since the rate of increase of this enzyme during glycerol induction is inhibited by chloramphenicol (4), a known inhibitor of mitochondrial-specific protein synthesis (13, 18, 27). These features of the G3P dehydrogenase are similar to those described for the cytochrome oxidase and ATPase of the mitochondrial inner membrane which have been shown to be heteropolymers consisting of polypeptide components synthesized on either cytosolic or mitochondrial ribosomes (1, 10, 11, 14, 18, 29-31). However, in contrast to these essential mitochondrial enzyme systems, G3P dehydrogenase does not appear necessary for growth in acetate medium (3, 5), and therefore mutants might be obtained which affect the structure and regulation of this inducible enzyme.

Glycerol-nonutilizing fungal mutants have been previously isolated, but these reports have been limited to a description of a selective procedure and genetic mapping of mutations affecting glycerokinase in *N. crassa* (20, 31), polyol utilization in strains derived from *N. tetrasperma* (31), and the isolation of glycerol nonutilizing mutants in *Saccharomyces cerevisiae* affecting either the glycerokinase or the G3P dehydrogenase (28), although the induced synthesis of this latter enzyme presented several differences from that of the homologous *Neurospora* enzyme. Glycerol-nonutilizing mutants have been previously isolated from mutagenized

N. crassa (20), but these procedures employed a slowly growing colonial mutant whose phenotype was not readily amenable to routine biochemical analyses necessary for the screening of numerous putative mutants for deficiencies of mitochondrial enzymes. Since strains defective in G3P dehydrogenase may present an opportunity to examine the involvement of nuclear and/or mitochondrial genes in the synthesis and assembly of this mitochondrial enzyme, we have set out to establish the necessary conditions and methodology for the isolation and characterization of novel *Neurospora* mutants which are solely deficient in G3P dehydrogenase. Consequently, this paper reports on the isolation and genetic analysis of G3P dehydrogenase-deficient mutants derived from *inl*^{ts} derivatives of *N. crassa*. The growth properties and morphological characteristics of these mutants are also presented and should prove useful in the isolation of additional Glp mutants.

MATERIALS AND METHODS

***Neurospora* strains.** A list of the genetic strains of *N. crassa* used in these experiments is given in Table 1. All *glp-2* mutant strains employed in these studies were heterokaryon compatible with the *arg-12* Cde tester, and strains 33-14 and 33-17 have been deposited with the Fungal Genetics Stock Center, Arcata, Calif.

Growth of cells. The wild-type and mutant *Neurospora* were grown in appropriately supplemented mineral salts medium of Vogel (32) containing either 40 mM Na-acetate, 100 mM respective sugar, or 100 mM glycerol as the sole carbon source as previously described (3, 5). Amino acids were autoclaved separately and added at a final concentration of 50 to 100 μg/ml. Where necessary, media were solidified with either 1.5% agar (Difco) or 0.5% agarose (Sigma Chemical Co.). Minimal acetate or glycerol plates contained 0.1% L-sorbose. Linear growth rates were measured in tubes (1.2 [inside diameter] by 32 cm) containing 15 ml of solidified agarose medium. Single conidial derivatives were routinely isolated; Glp-2$^+$ and Glp-2$^-$ strains can be readily distinguished on the basis of colony morphology on solidified antibiotic medium no. 2 (Difco) modified to contain Vogel-Bonner mineral salts, 0.15% yeast extract, 0.3% peptone, 0.075% beef extract, 1% glycerol, and 0.35% L-sorbose (added after autoclaving), termed SGS medium. Cornmeal agar plus glucose (Difco) was used for genetic crosses.

Isolation of glycerol-nonutilizing mutants. Several attempts were made to isolate glycerol-nonutilizing mutants by means of filtration enrichment followed by plating of the survivors on minimal acetate plates, but no glycerol-nonutilizing mutants were found by this procedure. Since these difficulties might

TABLE 1. *Neurospora* strains

Strain	Marker[a]	Mating type	Source
74A	Wild type	A	FGSC[b]
74-4	*glp-3(C1)*[c]	A	J. B. Courtright
285	*inl*^{ts}*(8320lt) ylo-1(Y30539y)*	A	FGSC
L26	*inl*^{ts} *ylo-1 glp-2(GL)*	a	This study (285)
33	*inl*^{ts} *ylo-1 glp-3*	a	74-4 × 285
33-14	*T(II-V)inl*^{ts} *ylo-1 glp-3 glp-2(D14)*	a	This study
33-17	*inl*^{ts} *ylo-1 glp-3 glp-2(D17)*	a	This study
14-44	*T(II-V)inl*^{ts} *glp-2*	A	33-14 × 1290
17-53	*glp-2*	A	33-17 × 1290
17-8	*glp-2*	a	33-17 × 1290
1750	*glp-2 pan-1*	a	17-53 × 2661
234	*glp-1(234)*	a	H. G. Kolmark
997	*T(I; II)4637 al-1; T(IV; V)R2355 cot-1; T(III; VI)1, ylo-1*	A	FGSC
312	*arg-5(27947) fl(L)*	A	FGSC
1290	*cys-3(P22) arg-5(27947)*	A	FGSC
2282	*un-5(b39t) al-2(15300) arg-13(RU3)*	A	FGSC
2661	*pan-1(5531) al-2(15300)*	a	FGSC
53	*aro-3(C163)*	a	FGSC
37	*pe(Y8743m)*	A	FGSC
1527	*arg-12(UM107) het Cde*	a	FGSC
2415	*T(II-V)ALS176*	a	FGSC
2533	*T(IIR-IL)NM177 aro-1 al-2*	A	R. L. Metzenberg
2537	*T(IIR-IL)NM177 aro-1 al-2*	a	R. L. Metzenberg

[a] Abbreviations: *al*, albino; *arg*, arginine; *aro*, aromatic; *cot*, colonial; *ts*, temperature sensitive; *cys*, cysteine; *fl*, fluffy; *glp*, glycerophosphate; *het*, heterokaryon compatibility; *inl*, inositol; *pan*, pantothenic acid; *pe*, peach; *un*, unknown; *ylo*, yellow; *T*, translocation.

[b] FGSC, Fungal Genetics Stock Center.

[c] Allele designations in parentheses.

be attributed to the poor vegetative growth of wild-type strains on minimal glycerol, a *glp-3 inl*^{ts} derivative (Table 1, strain 33) of the previously characterized *N. crassa* strain 74-4, which does not conidiate on liquid glycerol medium (5), was constructed, and additional attempts at mutant isolation were carried out with this strain by a modification of the inositolless death technique of Lester and Gross (15).

Strain 33 (Table 1) at a concentration of 5×10^8 conidia per ml was irradiated for 4 min with a GE germicidal lamp at an intensity of 2,000 ergs per cm per s, resulting in a survival of approximately 20%. Germination following irradiation was initiated by inoculating 10^8 conidia into 100 ml of minimal sucrose medium and incubating on a reciprocating shaker for 4 h. The conidial suspension was centrifuged, and the conidia were washed twice in mineral salts (without a carbon source) and finally inoculated at a concentration of 10^6 conidia per ml into several 250-ml Erlenmeyer flasks containing 50 ml of minimal glycerol without inositol. Incubation was continued with shaking at 35°C, and the culture was filtered whenever visible growth was noted. After 96 h, and at 12-h intervals thereafter, 1-ml aliquants were pipetted onto minimal acetate plates containing 0.1% L-sorbose. Colonies which grew on these plates were transferred to sucrose slants and subsequently tested for growth on liquid minimal glycerol tubes (2 ml per tube). Putative mutants which failed to grow on glycerol were rechecked for their ability to grow on acetate, sucrose, or glycerol media.

The search for glycerol-nonutilizing mutants after inositolless death of strain 33 on minimal glycerol medium (see above) resulted in the isolation of two derivatives, 33-14 and 33-17, which grew on acetate and glucose media but which were unable to use glycerol as a sole carbon source. The strains 14-44, 17-8, and 17-53 were later derived from these original glycerol nonutilizers and were similarly found defective in their ability to use glycerol as a sole carbon source.

In later experiments, it was found that semicomplete SGS medium provided another means of distinguishing glycerol-nonutilizing *glp-2* type mutants from wild type. Thus, in a separate experiment, conidia from strain 285 were similarly irradiated and subjected to inositolless death at 35°C in minimal glycerol. After incubation at 35°C, the survivors were plated on SGS medium, and those colonies which exhibited a morphology characteristic of strain 17-53 were transferred to sucrose slants and subsequently tested for their growth on minimal glycerol. Although several colonies exhibited reduced growth on glycerol, only one, strain L26, had properties which were similar to those of strains 14-44 and 17-53 (Table 2). The analysis of this mutant is also included in several experiments described in this paper.

Preparation of extracts. For the assay of glycerokinase and G3P dehydrogenase in cell extracts, mycelia were ground in sucrose-KCl-glycerol-mercaptoethanol (SKGB) (3) at 4 to 10°C with sand, and crude cytosolic and mitochondrial fractions were obtained after centrifugation (3). Mitochondria were suspended and centrifuged at 30,000 × *g* for 20 min before assay. All assays were completed within 15 h from the start of any given extraction.

TABLE 2. *Growth of wild type and glp mutants on various carbon sources*

Strain grown on:	Growth rate (cm/day)	Mycelial yield (mg/50 ml)
Acetate		
74A	2.9	37
14-44	1.8	18
17-53	2.4	28
L26	3.1	21
Glucose		
74A	6.7	148
14-44	4.8	107
17-53	4.1	97
L26	5.0	143
Glycerol		
74A	2.6	30
14-44	0.0	0
17-53	0.0	0
L26	0.0	0
Mannitol		
74A	3.7	125
14-44	0.9	4
17-53	0.9	8
L26	3.4	15

Enzyme assays. The activity of glycerokinase, G3P dehydrogenase, and succinate dehydrogenase (EC 1.3.99.1) were determined in the previously described spectrophotometric assays (3). The activities of the NADH-dependent dihydroxyacetone phosphate (DHAP) reductase (*sn*-G3P:NAD 2-oxidoreductase, EC 1.1.1.8) (5) and NADP-dependent glycerol dehydrogenase (glycerol:NADP 2-oxidoreductase, dihydroxyacetone forming, EC 1.1.1.6) (31) were determined in spectrophotometric assays. It should be noted that the DHAP reductase can be assayed only as the DHAP-dependent oxidation of NADH; no NAD-dependent G3P dehydrogenation can be demonstrated with any assays employed to date (3). The units of these assays are expressed in nanomoles per minute; specific activity is expressed as units per milligram of protein. Cytochrome oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) was determined by the method of Phan and Mahler (26) and is expressed as the first-order rate constant, *k*, per unit per milligram of protein. All values for NADH or reduced cytochrome *c* oxidation were corrected for oxidation which occurred in the absence of substrate or enzyme, respectively. Protein was determined with the Folin-Ciolcalteu reagent by using the method of Lowry et al. (17) and crystalline bovine serum albumin as a standard.

Chemicals. All biochemical reagents were obtained from Sigma Chemical Co. Inorganic chemical reagents and other supplies were purchased from either Fisher Scientific Co. or Malinkrodt Chemicals.

RESULTS AND DISCUSSION

Phenotype of Glp$^-$ mutants. Since the

growth properties of glycerol-nonutilizing mutants have not been completely described, the mycelial yield and hyphal growth rates of wild-type and *glp* mutant strains were measured on various carbon sources known to be utilized by *Neurospora*. As seen in Table 2, the mutants isolated in this study, as well as the previously isolated *glp-1*, totally failed to grow on minimal glycerol medium. The defect was specific for glycerol since cell yields comparable to wild type were obtained on acetate and glucose media. The mycelial yield and the growth rate on mannitol of mutants 14-44 and 17-53, but not L26, were significantly lower than that of wild-type cells (Table 2), suggesting a similarity to the polyol-defective strains. However, mutant strains 14-44, 17-53, and L26, as well as all other *glp-2 glp-3*$^+$ derivatives, are female fertile in genetic crosses, thereby indicating that they are not identical to the previously described polyol-protoperithecial defective derivatives of *N. tetrasperma* (31).

The lower rate of growth and mycelial yields of the glycerol-nonutilizing mutants on various carbon sources may be related to the morphological alteration which was present in the mutant strains and which differed markedly from that of wild type in that the surface of minimal sucrose slants was virtually devoid of conidia. This difference between mutant and wild type was especially pronounced on plates containing SGS medium on which wild-type cells formed compact rounded colonies in contrast to the flat spreading colonies of mutants (Fig. 1A and B). This morphological phenotype was present in all three independently isolated Glp-2$^-$ mutants as well as all glycerol-nonutilizing progeny derived from various crosses and, conversely, was absent in all Glp$^+$ recombinant progeny. However, revertants of these mutations have not yet been obtained, and it is not yet possible to ascertain whether this morphological feature represents the presence of a mutation in a closely linked morphology gene or is a consequence of metabolic alterations in the mutant similar to those documented for other morphological mutants in *Neurospora* (19).

Mapping of the *glp-2* locus. To determine if the Glp-2 phenotypes were the result of mutations of a single nuclear gene, crosses of strains 33-14 and 33-17 to the triply translocated "alcoy" strain (25) were performed, and the Glp, Albino, and Colonial temperature-sensitive phenotypes of the resulting ascospore progeny were determined. For both 33-14 and 33-17, there was segregation of *glp-2* from the *albino* marker (approximately 100 spore progeny examined for each cross), thereby suggesting linkage to either chromosome I or II. Follow-up crosses to strains 312 and 2282 showed no linkage to the chromosome I markers, but close linkage to the *arg-5* locus on chromosome II. The similarity in recombination percentages between *arg-5* and *glp(D14)* and *arg-5* and *glp(D17)* suggested that the same locus was mutated in both strains. In performing this analysis, 79 of 304 ascospores derived from the cross of 312 × 33-14 were nonpigmented and failed to germinate, a feature characteristic of translocation-bearing strains (23, 24). Since further analysis revealed that 86% of the Glp-2$^-$ type progeny also possessed the parental *inl*^{ts} allele, this indicated a chromosomal break point distal to the *glp* locus. Thus, the presence of this translocation on chromosome II may account for the small difference between the *arg-5—glp-2(D14)* and *arg-5—glp-2(D17)* map distances.

Since the other chromosome II markers, *fluffy* and *glp-3*, were also present in the parental types, it was possible to classify double crossover progeny and assign the *glp-2* locus to a position approximately 9 centimorgans (cM) to the right of *arg-5*. This location on IIR was further confirmed by finding a genetic separation of only 6.6

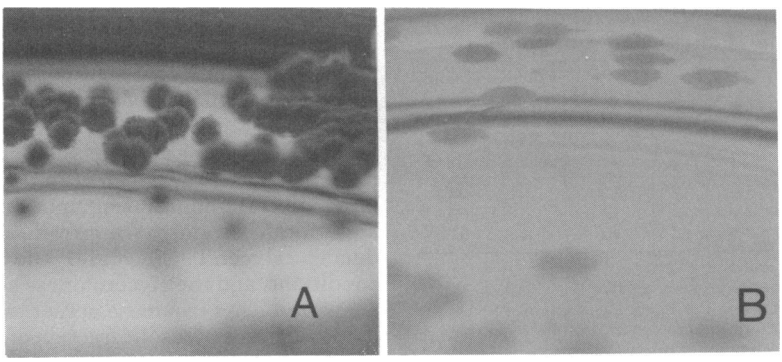

FIG. 1. *Differences in sorbose-induced colonial morphology of wild type and glp-2 mutants. Wild-type 74A (A) and glp-2 mutant 17-53 (B) were grown on SGS medium (see the text) for 3 days at 23°C.*

cM between *glp-2* and *pe* (Table 3). Finally, the recombinants obtained from a cross of *glp-2* to the *aro-3* marker gave a genetic map distance about 3.3 cM and indicated that the glycerol-nonutilizing locus was located between *arg-5* and *aro-3* (Table 3).

The location of the *glp-2* locus was also established by constructing partial diploids which contained duplications of chromosomal regions of IIR and determining the Glp phenotype among the progeny. In the first set of experiments, strain 17-53 was crossed to strain 2415, which possessed an insertional translocation of IIR. Approximately two-thirds of the surviving progeny were Glp$^+$ (Table 4), a result which is consistent with the number predicted for cases in which the duplicated fragment contains a functional wild-type gene and in which the mutational deficiency is recessive to the wild-type allele (24). The progeny containing the duplicated segment ALS176 are not, however, chromosomally stable, and haploid heterokaryons predominate among the vegetative isolates (24; D. D. Perkins, personal communication). For this reason, it was not possible to determine any effects of gene dosage on either basal or induced levels of the G3P dehydrogenase. An additional cross of strains 17-53 and 14-44 to strain 2537, which contains an *aro-1* mutation, yielded less than 11% Glp$^+$ progeny among the Aro-1 prototrophs (Table 4). Since all prototrophs would be Glp$^+$ if the *glp-2* locus were located in this region, this result indicates that the *glp-2* locus is not contained in the segment delimited by insertional translocation NM177. These genetic data place the *glp-2* locus to the right of *arg-5* and to the left of *nuc-2* on chromosome II.

TABLE 3. *Linkage of glp-2 to chromosome II loci*

Cross[a]	Region	N[b]	Percent recombinants[c]
33-14 × 312	*arg-5—glp-2*	285	7.7
33-17 × 312	*arg-5—glp-2*	286	9.4
33-14 × 312	*glp-2—glp-3*	285	15.4
33-17 × 312	*glp-2—glp-3*	286	16.1
17-53 × 37	*glp-2—pe*	241	6.6
17-53 × 53	*aro-3—glp-2*	442	3.2

[a] Strains given in Table 1.
[b] N, Total number of progeny analyzed.
[c] Percentage of total progeny with nonparental phenotypes for the indicated genetic region.

TABLE 4. *Results of crosses to strains with chromosome II insertional translocations*

Cross	Region duplicated[a]	N[b]	Fraction of Glp$^+$ progeny	
			Expected[c]	Obtained
17-53 × 2415	*arg-5—fl*	249	0.67	0.73
14-44 × 2537	*nuc-2—arg-12*	61	1.00[d]	0.11
17-53 × 2537	*nuc-2—arg-12*	51	1.00	0.06

[a] From Perkins and Barry (24).
[b] N, Total number of progeny analyzed.
[c] Fraction expected if the *glp-2* locus is covered by the duplication and if there is no recombination between the *glp-2* locus and the break point of the translocation.
[d] Parental normal sequence combinations of strain 2537 contain the *aro-1* mutation and are not obtained among survivors grown on minimal sucrose.

The allelism of mutant loci in the two mutant strains was proved by two additional tests. First, strain 17-53 was crossed to 33-14, and 200 viable spore progeny were collected and grown on sucrose slants. When tested on minimal glycerol medium, none of these proved to be Glp$^+$. Subsequently, direct plating of more than 5,000 ascospores on minimal glycerol sorbose medium also yielded no Glp$^+$ recombinants. Similarly, crosses of 17-53 to L26 produced no Glp$^+$ progeny among more than 2,000 spores tested.

As a second test of the possible allelism of these mutants, heterokaryons of the composition [*glp-2(D17)*; *pan-2* + *glp-2(D14)*; *inl*^{ts}] and [*glp-2(D17)*; *pan-2* + *glp-2(L26)*; *inl*^{ts}] were formed on minimal sucrose medium at 35°C and then tested at both 25 and 35°C for growth on minimal glycerol medium. Since no Glp$^+$ heterokaryotic conidia were obtained by this procedure, and since no Glp$^+$ recombinants were earlier detected, it seems likely that the *glp-2*$^-$ mutations in strains 33-14, 33-17, and L26 are allelic.

Glycerokinase activities in *glp-2* mutants. Although several glycerol- and/or glycerate-catabolizing enzymes have been described in *Neurospora* (31), the fact that only the glycerokinase and G3P dehydrogenase are specifically induced by growth on glycerol would suggest that these two enzymes are primarily involved in the initial catabolism of glycerol. In earlier studies, glycerol-nonutilizing (12) and polyol-nonutilizing (31) *Neurospora* mutants were shown to be deficient in glycerokinase, thereby demonstrating that a loss in the ability to phosphorylate glycerol could not be circumvented by the metabolism of glycerol through alternate pathways.

To determine if either glycerokinase or G3P dehydrogenase was deficient in these newly isolated glycerol-nonutilizing mutants, wild-type and mutant strains were grown under both inducing (glycerol + acetate) and noninducing conditions, and the glycerokinase activities were compared. Glycerokinase activities were clearly detected in the cytosolic fraction of *glp-2* mutant extracts but with specific activities substantially lower than those of fully induced wild type (Table 5). This reduction is apparently due to fewer

glycerokinase molecules synthesized during the glycerol-mediated growth inhibition (see below) since the glycerokinase cross-reacting material levels per unit of enzyme are essentially the same as those of wild type and since normal levels are detected when cells are induced for glycerokinase by incubation at a low temperature (9, 21). Furthermore, since we have found in separate studies (manuscript in preparation) that the glycerokinase in *glp-2* mutants is fully induced by deoxyribose and has identical electrophoretic mobility and thermal stability to that of wild-type glycerokinase, we conclude that the *glp-2* locus does not represent a structural or regulatory gene for this enzyme, an interpretation consistent with the proposed assignment of the glycerokinase structural gene to the *glp-4* locus (31).

G3P deficiency in *glp-2* mutants. A deficiency in G3P dehydrogenase activity was clearly noted in both cytosolic and mitochondrial extracts of strains 33-14, 33-17, and L26 as well as their derivatives (Table 6). This loss in activity was apparently complete as noted by the absence of detectable activity under all assay conditions employed to date. Mixtures of mutant and wild-type mitochondria showed complete additivity of G3P dehydrogenase activity, indicating that the loss of activity in mutants is not due to some freely diffusible inhibitor of the enzyme. This deficiency is specific for G3P dehydrogenase since mitochondrial extracts from mutant cells can also be shown to possess normal activities for succinate dehydrogenase and cytochrome oxidase (data not shown). Moreover, there was no detectable G3P dehydrogenase activity in cytosolic fractions (Table 7) from mutants as might be expected if the cytosolic G3P dehydrogenase activity represented a precursor or isoenzymic form of the mitochondrial G3P dehydrogenase as previously suggested (4, 6, 7).

This absence of G3P dehydrogenase activity was noted not only in extracts from glycerol-induced cells, but also in glucose-grown cells, where basal levels of this enzyme were readily detected in wild type. Since North has shown that several other carbohydrates can serve as effective inducers for the glycerokinase (21), we have tested the effect of growth on these sugars on the specific activity of the G3P dehydrogenase. Galactose, a highly efficient inducer for the glycerokinase, increased the basal levels in wild type approximately twofold (Table 6), whereas deoxyribose, the most efficient inducer for the glycerokinase (21), had no effect on the basal

TABLE 5. *Glycerol kinase levels in glp-2 mutants*

Strain	Sp act[a]			Relative CRM level[c]
	Acetate	Glycerol	Glucose[b]	
33	0.9	28.5	15.2	1.00
33-14	<0.02	7.8	16.7	0.96
33-17	<0.02	8.3	17.6	1.06

[a] Specific activity of glycerol kinase was determined as described in Methods.

[b] Cultures were grown 16 to 18 h at 4°C as previously described (3, 5).

[c] Extracts from strains 33, 33-14, 33-17, grown on minimal glycerol medium for 16 to 18 h, were adjusted to give 0.6 U of glycerol kinase in 0.08 ml. Varying amounts of antiglycerol kinase antisera were added and incubated for 15 min at room temperature. Control experiments indicated that there was no inhibition of glycerol kinase by preimmune sera.

TABLE 6. *G3P dehydrogenase specific activities in wild type and glp mutants grown on different carbon sources*[a]

Strain	Sp act			
	Acetate + glycerol	Galactose	Glucose	Sucrose
33	213.7	ND[b]	101.0	48.0
74A	351.0	94.0	46.6	58.1
33-14	<0.1	<0.1	<0.1	<0.1
33-17	<0.1	<0.1	<0.1	<0.1
14-44	<0.1	<0.1	<0.1	<0.1
17-53	<0.1	<0.1	<0.1	<0.1
L26	<0.1	<0.1	<0.1	<0.1
234	115.6	37.6	53.0	ND[b]

[a] Strains (see Table 1) were grown on the indicated carbon sources, and the specific activity was determined in the mitochondrial fraction as described in the text.

[b] ND, Not determined.

TABLE 7. *Cytosolic enzyme activities in wild type and glp-2 mutants*

Strain[a]	Sp act		
	G3P dehydrogenase	Glycerol dehydrogenase	DHAP reductase
74A	2.0	21.1	4.5
33	5.0	9.8	5.6
33-14	<0.05	9.6	5.1
33-17	<0.05	9.1	5.9
14-44	<0.05	41.6	4.3
17-53	<0.05	35.1	4.5
L26	<0.05	ND[b]	ND[b]

[a] All strains were grown on minimal sucrose medium. Homogenates were prepared and centrifuged at 100,000 × *g* in an SW50.1 rotor (Beckman) for 1 h. Specific activities were calculated as described in the text. G3P dehydrogenase was measured as the pyridine nucleotide-independent oxidation of G3P whereas DHAP reductase was measured as the DHAP-dependent oxidation of NADH.

[b] ND, Not determined.

level of the G3P dehydrogenase (data not shown). Although the extent of this induction was not as great as that noted during glycerol induction, this galactose-induced increase in G3P dehydrogenase did not occur in either mutant (Table 6), thereby suggesting that the deficiency in G3P dehydrogenase in these mutants is probably not due to defective genetic regulation. Although it is possible that the enzymatic deficiency in these strains might be due to a lesion in a positive acting regulatory gene with decreased affinity for effector, this seems unlikely since evidence from the positively controlled phosphate (16) and quinate (2) systems showed that the respective enzymes are synthesized at basal levels in regulatory mutants.

We have attempted to establish a structural role for the *glp-2* locus but have been unable to obtain Glp^+ revertants from any of the strains used in this investigation. Since neither UV-induced, nitrosoguanidine-induced, nor spontaneously arising revertants have yet been recovered among 10^{10} *glp-2* conidia, it is possible that all mutant strains selected to date possess deletions of all or part of the *glp-2* locus. In a separate study we are attempting to purify the G3P dehydrogenase from wild-type and mutant cells but to date have no information regarding the effect of the *glp-2* mutations on the structure of this enzyme.

Finally, it should be noted that separately the specific activities of the cytosolic enzymes, glycerol dehydrogenase (NADP dependent) and DHAP reductase, have been measured in these mutants and found to be comparable to those in wild-type strains (Table 7). The unique kinetic features for the NAD-dependent G3P dehydrogenase (see above) may be the reason for the inability of the G3P dehydrogenase-deficient mutants to grow on glycerol despite the formation of G3P. Thus, there is no reason at this time to suspect that the lesion in these strains directly affects any enzyme other than the mitochondrial G3P dehydrogenase.

G3P accumulation in culture medium. Tests were also performed with these mutants to determine if there was any accumulation of G3P during attempted growth on glycerol medium similar to accumulations in *glpD E. coli* mutants (8). For this purpose, cells were first grown in sucrose media and collected on sterile filter paper, and the resulting mycelial pad was suspended in mineral salts containing glycerol or sucrose as a sole carbon source. After the incubation in glycerol, there was a readily detected accumulation of G3P in filtrates of only the mutant cultures (Table 8). This accumulation clearly indicated not only that there is sufficient glycerokinase present to synthesize G3P, but also that there is no significant pathway available for an alternative catabolism of G3P. Moreover, this accumulation during attempted growth on glycerol indicated that the activity of the glycerokinase is not inhibited by G3P or related products, a finding which is consistent with the previously reported (3) insensitivity of the *Neurospora* glycerokinase to fructose 1,6-bisphosphate and in contrast to the inhibition of the *E. coli* glycerokinase by this glycolytic intermediate (34).

Growth stasis in *glp-2* mutants. Since noticeably reduced cell yields had been noted during the attempted induction in acetate plus glycerol media, it seemed possible that the glycerol resulted in G3P accumulation, which in turn inhibited cell growth. To test this, wild-type and mutant cultures were grown on minimal salts plus Casamino Acids until exponential-phase growth was attained. At various times, samples were collected and the relative increase in cell mass, N/N_0, from zero time, was determined. After 5 h, glycerol was added and growth was monitored by measurement of mycelial dry mass as above. As seen in Fig. 2, there was a marked reduction in the growth in mutant cultures upon the glycerol addition. Since mutants are able to synthesize G3P under these conditions (Table 8), it appears likely that this growth rate reduction is due to inhibition of one or more enzymatic steps by G3P or a related metabolite.

Genetic loci controlling glycerol dissimilation in *Neurospora*. The evidence presented in this report establishes the location of another genetic locus controlling the dissimilation of glycerol in *Neurospora*, thereby bringing to five the number of identified genetic loci involved in this system. Since the structural genes for the DHAP reductase and the NADP-linked glycerol dehydrogenase have not yet been identified, there is reason to believe that there are more loci to be identified in this system, including

TABLE 8. *G3P accumulation by glp-2 mutants*

Strain	Carbon source	nmol of G3P accumulated[a]	Wt of mycelial culture	Accumulation/mg (dry wt)
33	Sucrose	0	175	0
33	Glycerol	0	66	0
33-14	Sucrose	0	269	0
33-14	Glycerol	224	71	3.17
33-17	Sucrose	0	259	0
33-17	Glycerol	170	80	2.13

[a] Strains were grown for 24 h on minimal sucrose medium and then transferred to 50 ml of either glycerol or sucrose minimal medium and incubated at 25°C for 17 h. The cultures were filtered and the amount of G3P accumulated in filtrates was determined as described in the text (3).

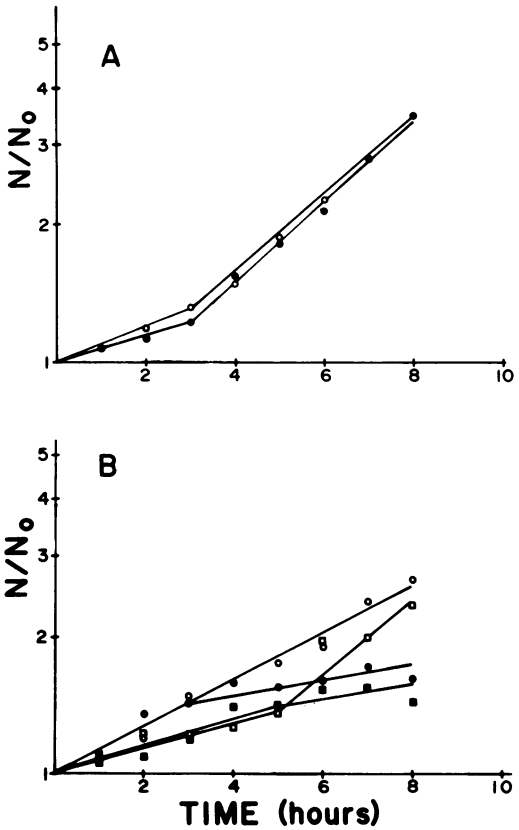

FIG. 2. *Growth stasis by accumulated G3P. (A) Two cultures of strain 33* (○, ●) *were grown on Casamino Acids as described in the text and, after 2 h, glycerol was added to one at a final concentration of 1%* (○). *(B) Strains 33-14* (□, ■) *and 33-17* (○, ●) *were grown as described in A, and glycerol was added to one culture of each after 2 h (33-14* [■]; *33-17* [●]).

possible regulatory gene(s) for the G3P dehydrogenase. However, the existing features of known *glp* mutants indicates that mutations affecting either the glycerokinase or G3P dehydrogenase are sufficient to prevent the utilization of glycerol by this organism. Consequently, it would appear that the dissimilation of glycerol requires phosphorylation of glycerol followed by the mitochondrial oxidation of the cytosolically formed G3P; the resulting dihydroxyacetone phosphate can enter the glycolytic pathway via the action of triose phosphate isomerase and aldolase. Furthermore, the accumulation of G3P in *glp-2* mutants would suggest that the dihydroxyacetone phosphate reductase is incapable of catalyzing the reverse reaction to form DHAP from G3P. The role of the glycerol dehydrogenase in glycerol utilization is less clear, since mutations at the *glp-3* locus which reduce the activity of this enzyme (Table 7) allow for growth on glycerol (5) but result in an inability of mutant strains to serve as protoperithecial parents in genetic crosses.

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