Novel *Neurospora crassa* mutants with altered synthesis of polyunsaturated fatty acids

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Five new mutants of *Neurospora crassa* that require supplementation with unsaturated fatty acids have been isolated. The mutants, designated *pfa*, are impaired in the synthesis of the polyunsaturated fatty acids α -linoleic or α -linolenic acid, but are able to synthesize oleic acid. The *pfa* mutants are thus distinct from previously described *ufa* mutants, which are unable to synthesize oleic acid. The five *pfa* mutants map to distinct loci, and have characteristic patterns of incorporation of [14 C]acetate and [14 C]oleate into their fatty acids.

Keywords: *Neurospora crassa*, fatty acid desaturation, lipids, polyunsaturates

INTRODUCTION

Polyunsaturated fatty acids (FA) are ubiquitous components of membrane lipids and are often components of storage lipids in eukaryotic cells. They are important determinants of lipid physical properties, such as membrane fluidity and the melting temperatures of triglyceride-based oils. The levels of polyunsaturates in membrane lipids usually increase in response to cold treatment, conferring greater membrane fluidity, and are believed to be an important factor in temperature adaptation (Weete, 1980; Martin *et al.*, 1981). The polyunsaturates α -linoleate (18:2 $\Delta^{9,12}$) and α -linolenate (18:3 $\Delta^{9,12,15}$) are also essential dietary components for human nutrition. The major source of these FA in the human diet is from plant-derived oils.

Aside from plants, few other organisms are able to complete the *de novo* pathway of α -18:3 biosynthesis. Among these organisms are some fungi (Weete, 1980). The biosynthetic pathway for polyunsaturated FA is thought to be similar for higher plants and fungi. Plants contain at least two routes for the synthesis of polyunsaturates (one localized in the chloroplast, one in microsomes, probably ER), and the ER route is apparently the major one for the synthesis of polyunsaturate-containing TG (Frentzen, 1993). In fungi, the synthesis of polyunsaturates is believed to occur in the ER (Weete, 1980). For fungi and the ER-localized pathway in plants,

desaturation of the immediate precursor 18:1 occurs while it is esterified to the phospholipid PC (Weete, 1980; Heinz, 1993).

Mutants in the pathway of FA desaturation would be useful for a better understanding of the regulation of these pathways and for gene isolation. In α -18:3-producing fungi, comparatively little information is available on the desaturation pathway. The filamentous fungus *Neurospora crassa* synthesizes α -18:3, and is amenable to classical and molecular genetic techniques. Additionally, some biochemical information on the pathway of desaturation in *Neurospora* is available, and mutants for the desaturation of 18:0 to 18:1 (*ufa*) (Scott, 1977a; Goodrich-Tanrikulu *et al.*, 1994) and for the biosynthesis of PC (*chol-1*, *chol-2*) (Horowitz & Beadle, 1943; Horowitz *et al.*, 1945; Scarborough & Nyc, 1967) have been isolated. Mutants defective in the desaturation of 18:1 or α -18:2 have not been described, aside from reports that mutants defective in the synthesis of NADPH have reduced levels of α -18:3 (Brody & Nyc, 1970; Nyc & Brody, 1971), and that *ufa* mutants may also be impaired in subsequent desaturation steps (Scott, 1977b; Gabrielides *et al.*, 1982; Goodrich-Tanrikulu *et al.*, 1994).

Mutants in the biosynthesis of polyunsaturated FA have been isolated based on impaired growth at low temperatures from the cyanobacterium *Synechocystis* PCC 6803 (Wada & Murata, 1989), which utilizes a chloroplast-like pathway to synthesize α -18:2, and from the fungus *Mortierella*, which synthesizes γ -linolenate (18:3 $\Delta^{6,9,12}$) and arachidonate (20:4 $\Delta^{5,8,11,14}$) (Jareonkitmongkol *et al.*, 1992). Using an α -18:3 requirement for growth at low temperatures, we have isolated several *Neurospora* mutants with altered synthesis of unsaturated FA. We have previously described two

Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; DG, diacylglycerol; ER, endoplasmic reticulum; FA, fatty acid; G-3-P, glycerol 3-phosphate; LPA, lyso-phosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TG, triacylglycerol.

mutants which synthesize only saturated FA (Goodrich-Tanrikulu *et al.*, 1994). Here, we describe FA composition, synthesis, and desaturation by mutants with defects in the synthesis of polyunsaturated FA.

METHODS

Cultures, media and crosses. The wild-type *Neurospora crassa* strains 74-OR23-1A (wt74A) and 74-OR8-1a (wt74a), and the strain *alcoy; csp-2* were obtained from the Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, KS, USA. The *a^{m1} ad-3B cyh-1* strain was a gift of David D. Perkins, Stanford University.

Cultures were grown as described by Goodrich-Tanrikulu *et al.* (1994); unless otherwise noted, FA supplementation for all media was 1 mM α -18:3 (87%, remainder α -18:2, prepared as described by Goodrich-Tanrikulu *et al.*, 1994), solubilized with 1% (v/v) of the detergent Tergitol NP40.

Preliminary genetic mapping was with the linkage tester strain *alcoy; csp-2* as described by Goodrich-Tanrikulu *et al.* (1994), except the crossing medium was supplemented with 1% Tergitol NP40 and 5 mM 18:3. Lines of interest which carried additional morphological mutations (three of five mutants) were backcrossed to wt74a to segregate out these characters.

Mutagenesis, selection and characterization of polyunsaturated FA mutants. Conidia of wt74A, suspended in tubes of sterile water at a density of 2×10^7 ml⁻¹, were mutagenized by exposure to γ -irradiation (⁶⁰Co source) for 4 h at 100 Gy h⁻¹, or by exposure to NTG or ICR-170 as described in Davis & deSerres (1970). Mutant enrichment and preliminary screening were at 15 °C in Vogel's minimal medium (Davis & deSerres, 1970) as described by Goodrich-Tanrikulu *et al.* (1994). Lines which reproducibly grew faster with 18:3 supplementation (two additional transfers) were considered potential mutants in the synthesis of unsaturated FA, and were further screened to identify total FA biosynthetic profiles by radiolabelling with [¹⁴C]acetate at 15 °C as described by Goodrich-Tanrikulu *et al.* (1994). In more detailed studies, liquid-grown cultures were radiolabelled with either 370 kBq [¹⁴C]acetate (sodium salt, ICN, 1.48–2.22 GBq mmol⁻¹) or 37 kBq [¹⁴C]oleate (ICN, 1.11–2.22 GBq mmol⁻¹) at 23 °C. Lipids were extracted and analysed as described by Goodrich-Tanrikulu *et al.* (1994).

RESULTS AND DISCUSSION

Mutant selection, genetic mapping, and growth characteristics

Following mutagenesis and filtration enrichment, 26 200 colonies (6000 from NTG, 2800 from ICR-170, and 17 400 from γ -irradiation treatments, respectively) were screened for growth in the presence and absence of supplemental 18:3. In the screening of ICR-170- and NTG-treated conidia, several hundred lines showed improved growth through the preliminary screen and an additional transfer, but failed to grow reproducibly upon subsequent transfers. We suspected that some promising lines were being lost to reversion, and attempted to obtain non-reverting lines using γ -irradiation. A similar proportion of the γ -irradiated lines were 'lost' in a similar manner.

Of the total colonies screened, 44 showed a requirement for unsaturated FA supplementation through two ad-

ditional transfers at 15 °C, and were further screened for ability to synthesize FA *de novo* from [¹⁴C]acetate. Based on this assay, 15 lines showed reduced synthesis of polyunsaturated FA. The levels of labelled polyunsaturated FA remained within the wild-type range for all but five of the lines assayed. We have selected the designation *pfa* for these five mutants. Four of the *pfa* mutants synthesized no 18:3 at 15 °C; all but one of these (*pfa*-1) also had reduced 18:2 (Table 1). The remaining mutant (*pfa*-5) synthesized only 40% of the 18:3 and 55% of the 18:2 of wild-type. Also at 23 °C, all five mutants showed impaired synthesis of polyunsaturated FA (detailed below).

The five mutations were independent as shown by mapping and tests for allelism (Table 2). After backcrossing, the morphology of all of the mutants was wild-type. The mutants required unsaturated FA supplementation at 15 °C, 23 °C, and 30 °C, except for the mutant *pfa*-1, which required supplementation at 15 °C, but grew very slowly (requiring several weeks to germinate) without supplementation at the higher temperatures. Germination of mutant conidiospores was delayed by several days (several weeks for *pfa*-3) compared to wild-type. Thereafter, the mutants grew at nearer wild-type rates. All of the mutants grew more slowly than wild-type despite supplementation, suggesting either that the level of supplementation was suboptimal or that FA supplementation only partially compensated for the mutation. A similar lack of complete restoration of wild-type phenotype by FA supplementation has been noted in the *cel* mutant of *Neurospora* (Coté & Brody, 1987), which is defective in formation of 16:0.

18:3 appeared to be the optimal FA supplement for four of the *pfa* mutants. The mutant *pfa*-4, however, unexpectedly grew more vigorously when supplemented with 1% (v/v) Tween 80 (which contains esterified 18:1) than with either 1–5 mM free 18:1 (96%) or 18:3 (87%). The improved growth may be due to the higher level of available FA when supplied as a Tween ester.

Stocks of the strains could be maintained by cryopreservation as heterokaryons, but not reliably as homokaryons. Crosses were usually fertile when the mutants were sheltered as heterokaryons with the helper strain *a^{m1} ad-3B cyh-1*, used as male parent, and supplemented with 18:3.

Each of the mutants was unstable, frequently reverting upon subculturing, as evidenced by regain of wild-type growth and FA biosynthetic profiles. Stocks of *pfa*-1 and *pfa*-2 also tended to become progressively impaired earlier in the desaturation pathway, either after subsequent transfers, or after backcrossing and selecting for FA supplementation requirement. This was evident from altered radiolabelling patterns with [¹⁴C]acetate; in extreme cases, the mutant patterns were identical to those of *ufa* mutants (Goodrich-Tanrikulu *et al.*, 1994), which lack the ability to desaturate 18:0. The five *pfa* mutants were non-allelic with *ufa* strains (Goodrich-Tanrikulu *et al.*, 1994), as determined by heterokaryon tests. The accumulation of additional defects is reminiscent of some adenine and cysteine auxotrophs, which spontaneously

Table 1. Results of screen for *Neurospora* mutants affecting the synthesis of polyunsaturated FA

Relative percentage incorporation of [^{14}C]acetate (37 kBq) into individual FA of total lipids after 24 h at 15 °C is shown. Cultures were supplemented with 1 mM 18:3. See Methods for further details of culture and labelling conditions. The range of wild-type values is expressed as mean incorporation $\pm$ SE for three determinations. Total label incorporated ranged from approximately 0.3 to 1.7×10^6 c.p.m.

Strain	[^{14}C]Acetate incorporation into:					
	16:0	18:0	18:1	18:2	18:3	Other*
wt74A	35.0 $\pm$ 5.1	9.7 $\pm$ 1.3	14.3 $\pm$ 0.3	32.0 $\pm$ 2.1	8.0 $\pm$ 3.6	1.0 $\pm$ 1.0
<i>pfa-1</i>	47	4	17	31	0	0
<i>pfa-2</i>	53	14	12	21	0	0
<i>pfa-3</i>	48	15	15	21	0	0
<i>pfa-4</i>	41	24	18	16	0	0
<i>pfa-5</i>	36	23	18	18	3	1

* Unidentified FA.

Table 2. Map location of *Neurospora pfa* mutants

Linkage is expressed as percentage of ascospores able to grow on unsupplemented medium that carry marker for given chromosome(s). Deviations from 50 % (no linkage) are significant (Perkins, 1994). The mutations mapping to the same chromosomes were shown to be distinct by heterokaryon tests (Davis & deSerres, 1970).

Strain	Mutagen	Chromosome*	Linkage
<i>pfa-1</i>	γ -Irradiation	VII	65 % (53/81)
<i>pfa-2</i>	γ -Irradiation	IV/V	68 % (32/47)
<i>pfa-3</i>	NTG	IV/V	71 % (25/35)
<i>pfa-4</i>	ICR-170	VII	67 % (52/78)
<i>pfa-5</i>	γ -Irradiation	I/II	93 % (40/43)

* Map location based on crosses to *aleo*; *csp-2*.

accumulate secondary mutations in their respective biosynthetic pathways, suggesting a selective advantage for the double mutants (Mitchell & Mitchell, 1950; Murray, 1965). The wild-type also tended to become impaired in 18:2 desaturation upon culturing in the presence of 18:3.

FA synthesis and desaturation

In *Neurospora*, 18:1 is most likely desaturated to 18:2 and 18:3 while esterified to PC (Baker & Lynen, 1971; Weete, 1980). A mutation in one of the two desaturases would most directly reduce the level of polyunsaturated FA. However, because the mutations we identified were independent, more than two genes must affect the biosynthesis of polyunsaturated FA. Likely candidates are genes in the pathway for substrate synthesis and factors involved in desaturation, e.g., cytochrome b_5 and associated reductases. In *Neurospora* and other α -18:3-producing fungi, the major pathway for biosynthesis of

phospholipids and triacylglycerols containing polyunsaturated FA is believed to be:

with the acylation steps utilizing acyl CoA thioesters (Weete, 1980; Chopra & Khuller, 1984; Baker & Lynen, 1971). In addition, enrichment in the level of polyunsaturates probably occurs, as it does in plants (Frentzen, 1993; Heinz, 1993), via alternative, reversible reactions forming PC: the reaction of DG with CDP-choline, and acylation of lysoPC (Weete, 1980).

Prior studies have shown that mutations in pathway enzymes, or in synthesis of cofactors involved in the desaturations, can affect the levels of polyunsaturates: *Neurospora* mutants (*chol-1*, *chol-2*) blocked in the formation of PC by the major pathway of PE methylation differ from wild-type in FA composition and in the relative amount of PC and PE, with the differences dependent on the level of choline supplementation (Hubbard & Brody, 1975; Johnston *et al.*, 1982). Also, mutants with reduced NADPH levels due to altered glucose-6-phosphate dehydrogenase produce only 20 % of wild-type levels of 18:3 (Brody & Nyc, 1970).

To obtain more information about where in the pathway the mutants we have identified might be affected, we examined the incorporation of polyunsaturated FA precursors into lipids of the mutants. First, we compared the incorporation of ^{14}C from acetate into individual phospholipids, glycolipids and neutral lipids of the mutants and wild-type under identical supplementation conditions. Incorporation patterns into lipids of each fraction were typically similar for the mutants and wild-type, and the majority of label was incorporated into TG, with one major exception: the mutant *pfa-3* incorporated more label into free FA than did wild-type or other mutants. The relative amount of label incorporation into free FA of *pfa-3* varied among experiments, ranging from about 25 to 100 % of the label incorporated into TG.

Fig. 1. TG synthesis by 18:3-supplemented *Neurospora*. (a) $[^{14}\text{C}]$ Acetate incorporation into TG; relative percentage incorporation into FA of TG 24 h after label addition to cultures grown at 23 °C. Cultures were radiolabelled and lipids extracted and separated as described in Methods and by Goodrich-Tanrikulu *et al.* (1994). FA methyl esters derived from the TG fraction were separated by HPLC, with radiodetection. (b) Relative percentage FA composition of TG by weight, based on GC analysis of methyl esters. Values are means of at least three determinations; error bars indicate SE. Absolute values for amount and ^{14}C incorporation per culture, approximately 1.5 mg FA and 1×10^7 c.p.m., respectively. Left to right: ▨, 16:0; ■, 18:0; ■, 18:1; ▤, 18:2; □, 18:3; ▩, other (unidentified).

$[^{14}\text{C}]$ Acetate incorporation into FA of TG showed distinctive differences for wild-type and mutant cultures (Fig. 1a). At 23 °C, the TG incorporation profiles differed somewhat from the total FA incorporation profiles used in screening at 15 °C (compare data of Table 1). At the lower temperature, more label remained in 16:0 for each of the strains, including two (*pfa-3* and *pfa-4*) that accumulated label in 18:0 at higher temperatures (Fig. 1a). The higher proportion of label in 16:0 may be due to a slower rate of elongation and desaturation at the lower temperature. As observed previously (Goodrich-Tanrikulu *et al.*, 1994), the FA composition of the TG was affected by the exogenous supplement, with the 18:3, and, to a lesser degree, 18:2 of the supplement being the predominant FA (Fig. 1b).

Since PC is believed to be the substrate for 18:1 and 18:2 desaturation, we compared the incorporation of $[^{14}\text{C}]$ acetate into FA of PC for each of the strains (Fig. 2). The amount of label incorporated into 18:2 was variable for the four mutants synthesizing the lowest levels of 18:3 *de novo*. The variable incorporation may result from mass-

or age-dependent levels of 18:2 produced in *Neurospora* cultures (Coté & Brody, 1987).

We also tested the ability of the mutants to desaturate exogenous 18:1 to polyunsaturated FA *in vivo*. At first inspection, the mutant phenotypes appeared to fall into three classes based on their ability to desaturate exogenous 18:1: *pfa-5* closely resembled wild-type; *pfa-1* and *pfa-2* appeared to be impaired in the synthesis of 18:3, and were potentially defective in 18:2 desaturation; and *pfa-3* and *pfa-4* appeared to be impaired in the synthesis of both 18:2 and 18:3 (Fig. 3). However, in preliminary experiments, *pfa-4* was able to convert the same proportion of labelled 18:1 to 18:2 in PC as wild-type (36%). Thus, the defect in *pfa-4* may be in 18:2 desaturation. For the *pfa-3* mutant, the amount of ^{14}C from 18:1 incorporated into FA of PC was below our detection limit, so the distribution of label in PC is unknown. The patterns of incorporation of ^{14}C from 18:1 into neutral or phospholipids were unchanged from wild-type for any of the mutants (autoradiographs not shown), although for some of them, much of the label remained in the free FA

Fig. 2. $[^{14}\text{C}]$ Acetate incorporation into PC of 18:3-supplemented *Neurospora*. Details as for Fig. 1(a), except FA were derived from PC. (Absolute incorporation estimates can be obtained from Table 3.) Left to right: ■, 16:0; ■, 18:0; ■, 18:1; ▨, 18:2; □, 18:3; ▩, other.

Fig. 3. $[^{14}\text{C}]$ Oleate incorporation into total FA of 18:3-supplemented *Neurospora*. Relative percentage incorporation into total FA of *Neurospora* cultures grown at 23 °C, 24 h after label addition, is shown. FA methyl esters were separated by HPLC, with radiodetection. Values are means of two (*pfa-3*, *pfa-5*) or three determinations; error bars indicate s.e. Left to right: ■, 18:1; ▨, 18:2; □, 18:3; ▩, other.

band. This could have occurred due to impaired transfer of label into other lipids, or from unincorporated label in slow-growing cultures.

We also noted the presence of an unknown glycolipid which contained a small percentage of the total hydrolysable FA, predominantly as 18:3, in both wild-type and mutants (including the *ufa* mutants previously described by Goodrich-Tanrikulu *et al.*, 1994). It also incorporated labelled acetate (less than 2% of total label)

at 24 h almost exclusively into 18:3, but did not incorporate exogenous 18:1. This lipid is perhaps involved in a minor pathway of FA desaturation.

Probable basis of the mutations

Several interpretations are possible for the data which we have gathered on these mutants. Below we describe further characteristic details of the individual mutants,

Table 3. Synthesis of major glycerolipids in *Neurospora pfa* mutants

Cultures were labelled with [^{14}C]acetate (370 kBq) for 24 h at 23 °C, and were supplemented with 1 mM 18:3; see Methods for details of culturing and labelling conditions. FA methyl esters were analysed following lipid class separation: amount (amt; relative weight percentage FA) was determined by GC with flame ionization detection, and [^{14}C]acetate incorporation (incorp) was determined by HPLC with radiodetection. The absolute amount of material harvested varied between experiments, so relative values are more indicative of differences between strains. Average results of three or more sets of determinations (two for *pfa-2* and *pfa-3* DG incorporation) are shown.

Strain	PE† (% TG)		PC† (% TG)		DG† (% TG)		TG‡ (% wt)	
	Amt	Incorp	Amt	Incorp	Amt	Incorp	Amt	Incorp
wt74A	4.5	10.9	3.8	12.1	0.8	2.8	100	100
<i>pfa-1</i>	6.4	10.0	8.6	15.8	3.7	4.8	27	64
<i>pfa-2</i>	4.1	9.6	6.1	12.8	1.2	2.6	81	70
<i>pfa-3</i>	3.7	12.3	6.1	9.6	3.0	3.3	43	82
<i>pfa-4</i>	3.2	15.5	8.0	22.0*	5.5	3.5	10	19
<i>pfa-5</i>	7.1	13.6	4.9	15.5	2.5	6.9*	41	121

* Relative values significantly different from wild-type (95 %, Student's *t*-test).

† Expressed as percentages relative to TG (normalized to 100 %).

‡ Expressed relative to wild-type TG (absolute values for amount and ^{14}C incorporation per culture, approximately 1.5 mg and 1×10^7 c.p.m., respectively).

and what we consider the most likely explanation for them.

The mutant *pfa-5* most resembled wild-type in lipid composition and incorporation of exogenous acetate and 18:1. The reduced levels of polyunsaturates in this mutant are apparently not due to impaired desaturase activity (see Fig. 3). In this mutant, both the amount and the incorporation of acetate-derived label in PE, PC and DG relative to TG were on average higher than for wild-type (Table 3). The higher incorporation into DG was significant. Additionally, the absolute amount of label in TG averaged 20 % higher than in wild-type.

A mutation reducing the level of PC biosynthesis by the minor CDP-choline:DG choline phosphotransferase (Kennedy) pathway (Weete, 1980) would cause reduced levels of polyunsaturates, since utilization of this pathway can return 18:1 or 18:2 from DG to PC, the substrate for further desaturation. This defect could cause the observed effects, namely accumulation of label in DG, and a compensating increase in the flux of FA through the main route of PC synthesis, via methylation of PE. Furthermore, since the rate-limiting step in TG biosynthesis in some organisms is DG acyltransferase (Frentzen, 1993), an accumulation of DG could enhance TG biosynthesis. It is unclear why this mutant might require 18:3 supplementation. Higher 18:3 levels may be required for an unknown biochemical function, or supplementation may compensate structurally for the higher level of saturates incorporated into lipids of the mutant.

pfa-3, as noted above, incorporated an extremely high proportion of the total acetate- or 18:1-derived label into

free FA. *pfa-3* cultures also had eight times the wild-type level of free FA, relative to the amount of TG produced. The acetate-derived label in free FA was mostly found in 16:0 and 18:0, as for wild-type; however, the free FA bulk composition differed: in wild-type, 38 % of the free FA was 16:0, and 10 % 18:3, whereas in the mutant *pfa-3*, 15 % was 16:0, and 37 % 18:3. The 18:3 in the free FA pool, since unlabelled, was probably from the supplement. These observations suggest that *pfa-3* most likely has a defect that interferes with acylation, such as a defective acyl-CoA synthase.

The label incorporation profiles of mutant *pfa-4* superficially resembled those of *pfa-3*, but *pfa-4* was distinctive in several respects. This mutant was capable of desaturation of 18:1 to 18:2 (see above), but had difficulty in further desaturating 18:2, and in its transfer to DG and TG: [^{14}C]acetate-derived 18:2 was higher than for wild-type in PC (Fig. 2), but lower than wild-type in DG (not shown) and TG (Fig. 1a). The levels of the exogenously added 18:3 in TG (Fig. 1b) and PC (not shown) were also significantly low for this mutant. Other unusual characteristics of the mutant are that [^{14}C]acetate-derived label was high (1.8 times wild-type) in PC relative to TG, and the amount of PC and DG also averaged comparatively more than for wild-type (Table 3). Also, as noted above, supplementation with high levels of exogenous 18:1 in the form of Tween 80 restored near wild-type growth. We suspect that this mutant either has a defect in the incorporation of unsaturated FA into complex lipids, most likely at the second acylation step, or that it has a mutation affecting electron transport which affects the second desaturation step to a greater degree. Mutations in

the NADPH biosynthetic pathway reduce 18:3 levels in lipids of *Neurospora* (Brody & Nyc, 1970). Either of these alternatives might be corrected by culture supplementation with high levels of unsaturated FA.

The mutants *pfa-1* and *pfa-2* both appear to be impaired in the desaturation of 18:2 to 18:3. The mutants differed in one respect, the incorporation of [¹⁴C]acetate-derived label into DG. In *pfa-1*, the label was primarily in the saturates (16:0 had an unusually high percentage of the total label), with some label detectable in 18:2. In contrast, *pfa-2* had an unusually low amount of 16:0, both compared to wild-type and compared to the levels in PC. The label in 18:2 of DG was higher than for wild-type. Apparently, the two mutants have different selectivity for those molecular species of PC (or an alternative precursor) which are converted to DG. We suspect that one of these two is a 18:2 desaturase mutant, but do not know which DG incorporation profile is more likely for such a mutant.

Implications for the control of polyunsaturated FA biosynthesis in *Neurospora*

Four of the mutants we have isolated made no 18:3 in the 15 °C screening, but did make 18:3 at reduced levels at 23 °C (compare Table 1 and Fig. 1a). Alternative desaturation or elongation products of α -18:2 were not detected in wild-type or mutant lipids. Isolation of a set of four temperature-sensitive mutations, or mutations leaky at high temperature, is possible given the selection protocol. The results could alternatively be attributable to a second 18:2 desaturase or desaturation pathway in *Neurospora*. The substrate for desaturation to α -18:3 is believed to be 18:2-PC, but studies of 18:2 desaturation in *Neurospora* have been rare (Weete, 1980), and most have examined effects of temperature (Martin *et al.*, 1981; Aaronson *et al.*, 1982; Martin & Johnston, 1983). It is not known which acyl group(s) undergoes the desaturation, and alternative substrates, such as CoA esters, which might be utilized in a minor pathway, have not been ruled out (Baker & Lynen, 1971). Since NADPH biosynthetic mutants have reduced 18:3 levels (20% of wild-type), it has been postulated that the 18:2 desaturase may utilize NADPH preferentially for the desaturation (Brody & Nyc, 1970), whereas the 18:1 desaturase utilizes NADH (Gabrielides *et al.*, 1982). A small amount of the total 18:3 may thus be formed by a second desaturase or using an alternative reductase. Most likely, given the absence of 18:3 synthesis by the mutants at 15 °C, only one desaturation system is utilized at this temperature. The preferential use of an 18:1-CoA desaturase over an 18:1-PC desaturase at low temperature has been reported for the fungus *Candida lipolytica* (Pugh & Kates, 1975). Evidence for an alternative desaturation pathway in *Rhodotorula gracilis* that is resistant to sterulate inhibition has also been presented (Rolph *et al.*, 1990).

Our study has found that several mutations in *Neurospora* reduced the synthesis of polyunsaturated FA, indicating that multiple genes influence the polyunsaturated FA composition of membrane and storage lipids. Each of these mutations also showed genetic instability. The

strong selection pressure against the mutants may be due to the specific biochemical defects or to the resulting effect on FA composition. Supplementation of the medium with 18:3 allowed the mutants to grow, and produced fertile crosses, suggesting that at least part of the phenotype resulted from failure to produce adequate levels of 18:3. However, even with supplementation, the mutants grew more slowly than wild-type. It is possible that some of the differences in precursor incorporation into the polyunsaturates result from the slower growth rate. Most notably, reduced levels of both 18:2 and 18:3 were synthesized *de novo* by each of the mutants at 23 °C, even those which, judged by the criterion of ability to further desaturate [¹⁴C]oleate, were selectively impaired in the synthesis of 18:3. This effect contrasts with observations made in studies of *Neurospora* phospholipid composition during temperature changes, in which the amount of 18:2 and 18:3 appear to be controlled independently (Martin *et al.*, 1981). However, the effect resembles one observed for *ufa* mutants, which do not desaturate 18:0, but may also show impaired 18:1 or 18:2 desaturation (Scott, 1977b; Gabrielides *et al.*, 1982; Goodrich-Tanrikulu *et al.*, 1994).

More definitive characterization of the defects in these mutants requires additional study. Unfortunately, only limited information is available on the relevant biochemical pathways in *Neurospora* (Weete, 1980; Chopra & Khuller, 1984). Mutants in the FA desaturation pathway of *Arabidopsis* have been extremely valuable for identifying the control of the corresponding plant pathway, for observing physiological effects of altered FA composition, and for isolation of the genes involved (reviewed by Heinz, 1993; Gibson *et al.*, 1994). Likewise, further studies in *Neurospora* will identify similarities and differences in FA biosynthesis of filamentous fungi, and should provide insight into related processes in higher plants.

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