EVIDENCE FOR NONSENSE MUTATIONS IN THE *AROM* GENE CLUSTER OF *NEUROSPORA CRASSA*[1]

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THE *arom* gene cluster in *Neurospora crassa* contains five structural genes coding for an aggregate of five enzymes catalyzing steps two through six in the aromatic synthetic pathway prior to chorismic acid (GILES, CASE, PARTRIDGE, and AHMED 1967a). A major category of *arom* mutants consists of those which do not complement any other *arom* cluster mutants (non-complementing types), lack activities for all five enzymes, and map at one end of the cluster within the structural gene coding for the enzyme dehydroquinic acid (DHQ) synthetase. On the basis of this evidence, these mutants have been interpreted as pleiotropic polar mutants resulting from mutations to nonsense (chain-terminating) codons within the proximal structural gene (*arom-2*) of the *arom* gene cluster, which is assumed to be transcribed in a polarized fashion via a single polycistronic messenger RNA. The present paper presents evidence in support of this interpretation of these pleiotropic mutants based on genetic and biochemical studies employing suppressors of nonsense mutations (SEALE 1968). A brief report of certain aspects of these studies has been presented previously (CASE, GILES, and PARTRIDGE 1967).

MATERIALS AND METHODS

Origin of the strains: The genetic localization, complementation pattern, and biochemical characteristics of the polyaromatic amino acid-requiring strains (*arom* cluster mutants) used in these studies have been described previously (GILES, *et al.* 1967a). The origin of the glutamate dehydrogenase (*am*) suppressor strains has been described by SEALE (1968) who kindly supplied these strains. In this paper the two suppressor strains of *am 17* used are designated as follows: *am 17 su 17RN22* (subsequently referred to as *am su22*) and *am 17 su 17RN33* (referred to as *am su33*). An additional strain having a suppressor without the *am* mutation was extracted from a cross of *am su22* with the non-complementing strain *arom M54*. This strain will be referred to subsequently as *am*+ *su22*. Positive identification of the presence of the suppressor combined with wild type (W.T.) in the strain was made by means of a cross of the strain to *am 17* (Table 1, cross type C).

Media and crossing procedures: The normal growth media for the *arom* mutants (arom medium) and the crossing procedures have been described previously (GILES, *et al.* 1967a). 500 μg monosodium glutamate/ml plus 100 μg DL alanine/ml (am medium) were used for growth of the *am* mutants. In crosses, strains lacking the suppressor were always used as protoperithecial parents, and crosses were initiated after growth for 4 to 5 days at 25°C by adding a conidial suspension of the strain carrying the suppressor.

Genetic procedures: Genetic analysis of the crosses was made by tetrads, by individual random ascospore isolations, or by random ascospore platings on sorbose, fructose, glucose agar with

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TABLE 1

Tetrad analysis of crosses involving the am 17 *suppressor*

Type of cross	*arom* mutant category	*arom* ascus types wild type: mutant			*am* ascus types wild type: mutant		
		8:0	6:2	4:4	8:0	6:2	4:4
(A) *arom am*+ *su*+							
×							
arom+ *am su22*							
Suppressed mutants							
M54	E	3	7	2	5	6	1
M1065	F	1	7	1	7	2	0
M1108	E	0	2	2	.	.	
M1172	E or F	0	1	3	2	1	1
Non-suppressed mutants							
M58	A	0	0	9	4	3	2
M14	B	0	0	6	4	2	0
M1199	D	0	0	5	0	2	3
M25	F	0	0	10	0	3	6
M34	F	0	0	6	2	3	1
(B) *arom am*+ *su*+							
×							
arom+ *am*+ *su22*							
Suppressed mutants							
M26	E	0	2	2			
M1039	E	1	4	4			
M1162	E or F	0	4	2			
(C) *arom*+ *am su*+							
×							
arom+ *am*+ *su22*					2	5	2

appropriate supplementation for *arom* and *am* mutants. With random ascospore platings, no estimate of the viability of the crosses was made.

arom D, *E*, and *F* polar mutants (GILES, *et al.* 1967a) are distinguishable morphologically from wild type by their semi-colonial growth and production of yellowish orange conidia. The phenotypes of these polar mutants and of the other *arom* mutant types were checked by testing on minimal and on arom supplemented media. The major problem in mutant identification arose in the classification of *arom am* double mutant isolates. *am* isolates can grow on the usual polyaromatic amino acid supplement by utilizing phenylalanine and tryptophan (FINCHAM 1950), and will also grow slowly on minimal medium. Growth on minimal medium can be considerably delayed by the addition of glycine (1500 μg/ml). However, *arom* isolates are also inhibited by glycine, even in the presence of the usual aromatic amino acids. Identification of the *am* isolates in 1-2 days is possible on the basis of their rapid growth on the usual am supplement, slower growth on the arom supplement, and no growth on glycine. The *arom am* double mutants show no growth in two days on the am supplement, slight growth on the arom supplement, and none on glycine. Because of problems in the positive identification of the *am* and *arom am* isolates, particularly in random populations, the segregation of the *am* marker was not followed in all crosses. In the random isolates, only the phenotypically wild-type colonies were tested on glycine.

Biochemical procedures: Enzyme assay methods, heat inactivation procedures for distinguishing constitutive and inducible dehydroquinases, and sucrose density gradient procedures have been described previously (GILES, *et al.* 1967a, 1967b).

RESULTS

Evidence for suppression of arom mutant M54 *by the suppressor* am su22: The initial part of this analysis involved a selected group of polar mutants in the *arom* cluster. These mutants are characterized by a reduced complementation response with all of the other mutant groups (*A* type, *M58*), or by failure of complementation with two (*B* type, *M14*), three (*D* type, *M1199*), or all four groups (*E* type, *M54*; and *F* types, *M25* and *M34*). (GILES *et al.* 1967a). The mutants were crossed with *am su22* and serial ascospore isolates from these crosses were analyzed (Table 1). Only mutant *M54* showed a typical suppression pattern for the arom requirement (Table 1 also contains data from crosses with other *E* and *F* type mutants which will be discussed later). The presence of 7 asci with 6 wild type:2 *arom* constitutes positive evidence for suppression. The numbers of asci with 8 wild type:0 *arom* and with 4 wild type:4 *arom* indicate that the suppressor segregated independently of or is only very loosely linked to the *arom* region. In the same asci the *am* requirement showed a similar 6 wild type:2 *am* suppression pattern. Although the other five *arom* polar mutants referred to in this section were not suppressed by the *am* suppressor, the *am* segregation in these asci also exhibited a typical suppression pattern indicating that there were no factors in these crosses inhibiting the expression of the *am* suppressor in mutants derived from wild type 74. The suppressed *arom M54* strain is morphologically indistinguishable from wild type.

Confirmation of the suppression of arom M54: The phenotypically non-arom isolates from two asci, numbers 1 and 7, from the cross of *arom M54* with *am su22* were analyzed further in crosses with wild type 74. The phenotypes of these two asci are given in Table 2. Only three of the four isolates from ascus 1 were crossed since isolate 5 was an *am* mutant. Random data from these crosses

TABLE 2

Backcrosses to wild type 74 of isolates from asci containing suppressors obtained in the cross arom M54 × am su22. *Random ascospore isolations*

Isolate number and phenotype	Percent ascospore germination	Classification of isolates			Indicated genotype of ascus isolate
		Wild type	*arom**	*am*†	
Ascus 1					
1a wild type	84%	65	19	0	*arom am*+ *su*
4a wild type	92%	50	19	23	*arom am su*
5A am	..	..	..	..	*arom*+ *am*+ *su*+‡
7A wild type	90%	50	0	0	*arom*+ *am*+ *su*+ (W.T.)
Ascus 7					
1a wild type	94%	84	6	4	*arom am su*
4a wild type	90%	38	22	12	*arom am su*
5A wild type	73%	50	0	0	*arom*+ *am*+ *su*+ (W.T.)
7A wild type	27%	27	0	0	*arom*+ *am*+ *su*+ (W.T.)

* *arom* isolates detected by morphological phenotype.
† *am* isolates detected by testing 50 phenotypically wild-type isolates on glycine.
‡ Cross not made; genotype inferred on basis of phenotype and other segregation evidence.

TABLE 3

Origin of non-complementing (E *and* F) arom *mutants tested for suppression by* am 17 *suppressor strains* am+ su22 *and* am su33. *Tests for suppression based on random ascospore isolations*

Number of mutants	Origin	Non-suppressed	Suppressed	Percent suppressed
43	UV	40	2	5.0
17	EMS	13	4	30.7
1	Spontaneous	..	1	...

indicated that in each ascus 2 *arom* and 2 *am* isolates were present. These results confirm the original evidence for the suppression of both the *arom* and the *am* requirements by suppressor *am+ su22*.

Suppression of additional arom *non-complementing* (E *and* F) *polar mutants:* After evidence was obtained for the suppression of *arom M54*, 60 additional *E* and *F* polar mutants were crossed to *am+ su22* and to another independently isolated *am 17* suppressor, *am su33* (SEALE 1968). The origin of the mutants tested and a summary of the suppression results are given in Table 3. It was found that two (5%) of the ultraviolet-induced mutants and four (30.7%) of the mutants derived from ethyl methane-sulfonate treatment were suppressed. In addition, the only mutant of spontaneous origin was suppressed. These data suggest that the mutagenic origin of these *arom* mutants may be significant with respect to their suppressibility. The random ascospore isolation data indicating suppression of an additional six *arom E* and *F* mutants (plus comparative data for *M54*) are presented in Table 4. In most crosses in the Table the observed segregations are not significantly different from the expected ratio of 3 wild type: 1 mutant characteristic of an unlinked suppressor. In three of the six crosses involving *am su33* a significant deficiency of *arom* segregants was obtained. These distorted ratios presumably result from the lower viability of *arom* ascospores. Tetrad analysis with all six additional suppressible mutants confirms the

TABLE 4

Random ascospore isolation data indicating suppression of certain arom *non-complementing* (E *and* F) *mutants by two* am 17 *suppressors*

		Crossed to *am+ su22*		Crossed to *am su33*	
arom mutant	Origin	Percent germination	WT:arom	Percent germination	WT:arom
M26	UV	86%	64:22	86%	79:17
M54	EMS	82%	64:18	82%	60:22
M1065	EMS	94%	78:16	..*	91:9
M1108	UV	86%	71:15	..*	84:15
M1162	EMS	78%	66:12	76%	59:17
M1172	EMS	..	....	..*	48:12
M1039	Spontaneous	94%	77:17	..	....

* Data from random ascospore platings.

TABLE 5

Random ascospore isolation data indicating no suppression of certain single gene mutants in the arom cluster by the am17 *suppressor* am+ su22

Mutant category and strain	Percent ascospore germination	Ratio wild type:arom
arom-1		
M80	78%	32:46
M1183	87%	42:35
arom-2		
M81	80%	41:39
M82	83%	47:34
arom-4		
M49	79%	52:27
M1050	57%	34:23
arom-5		
M56	56%	27:29
M1146	76%	48:21

random ascospore data in indicating the occurrence in each cross of the expected 6 wild type:2 *arom* segregations (Table 1). Although not all crosses produced viable ascospores, the available tests indicate that *am*+ *su22* and *am su33* suppress the same mutants.

Absence of suppression of single gene mutants in crosses with am+ su22: Selected *arom* single gene mutants which lack only one of the five enzyme activities, produce *arom* aggregates having a molecular weight equivalent to that of the wild-type aggregate, and which have been interpreted as resulting from missense mutations (GILES, *et al.* 1967a) were crossed to *am*+ *su22*. The random ascospore isolation data from these crosses are given in Table 5. In most crosses the observed ratios do not differ significantly from the 1:1 ratio expected in the absence of suppression. In two crosses (*M49* and *M1146*) ratios significantly different from 1:1 were obtained. However, these two ratios also differ significantly from the 3:1 ratio expected if suppression is occurring. Presumably the observed deficiency of *arom* segregants (resulting in ratios close to 2:1) is the result of reduced viability of *arom* ascospores. As noted previously, a similar result was observed in random ascospore isolations in certain crosses of non-complementing mutants in which suppression does occur (Table 4) and where the inviability interpretation is directly supported by tetrad data (Table 1). Consequently, the present results are taken as indicating that none of the eight *arom* single gene mutants are suppressed.

Biochemical characterization of a suppressed arom *mutant:* Three types of data have been utilized in the biochemical characterization of one of the suppressed *arom* mutants, *M54*, strain *54-1.1* (*arom am*+ *su22*): (1) Quantitative assay data for the five enzyme activities coded for by the *arom* gene cluster indicate that this suppressed mutant possesses all five activities, each at a level approximately 70% of wild type (Table 6); (2) Sucrose density gradient studies (Figure 1) demonstrate that the five activities present in the suppressed mutant

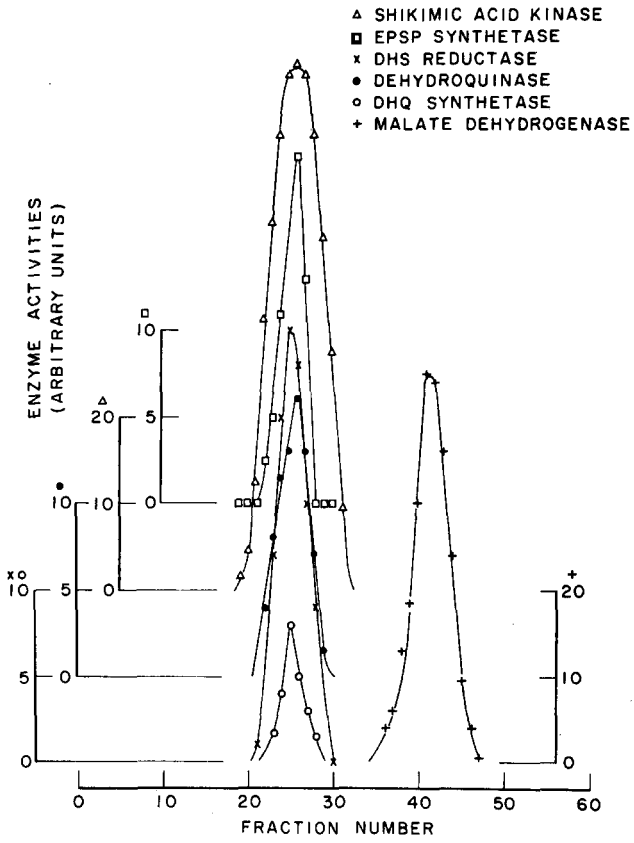

FIGURE 1.—Distribution, after centrifugation in a sucrose density gradient, of the five aromatic synthetic enzyme activities coded for by the *arom* gene cluster in *Neurospora crassa* (and of the reference activity—malate dehydrogenase) from a non-complementing arom mutant (*M54*) combined with a nonsense suppressor (*su22*). Procedures described in GILES, *et al.* 1967a. Assays of shikimate kinase and EPSP synthetase were performed by method (b).

TABLE 6

Specific activities, as percent of wild type 74A, *for the five enzymes coded for by the* arom *gene cluster in a suppressed* arom-54 *strain,* 54–1.1 (arom am+ su22)*

Dehydroquinase	DHS reductase	SA kinase	EPSP synthetase	DHQ synthetase
70%	75%	71%	75%	66%

* Assays were performed on protein precipitating in the 30–50% $(NH_4)_2SO_4$ saturation range. Mycelium was extracted in and protein precipitates dissolved in 100 mM, pH 7.4 KPO_4 plus 0.1 mM α thioglycerol. SA kinase was measured by method (a) and EPSP synthetase by method (a) as described in GILES, *et al.* (1967a).

TABLE 7

Assays to detect inducible dehydroquinase activity in wild type 74A *and in the suppressed* arom-54 *strain,* 54–1.1 *(*arom am$^+$ su22*)**.

Strain	Dehydroquinase activity		Percent inducible activity
	Extract	Heated Extract	
74A	10.0†	0	0
54–1.1 (54)	9.7	0.57	5.8%

* Assays were performed on material precipitated by 50% saturated $(NH_4)_2SO_4$. The induced dehydroquinase activity is defined as that portion of the total which is stable at 71° for 10 minutes in 100 mM pH 7.4 Tris HCl plus 1 mM EDTA and 0.1 mM α-thioglycerol (cf. GILES, *et al.* 1967b).

† Activities are given as mμ moles/min./mg. protein.

sediment together as an aggregate having a molecular weight indistinguishable from that of the normal *arom* enzyme aggregate present in wild type (GILES, *et al.* 1967a); (3) Tests to detect the presence of any thermostable inducible dehydroquinase activity, in addition to the thermolabile constitutive dehydroquinase activity, which occurs as part of the aggregate, indicate that in the suppressed *arom* mutant grown on minimal medium approximately 6% of the total dehydroquinase activity is of the inducible (thermostable) type (Table 7). Furthermore, additional studies indicate that this thermostable activity is found in material precipitating in the 0–30% ammonium sulfate fraction, whereas the thermolabile activity precipitates in the 30–50% fraction. Extracts of wild type grown under the same conditions contain no detectable inducible dehydroquinase activity (cf. also GILES, *et al.* 1967b).

DISCUSSION

SEALE (1968) has summarized the prior evidence from his studies that the *am* suppressors used in these investigations act to suppress nonsense codons in Neurospora. His observations indicate that a CRM$^-$ non-complementing glutamate dehydrogenase mutant (*am17*) (a strain forming no crossreacting material) reverted by suppressor mutations which also suppressed mutants at two different tryptophan loci—a *tryp-1* mutant and a *tryp-3* mutant (*td 140*). TERRY (1966) also reported that the latter mutant, which is CRM$^-$ and non-complementing, reverted by unlinked suppressor mutations. Nonsense suppressors were first well characterized in bacteriophage and bacterial system (cf. review by GORINI and BECKWITH 1966). Similar results have also been obtained with eucaryotes; e.g., super-suppressors, or multisite suppressors, which have the ability to revert several loci simultaneously, have been known for some time in yeast (HAWTHORNE and MORTIMER 1963; MANNEY 1964; GILMORE 1967).

In the present studies, genetic evidence is presented, based on both tetrad analysis and random ascospore isolations, that the two *am17* suppressors *am*$^+$ *su22* and *am su33* can suppress seven different non-complementing (*E* and *F*) *arom* mutants out of a total of 61 such mutants tested, whereas suppressor *am*$^+$

su22 failed to suppress any of eight single gene mutants tested—two in each of the four structural genes for which mutants are available in the *arom* cluster. These results are consistent with the conclusion that some non-complementing pleiotropic *arom* mutants are a consequence of nonsense mutations within the proximal structural gene (*arom-2*) of the *arom* gene cluster resulting in chain-terminating triplets in the polycistronic m-RNA. By contrast, single gene mutants lacking only one of the five enzyme activities—presumably the result of missense mutations—are not suppressed by the nonsense suppressors.

The biochemical characteristics of the suppressed mutant *M54* are compatible with the interpretation just discussed. In this suppressed strain each of the five aromatic synthetic enzyme activities is present at a high level, approximately 70% of that in wild type as is characteristic of *amber* nonsense suppressors (cf. GORINI and BECKWITH 1966). Furthermore, the five activities are associated in an aggregate having a molecular weight indistinguishable from that of the normal *arom* enzyme aggregate in wild type. Thus, the amino acid presumably inserted by the action of the nonsense suppressor in the DHQ synthetase polypeptide chain at a position corresponding to that of the nonsense codon results in the formation of an intact functional *arom* enzyme aggregate. The fact that the suppressed *M54* mutant produces an appreciable level of inducible dehydroquinase could indicate that this aggregate is not identical to the one in wild type, which produces no inducible dehydroquinase. However, the original *M54* mutant is known to produce a relatively high level of inducible dehydroquinase (GILES, *et al.* 1967b). On this basis the inducible dehydroquinase in the suppressed mutant may result from the production by this strain of some incomplete DHQ synthetase chains possessing sufficient activity to permit some synthesis of the inducer, DHQ, outside the *arom* aggregate. Evidence from bacterial and bacteriophage systems (WEIGERT, LANKA, and GAREN 1965; KAPLAN, STRETTON, and BRENNER 1965) indicates that suppression results in the production of partial chains in addition to complete and functional protein products in which the amino acid inserted by the action of the suppressor may be either identical to or different from that in the wild-type protein.

Failure to detect suppression by *am su22* of the small number of partially complementing polar mutants (*A*, *B*, *C*, and *D* types) as well as of the additional 54 non-complementing mutants (*E* and *F* types) tested does not prove that these mutants are not the result of nonsense mutations. Such non-suppressed mutants may either contain nonsense codons different from the ones suppressed by *am su22* or the amino acid inserted in these mutants may not be compatible with restoration of the five aromatic synthetic enzymic activities. The possibility also exists that some of these apparent polar mutants may result from other types of mutations, e.g., from frame shifts or from missense mutations affecting aggregation. Additional studies will be required to determine whether some or all of these mutants are suppressible by other nonsense suppressors. Preliminary evidence obtained in reversion studies with *arom M54* (CASE and GILES, unpublished) indicates that other types of nonsense suppressors exist which differ from the two *am 17* suppressors, since these new suppressors fail to suppress

am 17 but do suppress *td 140*. These suppressors presumably insert in *arom M54* an amino acid different from that inserted by the *am* suppressors. The action of these suppressors on the polar mutants used in the present investigations has not yet been tested.

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SUMMARY

Genetic analyses have been made to test for the suppression by nonsense suppressors, first detected in glutamate dehydrogenase (*am*) mutants, of various types of mutants in the *arom* gene cluster in *Neurospora crassa*. The results indicate that of 61 non-complementing mutants tested, seven are suppressed, whereas eight different single gene mutants (of four types) and three other types of presumptive polar mutants are not suppressed. Biochemical studies of one suppressed non-complementing mutant indicate that this strain possesses high levels (ca. 70% of wild type) of all five activities coded for by the *arom* gene cluster as an enzyme aggregate very similar to (but not necessarily identical with) that in wild type. These overall results support the previous hypothesis that non-complementing pleiotropic mutants in the *arom* gene cluster are the result of nonsense (chain-terminating) mutations in the proximal structural gene (*arom-2*) of this cluster of five structural genes which is transcribed in a polarized fashion via a single messenger RNA.

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