MUTATION AT THE *am* LOCUS OF *NEUROSPORA CRASSA*

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ABSTRACT

Forty-eight new mutations at the *am* locus of *Neurospora crassa* have been characterized. Nineteen mutations were induced by UV; of these, eight were missense, two were frameshifts, two were nonsense, three were deletions and four were unidentified. Twenty-nine mutations were induced with nitrous acid; of these, twenty-one were missense, three were frameshifts, one was nonsense, two were deletions and one was genetically unstable.

THE amination (*am*) gene of Neurospora codes for the enzyme NADP-specific glutamate dehydrogenase (GDH). This locus and its gene product have proved particularly favorable for genetical and biochemical analysis. The enzyme is a hexamer of six identical monomers, each 452 amino acid residues long. The complete sequence of the monomer has been determined (WOOTTON *et al.* 1974), and amino acid mutational replacements have been determined in a number of cases (BRETT *et al.* 1976; FINCHAM and BARON 1977; SEALE *et al.* 1977; SIDDIG *et al.* 1980; KINSEY *et al.* 1980). The sequence of mutational replacements in the polypeptide chain is co-linear with the sequence of mutational sites in the genetic map (FINCHAM 1967; SMYTH 1973; KINSEY *et al.* 1980). The sequence of the first 17 bases of the messenger RNA has also been deduced by comparing the amino acid sequences of double frameshift revertants of the mutant am_6 to that from the wild type (SIDDIG *et al.* 1980). With the introduction of a technique for the direct selection of mutants defective in GDH (KINSEY 1977), studies on mutation at the *am* locus have been facilitated. In this communication, we shall describe the characterization of 48 new mutant *am* alleles and discuss the frequency with which certain kinds of mutations occur. Some previous reports have been concerned with the characterization of certain individual mutants included in this study (KINSEY and FINCHAM 1979; KINSEY *et al.* 1980). The broad spectrum of mutational lesions found in this study confirms the general nature of the *am* selection procedure.

MATERIALS AND METHODS

All mutants were isolated in a *lys-1* stock that was obtained from a backcross of *lys-1* (r16)a to 74-OR-23-1A. The *lys-1* (r16)a strain was from the authors' stock collection. 74-OR-23-1A was obtained from the Fungal Genetics Stock Center, Arcata, Calif. The media used were those previously described by KINSEY (1977). The immunologically cross-reacting material (CRM)

test used was the variation of the technique of ROBERTS and PATEMAN (1964) previously described by KINSEY (1977). Enzyme assays used were those of CODDINGTON, FINCHAM and SUNDARAM (1966).

Tests for complementation between *am* mutants were made by inoculating tubes containing 1 ml of VOGEL's liquid medium (VOGEL 1956) (supplemented with 0.02 M glycine) with about 10^5 conidia from doubly inoculated tubes of complete medium. Growth was assessed by eye after 3 days at 25°. All strains had been made heterocaryon-compatible by repeated crossing to the wild type IVA (Fungal Genetics Stock number 2489) or ORSa (Fungal Genetics Stock number 2490).

Reversion frequencies were determined using conidia from 5-day cultures started as single conidial colony isolates. The technique used was that of STADLER (1966) for UV treatment. The technique for ICR-170 treatment was that of WHONG and BROCKMAN (1974). The ICR-170 concentration used was 5 μg per ml.

RESULTS

Mutant isolation: Mutant strains numbered am_{100} to am_{114} and am_{145} to am_{148} were isolated after treatment with ultraviolet light. The *am* mutant frequency obtained was 0.89×10^{-6}. Mutant strains numbered am_{116} to am_{144} were isolated after nitrous acid treatment. The *am* mutant frequency here was 4.1×10^{-6}. All mutants were isolated using the direct selection procedure (KINSEY 1977). Each mutant was crossed to a strain containing a wild-type allele at the *am* locus and re-isolated as a single *am* spore from this cross. Most characterization tests were performed on these isolates; however, complementation tests and ICR-170 reversion experiments were performed on stocks that had been extensively backcrossed (six or more generations) to the standard IVA or ORSa strains.

CRM determination: As a first step in their characterization, the new *am* mutants were examined for the production of either an active form of GDH or an immunologically related protein. Three of the mutants (am_{122}, am_{130} and am_{131}) produced GDH that was active in the reaction C (high substrate concentration) of CODDINGTON *et al.* (1966). These mutants have been the subject of a previous report (KINSEY *et al.* 1980).

Table 1 lists the results of CRM determinations for the 48 new mutants. As controls, am_{15}, a CRM negative mutant, and am_2, a CRM-positive mutant, were included. For purposes of standardization, we arbitrarily chose 25% protection as the cut-off point between mutants assigned as CRM-negative (25% or less protection) and CRM-positive (26% or more protection). By this criterion 31 of the mutants (65%) were CRM-negative and 17 (35%) were CRM-positive. When the mutants were divided according to the mutagen used to induce them, the results, even though the numbers were small, are interesting. There were 19 UV-induced mutants, but only 3 of them were CRM-positive (16%); whereas, 16 (84%) were CRM-negative. In contrast, of 29 nitrous acid-induced mutants, 14 (48%) were CRM-positive and 15 (52%) were CRM-negative.

Temperature sensitivity tests: The mutant isolation procedure was carried out at 33°, which represents a nonpermissive temperature for most Neurospora temperature-sensitive mutants. Therefore, all of the mutants were screened for mutant phenotype at 33° and 25°. The basis for the determination was the ability to grow on VSGG (Vogel's minimal agar containing 1.5% sorbose, 0.2% glucose and 0.2% glycerol) containing 0.02 M glycine (*am* mutants are inhibited

TABLE 1

Cross reacting material production by am *mutants*

Strain	% Protection*	CRM†	Strain	% Protection*	CRM†
15	15	—	124	22	—
2	77	+	125	72	+
100	0	—	126	0	—
101	5	—	127	0	—
102	11	—	128	0	—
103	16	—	129	0	—
104	87	+	130	81	+
105	9	—	131	90	+
106	47	+	132	0	—
107	6	—	133	66	+
108	3	—	134	51	+
109	0	—	135	68	+
110	89	+	136	7	—
111	8	—	137	21	—
112	0	—	138	0	—
113	12	—	139	0	—
114	12	—	140	19	—
116	84	+	141	0	—
117	71	+	142	0	—
118	100	+	143	29	+
119	6	—	144	87	+
120	17	—	145	23	—
121	8	—	146	92	+
122	78	+	147	0	—
123	71	+	148	5	—

* Percent protection = 100 $\times \frac{(\Delta OD_{340} \text{ mutant extract + antiserum + wild-type extract}) - (\Delta OD_{340} \text{ wild-type extract + antiserum})}{(\Delta OD_{340} \text{ wild-type extract alone}) - (\Delta OD_{340} \text{ wild-type extract + antiserum})}$

† CRM-negative is defined as 25% or less protection.

on this medium; wild type is not). Five of the 48 mutant strains (am_{105}, am_{117}, am_{120}, am_{123} and am_{136}) were able to grow on glycine medium at 25°, but not at 33°. All other mutants were inhibited at both temperatures. One of the 19 (5%) UV-induced mutants was temperature sensitive as were 4 of 29 (14%) of the nitrous acid-induced mutants.

Complementation tests: In complementation tests among the original isolates of these *am* mutants, only 5 appeared active. Both am_{119} and am_{126} would complement with either am_{122}, am_{130} or am_{131}, but not with each other. After an extensive backcrossing program to the standard IVA or ORSa strains, mutants were again tested for complementation. Because of the inordinate size of the complementation matrix required to test all mutants against each other, a somewhat simpler test was used. Including the 5 mutants listed above, 11 complementing *am* mutants have been previously described (see KINSEY *et al.* 1980). Therefore, all of the new *am* alleles were tested against each of these 11 alleles, as well as a heterocaryon-compatible *lys-1* allele. Table 2 displays a complementation matrix that includes only those mutants that showed complementation with at least one other mutant. In all, 20 of the 48 mutants (42%) are complementing.

Of the UV-induced mutants, 5 of 19 (26%), and of the nitrous acid-induced mutants, 15 of 29 (52%) were complementing. With the addition of these mutants, the complementation map for the *am* locus ceases to have any simple configuration.

Reversion by UV mutagenesis: All of the mutants, with the exception of those that were temperature sensitive, and am_{126} were tested for reversion after treatment with UV. The results (Table 3) are ordered, from lowest to highest UV-induced reversion frequency, within groups that are divided on the basis of CRM production. am_1, which reverts only by true reversion (STADLER 1966; SEALE 1968), and am^{17}, a nonsense mutant (SEALE 1968), were included as controls.

Deletion analysis: There were 12 mutants that showed no apparent reversions after UV treatment. All but three of these were induced by nitrous acid. We had initially used nitrous acid because of its reported effectiveness as a deletion-generating mutagen (ALPER and AMES 1975). Since mutations that are not reverted by UV constitute possible deletions, all of the UV nonreverting mutants were tested in an extensive crossing matrix with each other and with previously mapped *am* mutants. (FINCHAM 1967; SMYTH 1973). Two of the nonreverting mutants proved to be deletions on the basis of the simple genetic test of failure to give recombinant progeny when crossed to two mutants that give recombinant progeny when crossed to each other. Mutant am_{108} failed to show recombination with either am_{137} or am_{143}; whereas, am_{137}, when crossed to am_{143}, gave recombi-

TABLE 2

Complementation between am *mutants**

Mutant strain	1	2	3	7	14	19	119	122	126	130	131	*lys-1*
102	+	0	0	0	0	0	++	0	0	0	0	++
104	0	0	0	—	+	+	+	—	0	0	±	++
105†	+	0	0	0	0	0	0	0	0	0	0	++
107	0	0	—	0	0	0	0	0	0	0	—	++
117†	0	0	+	0	0	+	0	0	0	+	+	++
118	0	0	—	0	±	+	0	0	—	0	0	++
120†	+	0	+	+	0	+	0	0	0	±	+	++
123†	+	0	+	0	0	+	0	0	0	+	+	++
124	+	0	+	+	+	+	+	—	0	0	±	++
127	+	0	0	0	0	0	+	0	0	0	0	++
134	0	0	0	0	±	+	0	0	0	0	—	++
135	±	0	0	0	+	+	0	0	—	0	0	++
136†	+	0	0	0	0	0	0	0	0	0	0	++
137	+	0	0	0	0	+	0	0	—	0	0	++
138	+	±	+	+	+	0	+	—	0	±	+	++
139	+	0	0	0	0	0	+	0	0	0	0	++
143	+	0	0	0	0	0	+	0	0	0	0	++
146	+	+	+	0	+	+	0	+	+	+	+	++
148	±	0	±	—	—	±	—	0	0	0	—	++
lys-1	++	++	++	++	++	++	++	++	++	++	++	++

* 0 indicates no growth, — indicates trace of growth, ++ indicates wild-type growth.

† am_{105}, am_{117}, am_{120}, am_{123} and am_{136} complementation tests were performed at 33° because they are temperature sensitive. Other tests shown here were performed at 25°.

TABLE 3

Reversion frequencies for am *mutants after UV treatment*

Strain	Total live conidia tested	Revertants per 10^6 live conidia	Strain	Total live conidia tested	Revertants per 10^6 live conidia
118	1.04×10^6	0	125	14.4×10^6	2.3
135	23.34×10^6	0	110	2.8×10^6	2.8
143	13.4×10^6	0	116	2.0×10^6	8.0
122	2.4×10^6	0.41	104	1.28×10^6	11.7
144	6.7×10^6	0.45	130	2.48×10^6	12.1
133	12.2×10^6	0.49	146	1.9×10^6	16.3
1	11.7×10^6	0.6	131	1.7×10^6	20.2
134	14.7×10^6	0.75	106	0.96×10^6	55.2
		CRM Positive mutants			
103	8.8×10^6	0	111	4.7×10^6	1.06
107	4.5×10^6	0	139	10.9×10^6	1.2
108	16.6×10^6	0	138	2.6×10^6	3.8
112	9.1×10^6	0	119	3.7×10^6	4.3
114	9.8×10^6	0	142	4.5×10^6	5.3
127	1.3×10^6	0	100	18.3×10^6	7.04
128	13.6×10^6	0	124	3.6×10^6	12.1
132	30.0×10^6	0	102	7.0×10^6	15.8
137	15.9×10^6	0	17	4.0×10^6	29.9
121	16.3×10^6	0.12	109	1.3×10^6	36.2
140	7.05×10^6	0.14	145	0.89×10^6	42.7
129	2.9×10^6	0.34	101	0.9×10^6	50.4
141	9.4×10^6	0.42	148	1.8×10^6	54.0
113	6.3×10^6	0.94	147	1.0×10^6	108.0
		CRM Negative mutants			

nants. Mutant am_{132} failed to give recombinants when crossed to any of the tested *am* mutants, which included the most centromere-proximal allele known, am_6, as well as the most centromere-distal allele, am_9. Thus am_{132} probably deletes at least the entire *am* gene.

Suppression by super-suppressors: To facilitate detection of possible super-suppressible mutants, stock were constructed that consisted of the large deletion am_{132} and either *ssu-1* or *ssu-2* (SEALE, CASE and BARRATT 1969). When either of these stocks is crossed to an *am* mutant of unknown nature, all of the progeny will have an *am* phenotype unless the new *am* allele is suppressible. In this fashion, all 47 of the remaining mutants were tested. Only three mutants were found that were suppressible: am_{101}, am_{109} and am_{142}. All three were suppressed by both *ssu-1* and *ssu-2*. Only one of the suppressible mutants was induced by nitrous acid.

Reversion after treatment with ICR-170: The compound ICR-170 has been thought to cause both one-base additions and one-base deletions in Neurospora (MALLING 1967; BRUSICK 1969). It has been used to isolate yeast mutants that behave in many respects like the known frameshift mutants of bacteria (CULBERTSON *et al.* 1977). We therefore attempted to revert our CRM-negative *am*

mutants, not otherwise identified, with this mutagen. The adenine-3 mutant N23 (ONG 1978), which is known to revert with ICR-170, and the defined frameshift mutant am_6 (SIDDIG *et al.* 1980) were included as controls. In addition, three previously described mutants, am_9, am_{15} and am_{16}, that are CRM-negative (ROBERTS and PATEMAN 1964) are not reverted by UV treatment (PATEMAN 1960; STADLER 1966) were also included. Of these, am_{15} had previously been found to revert with ICR-170 (FINCHAM, personal communication). The results are shown in Table 4.

DISCUSSION

Our goal at the onset of this project was to try to characterize a set of *am* mutants isolated after UV and nitrous acid mutagenesis, for two principal reasons. First, to assure ourselves that the direct selection procedure was indeed nonbiased, and second, to provide a well-defined set of mutants for use in subsequent studies of recombination at the *am* locus. The basic strategy used was to employ a series of tests, that could be applied with some facility to this large group of mutants and would allow us to infer the nature of the mutational lesion.

TABLE 4

Reversion frequencies for am *mutants after ICR-170 treatment*

Strain	Percent survival	Total live canidia tested	Revertants per 10^6 live canidia
N24	88	9.3×10^6	68.2
6	100+*	2.3×10^7	0.5
16	29	8.6×10^6	0
102	30	7.8×10^6	0
103	54	1.5×10^7	0
107	33	1.1×10^7	0
108	21	4.7×10^6	0
111	52	1.8×10^7	0
112	49	1.6×10^7	0
113	40	2.6×10^6	0
124	32	1.3×10^6	0
128	48	1.5×10^7	0
139	34	3.8×10^6	0
140	46	1.3×10^7	0
137	26	5.2×10^6	0.38
138	62	1.6×10^7	0.6
9	43	1.3×10^7	0.7
127	50	1.1×10^7	0.7
143	36	8.4×10^6	0.7
114	36	1.3×10^7	2.7
15	31	1.0×10^7	2.8
100	64	1.6×10^7	7.4
121	39	1.4×10^7	13.5
141	53	1.2×10^7	27.8
129	26	3.6×10^6	34.2

* In two independent experiments, the survival was apparently greater than 100% as though ICR-170 increased the viability of this particular stock. We have no explanation for this at present.

The first broad category that we tried to establish was the subset of mutants that contained missense base-pair substitution mutations (Table 5). First, we made the assumption that most mutants that produce a polypeptide immunologically cross-reading with GDH are missense mutants. Previous experience with labile enzyme from certain missense mutants had suggested that nonsense and frameshift polypeptide fragments are unlikely to be cross-reacting. A second assumption was that mutants that are temperature sensitive are missense mutants, whether or not they produce CRM. We also made the assumption that mutants that are capable of complementation are missense mutants. The latter assumption is probably the weakest since it is possible that polypeptide fragments generated by nonsense or frameshift mutations would be capable of complementation. With regard to this argument, none of the mutants assigned as missense on the basis of complementation alone were suppressed by either *ssu-1* or *ssu-2*; however, four of the mutants were reverted at low frequencies with ICR-170. ICR-170 is known to revert base-pair substitution mutants at a low frequency (MALLING 1967), but it should be pointed out that the reversion frequency seen with these mutants approximates that seen with am_6, the only rigorously defined frameshift mutation in Neurospora (SIDDIG *et al.* 1980). Therefore, the assignment of these four mutants (am_{127}, am_{137}, am_{138} and am_{143}) as missense can be considered only tentative.

Missense mutants that have high reversion frequencies after UV mutagenesis probably have GDH's altered in tertiary structure, as was suggested for am_{19} (SEALE 1968). The implication is that a number of second-site amino acid substitutions can result in restoration of a normal or near-normal tertiary structure. Mutants $am_{116, 104, 130, 146, 131, 106, 124}$ and $_{148}$ fall into this category. The products of the last two of these mutants are presumably unstable, as they failed to produce CRM.

Missense mutants that, like am_1, have a very low reversion frequency presumably represent lesions for which reversion can occur either only as true reversion or by a very small number of alternate events. These mutants possibly represent lesions in the active site of the enzyme.

The second category that we have established consists of those mutations that are probably frameshift mutations. The assumption here is that a high reversion frequency after treatment with ICR-170 is indicative of a frameshift mutation. The converse is obviously not true, as indicated by our experience with am_6. If the apparent tendency for ICR compounds to affect runs of G-C pairs (ROTH 1974) holds for ICR-170, then the ability of ICR-170 to revert frameshift mutant would depend upon having a run of G-C pairs within sufficiently close proximity to the original lesion that a double-frameshift region is tolerable for a functional protein. It is possible that the four mutants designated as questionable missense mutants in fact represent frameshifts and that their complementation is due to the production of a polypeptide fragment. The strongest argument against this idea is that all of these mutants map in the "amino-terminal" half of the gene (unpublished results).

Two previously described mutants, am_9 and am_{15}, both of which are CRM-negative, non-complementing and not revertible by UV treatment, also showed reversion with ICR-170. Of these, am_{15} probably results from a frameshift; am_9,

TABLE 5

Designation of mutation type

Allele no.	Mutagen	CRM	Complementing	UV reversion*	ICR reversion*	Suppression†	Designation
102	UV	—	Yes	High	0	No	Missense
104	UV	+	Yes	High	ND	No	Missense
105	UV	—	No	ND	ND	No	Missense (ts)
106	UV	+	No	High	ND	No	Missense
107	UV	—	Yes	0	0	No	Missense
110	UV	+	No	Medium	ND	No	Missense
116	NA	+	No	Medium	ND	No	Missense
117	NA	+	Yes	ND	ND	No	Missense (ts)
118	NA	+	Yes	0	ND	No	Missense
119	NA	—	Yes	Medium	ND	No	Missense
120	NA	—	Yes	ND	ND	No	Missense (ts)
122	NA	+	Yes	Low	ND	No	Missense
123	NA	+	Yes	ND	ND	No	Missense (ts)
124	NA	—	Yes	High	ND	No	Missense
125	NA	+	No	Medium	ND	No	Missense
127	NA	—	Yes	0	Low	No	Missense ?
130	NA	+	Yes	High	ND	No	Missense
131	NA	+	Yes	High	ND	No	Missense
133	NA	+	No	Low	ND	No	Missense
134	NA	+	Yes	Low	ND	No	Missense
135	NA	+	Yes	0	ND	No	Missense
136	NA	—	No	ND	ND	No	Missense (ts)
137	NA	—	Yes	0	Low	No	Missense ?
138	NA	—	Yes	Medium	Low	No	Missense ?
139	NA	—	Yes	Medium	0	No	Missense
143	NA	+	Yes	0	Low	No	Missense ?
144	NA	+	No	Low	ND	No	Missense
146	UV	+	Yes	High	ND	No	Missense
148	UV	—	Yes	High	ND	No	Missense
100	UV	—	No	Medium	Medium	No	Frameshift
114	UV	—	No	0	Medium	No	Frameshift
121	NA	—	No	Low	High	No	Frameshift
129	NA	—	No	Low	High	No	Frameshift
141	NA	—	No	Low	High	No	Frameshift
101	UV	—	No	High	ND	Yes	Nonsense
109	UV	—	No	High	ND	Yes	Nonsense
142	NA	—	No	Medium	ND	Yes	Nonsense
103	UV	—	No	0	0	No	Deletion ?
108	UV	—	No	0	0	No	Deletion
112	UV	—	No	0	0	No	Deletion ?
128	NA	—	No	0	0	No	Deletion ?
132	NA	—	No	0	ND	No	Deletion
111	UV	—	No	Medium	0	No	?
113	UV	—	No	Medium	0	No	?
145	UV	—	No	High	ND	No	?
147	UV	—	No	High	ND	No	?
126	NA	—	Yes	?	?	No	unstable

* Induced reversion frequencies; Low indicates less than 1; Medium indicates between 1 and 10, and High indicates more than 10 revertants per 10^6 live conidia. ND indicates not done.

† Suppression by *ssu-1* and *ssu-2*.

which has a reversion frequency similar to am_6, possibly contains a frameshift mutation as well.

Only three of the 48 mutants were suppressed by *ssu-1* and *ssu-2*. This represents minimum estimate of chain-terminating mutation at the locus, for apparently both *ssu-1* and *ssu-2* represent the same class of super-suppressor (presumably either amber or ochre). To date, our attempts to build tester stocks with other described super-suppressors (SEALE, CASE and BARRATT 1969) have been unsuccessful.

There are five mutants that behave as we would expect for deletions. Only two of these (am_{108} and am_{132}) have been shown by rigorous genetic tests actually to be deletions. Two of the others (am_{112} and am_{128}) fail to recombine with each other; however, they both show recombination with other mutants in the same area of the genetic map (unpublished results) so that, if they are deletions, they must be quite small. The last mutation in this category (am_{103}) also shows recombination with all other tested mutations that map in the same region of the locus; if it is a deletion, it also must be a small one.

There were five mutants in this study that have so far defied simple categorization. Of these, one is genetically unstable (KINSEY and FINCHAM 1979), and could possibly represent either a duplication or an insertional mutation. The other four mutants are all CRM-negative, noncomplementing and not suppressed by *ssu-1* or *ssu-2*. They have medium or high reversion frequencies after UV treatment, and the ones tested failed to revert with ICR-170. One straightforward explanation for this group of mutants is that they contain chain-terminating codons not suppressed by *ssu-1* or *ssu-2*. However, it is possible that they represent either frameshift mutations in a region where ICR-170 is ineffective or a new class of missense mutants.

Assuming them to be correct, our assignments can be used to indicate the spectrum of mutations caused by UV or nitrous acid at the *am* locus (they certainly confirm the general nature of the *am* selection procedure). The breakdown of mutations was as follows: of those induced by UV (19), 8 were missense (42%), 2 were frameshifts (11%), 2 were nonsense (11%), 3 were deletions (16%) and 4 were unidentified (20%). Of those induced by nitrous acid (29), 21 were missense (73%), 3 were frameshifts (12%), 1 was nonsense (4%), 2 were deletions (7%) and 1 was unstable (4%).

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