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Identification of Nonsense Mutations in *Neurospora*: Application to the Complex *arg-6* Locus

Rowland H. Davis[1] and Richard L. Weiss[2]

[1] Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California 92717, USA
[2] Department of Chemistry and Biochemistry, University of California, Los Angeles, Los Angeles, California 90024, USA

Summary. The *arg-6* locus of *Neurospora crassa* encodes two enzymes of arginine synthesis, acetylglutamate kinase and acetylglutamyl phosphate reductase. Mutants lacking one or the other enzyme fall into two different complementation groups; a large non-complementing group lacks both enzymes. We wished to survey over 50 alleles for suppressibility by a nonsense suppressor. We compared two methods of assessing suppressibility. One, based on *trans*-action of a nonsense suppressor, was simple, but not efficient in detecting all suppressible alleles. The other, based on crosses involving a marker linked to the locus surveyed was very efficient in methodology and is suited to all cases, such as the *arg-6* locus, in which allelic crosses are sterile. The data indicate that the *arg-6* locus encodes a bifunctional protein.

Introduction

The *arg-6* locus of *Neurospora crassa* appears to be a complex genetic region which controls two early steps in arginine biosynthesis. On the basis of complementation analysis, a large group of mutants recently isolated (Davis 1979) can be grouped into three categories. Two groups, A and B, fail to complement within themselves, but complement readily with each other. A third, non-complementing (NC) group does not complement within itself, nor with groups A or B. Enzymatic tests (Davis 1979; Wolf and Weiss 1980, and unpublished observations) reveal that the NC group lacks activity for acetylglutamate kinase (ATP: N-acetylglutamate 5-phosphotransferase, EC 2.7.2.8) and acetylglutamylphosphate reductase (N-acetyl-L-glutamyl-5-semialdehyde: $NADP^+$ oxidoreductase [phosphorylating], EC 1.2.1.38) (Wolf and Weiss 1980). Group A mutants lack the kinase; group B mutants lack the reductase. The locus is very similar in its functions and complementation behavior to the homologous "argBC" region of *Saccharomyces cerevisiae* (Minet et al. 1979; Jacobs et al. 1980). The questions posed by observations on the two fungal systems are whether the two singly-deficient groups of mutants represent two genes transcribed into separate mRNA's; whether the entire region is transcribed into one mRNA and, if so, whether one or two proteins finally emerge from the translation of this mRNA.

Offprint requests to: R.H. Davis

The action of a genetic locus is most clearly demonstrated with null mutants of the nonsense or frameshift type, particularly if mutations reveal translational polarity with respect to component functions (Manney 1964; Radford 1969, 1970; Case and Giles 1968). The identification of nonsense mutations usually requires crosses of mutants with a strain carrying an informational suppressor gene. Present methods for such analysis are not well developed in *Neurospora*, in part because of sterility in crosses called for by certain direct rationales, and in part because of the lack of a vegetative diploid phase. Accordingly, in this paper, two other tests of nonsense suppressor action were made on an array of *arg-6* mutants. One was based on a heterokaryon test for suppressor action, in which trans-dominance of the suppressor mutation is required. The other was based on a crossing regime in which a visible marker linked to *arg-6* is used to facilitate assessment of the progeny genotypes of a sexual cross. The *arg-6* nonsense mutations identified are used to test hypotheses of *arg-6* gene structure and action.

Materials and Methods

Strains. A list of tester strains, with constituent loci and alleles, is given in Table 1. All are related by origin or backcrossing to the standard wild-type strain 74A of *Neurospora crassa*, and were constructed by appropriate crosses. The mutations in these testers are as follows. (a) Y319-44 ("ssu-1?"), isolated by Case and Giles (1974) is a nonsense suppressor on linkage group VIIR, similar in its linkage relations and action spectrum to the classic *ssu-1* allele, WRN 33 (Seale 1968, 1972, 1976). Y319-44 has not been

Table 1. List of tester strains

Strain	Genotype, mating type	Alleles	Origin[a]
IC10	*ssu-1, al-2 a* R1a	Y319-44, 15300	This work
IC11	*ssu-1, al-2 A* R3A	Y319-44, 15300	This work
IC12	*arg-6, ssu-1 a* R1a	CD-63, Y319-44	This work
IC13	*arg-6, ssu-1 A* R2A	CD-63, Y319-44	This work
IC14	*arg-6 A* R1A	CD-63	Davis (1979)
IC15	*arg-6 a* R3a	CD-63	Davis (1979)

[a] Strains carrying all mutations listed above are obtainable from Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, CA 95521

definitively proven to be allelic to WRN 33, but for convenience will be called *ssu-1* here. The symbol ssu^+ will designate the wild-type (inactive) form of the gene. Y319-44 was previously shown to restore enzyme activity and a polypeptide of appropriate molecular weight to nonsense mutants of the *arg-3* locus (Davis et al. 1981). Strains carrying *ssu-1* can be recognized by a 1–2 day delay in the onset of vigorous growth after germination of ascospores, and by a less fluffy conidial mass than Ssu^+ strains. (b) The al-2 allele, 15300, lies 0.5 map unit to the left of *arg-6* on linkage group IR and imparts an albino (white) phenotype to conidia. (c) The *arg-6* allele, CD-63, is a non-complementing mutant (in tests with all other *arg-6* alleles); it is not suppressible by *ssu-1*, and it is not rendered complementing by *ssu-1* (see *Results*). Strains carrying it were used in heterokaryon tests for suppression. Strains carrying all mutations given above are available from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, CA 95521.

Genetic Methods. Heterokaryon tests (Davis and de Serres 1970) were made by combining drops of conidial suspensions in 1 ml of minimal medium N of Vogel (1964) in 13 mm × 100 mm culture tubes with loose aluminum caps. Growth was scored each day for 10 days. Genetic analysis involving crosses were done by standard methods (Davis and de Serres 1970), and rationales are given in the Results. Intercrosses among *arg-6* mutants were sterile, which precluded fine-structure analysis of the locus. Sterility also precluded a straightforward test of suppressor action; namely, mating a tester strain carrying a non-suppressible *arg-6* allele (e.g., CD-63) and *ssu-1* to all other *arg-6* strains, and plating each cross on minimal medium. While this rationale works well in other systems (e.g. Kinsey and Hung 1981), it was impossible to apply here.

Results

Complementation Analysis of Mutants

All but two auxotrophic *arg-6* mutants, as well as several that displayed extremely poor growth on minimal medium, could be classified into the A (4 mutants), B (28 mutants) and NC (12 mutants) groups by complementation tests. The two exceptions were CD-189, which complemented with all A mutants and a few B mutants, and CD-219, an A mutant which complemented with only some B mutants. (Neither, as later tests showed, was suppressible.) There were 9 leaky mutants which were impossible to classify by complementation.

Crosses to Detect Nonsense Mutants

In this method, crosses are made to reveal whether *arg-6, ssu-1* double mutants will grow without an arginine supplement. The efficiency of the technique was greatly enhanced by use of the *al-2* (albino) marker, which is approximately 0.5 map units from *arg-6*. In practice, the *arg-6* mutants to be tested were mated individually to either IC10 or IC11 (*ssu-1, al-2*). Ascospores were plated on arginine-containing plating medium, and 50 colonies were transferred the next day to 10 mm × 75 mm slants of minimal medium. Because of tight linkage of *arg-6* and *al-2*, only four genotypic categories were expected (Table 2). In essence, all strains carrying arg-6^+ (with the associated *al-2* mutation) should grow and will be white. Progeny with *arg-6*$^-$ alleles, in combination with ssu^+, should fail to grow. However, progeny having *arg-6*$^-$ alleles in association with *ssu-1* should grow into orange cultures if they are suppressible, and should not grow at all if they are not suppressible. Thus, any set of spores yielding orange cultures signifies that the *arg-6* allele in question is suppressible. The test was strengthened by initial recognition of Ssu^- (slow growing) strains (see Materials and Methods). When orange progeny were found, they always appeared in the Ssu^- class.

The crosses detected twelve suppressible alleles. Eleven were group B, and one was non-complementing (Table 3). Several points regarding the crossing tests should be made. (a) "Leaky" alleles distinguished themselves from weakly suppressible alleles by the absence of complete auxotrophs among progeny in the minimal tubes. No leaky alleles were suppressible (i.e., less leaky with *ssu-1*). (b) Weakly suppressible alleles which failed to produce conidia could be transferred from the minimal tubes to arginine-supplemented media, where expectations about conidial color could be confirmed. (c) A slight orange tinge of *ssu-1, al-2* double mutants indicated that the *al-2* allele used was slightly suppressible. This did not confuse classificatin. (d) Trials in which ascospores were plated directly on minimal plating

Table 2. Major progeny types expected in *arg-6*$^+$, *al-2*; *ssu-1* × *arg-6*, *al-2*$^+$; ssu^+ crosses[a]

Progeny genotype	Percent expected	Phenotype
arg-6$^+$ *al-2*; ssu^+	25	Albino prototroph
arg-6$^+$ *al-2*; *ssu-1*	25	Albino prototroph
arg-6 al-2$^+$; ssu^+	25	Orange auxotroph
arg-6 al-2$^+$; *ssu-1*	25	Orange; prototroph if *arg-6* is suppressible, auxotroph if not

[a] The distance between *arg-6* and *al-2* is 0.5 cM; recombinants appear very infrequently in small populations of progeny

Table 3. Classification of *arg-6* mutants for leakiness and nonsense suppression

Group	Mutation	Leakiness	Suppressibility	
			Heterokaryon	Mating test
A	29, 88, 136, 174	–	–	–
B	34, 36, 50, 92, 116, 118, 127, 142, 185, 210	–	–	–
	73, 120, 126	–	–	Sterile
	25, 44, 46, 62, 137, 161, 222	–	–	+
	65, 78, 111, 199	–	+	+
	184	–	+	Sterile
	48, 204	+	ND[a]	–
NC	63, 68, 76, 121, 128, 132, 133, 138, 147, 217, MEP-1	–	–	–
	129	–	–	+
Unclassified	189, 219	–	–	–
	23, 33, 42, 49, 54,	+	ND	ND
	162, 169, 211	+	ND	–

[a] ND – not determined

Table 4. Complete analysis of *arg-6* crosses involving the nonsuppressible *CD-63* and four suppressible alleles: *arg-6, al*$^+$; *ssu*$^+$ × *arg-6*$^+$, *al-1*; *ssu-1*

arg-6 allele in cross	Progeny phenotypes[a]						
	Ssu$^+$[b]		*Ssu*$^-$				
	Al$^+$	*Al*$^-$	*Arg*$^+$ *Al*$^+$	*Arg*$^-$ *Al*$^+$	*Arg*$^+$ *Al*$^-$	*Arg*$^-$ *Al*$^-$	*Suppression*
CD-63	17	16	0	8	8	0	No
CD-65	19	11	8	0	11	0	Yes
CD-78	15	12	5	0	11	0	Yes
CD-111	12	18	11	1[c]	8	0	Yes
CD-199	14	19	10	0	7	0	Yes

[a] Ratio of *ssu*$^+$: *ssu-1* in pooled data: 153:88. Classification of *ssu* alleles was done first on the basis of retarded growth. Color and auxotrophy were determined later

[b] Not tested for arginine requirement; Al$^+$ are presumed to be Arg$^-$

[c] This isolate is presumed to be misclassified for *ssu-1*

medium, followed by observations of colony color on plates were not sufficiently rigorous in detecting suppressible *arg-6* alleles. (e) All *arg-6* alleles tested were found to show linkage to *al-2* in the crosses; at most, only one of the Ssu$^+$ prototrophs was orange. Thus, the method confirmed the genetic location of the mutations in the *arg-6* region. (f) The progeny bearing *ssu-1* were selected against to some extent (Table 4); this would compromise tests for suppressible alleles on the basis of Arg$^+$:Arg$^-$ ratios alone.

Heterokaryon Tests for Nonsense Suppression

This method depends upon forming heterokaryons of *arg-6, ssu-1* strains, IC12 or IC13, in which the *arg-6* allele is non-complementing and non-suppressible, with an array of *arg-6* single mutants to be tested. Growth of the heterokaryon indicates that the dominant *ssu-1* mutation in the tester nucleus suppresses the *arg-6* mutation in the other nucleus. The heterokaryon test identified five mutations as suppressible: CD-65, -78, -111, -199, and -184 (Table 2). The first four were suppressible by the crossing test. The last mutant was sterile in the cross, and the heterokaryon test is the only evidence of its suppressibility. Three controls accompanied the heterokaryon tests. (a) The *arg-6* mutants were tested alone to detect leakiness, a factor which must be excluded as a reason for growth in the test condition. In fact, some mutants were leaky and are listed as such in Table 2. (b) The *arg-6* mutants were tested against the IC14 or IC15 strain to confirm non-complementarity with CD-63. (c) IC12 and IC13, the tester strains, were themselves tested against *bona fide* group A and group B mutants to prove that *ssu-1* has not converted CD-63 to a complementing form. (This control is inherent in the survey of *arg-6* strains.) The second and third controls showed, as expected, that CD-63 was non-complementing, both in the presence and in the absence of *ssu-1*.

Test for Suppression of Translational Polarity Effects

Nonsense mutations are polar in their effect on translation. In a bifunctional locus, a nonsense mutation in a translational-proximal region may eliminate both proximal and distal functions. A nonsense suppressor may relieve the polarity effect without relieving the deficiency for the proximal function. If so, some non-complementers (doubly deficient) may be converted to complementers (singly deficient) by *ssu-1*, rather than to prototrophs. This phenomenon occurs in the *argBC* locus of yeast (Minet et al. 1979).

To test this possibility, all non-complementing (NC) alleles, alone or in combination with the *ssu-1* mutation, were tested for complementation by forming heterokaryons with CD-29 (A), CD-34 (B), and CD-63 (NC). (None of these testers is suppressible.) No heterokaryon displayed growth, indicating that *ssu-1* did not relieve polarity of the mutations sufficiently for growth to occur. The test is not rigorous, however, given the proven inefficiency of suppressor action in heterokaryons.

Discussion

The major goal of this work was to classify *arg-6* mutants and, in the process, to compare two methods of identifying nonsense mutations. The heterokaryon test, formally equivalent to diploid formation in yeast (e.g., Minet et al. 1979), identified positively only five of the 13 suppressible alleles. This probably reflects the inefficiency of forming heterokaryons involving a suppressor gene, or of achieving a nuclear ratio allowing the dominance of the *ssu-1* allele to be expressed at the level of growth. In yeast, diploid formation assures a 1:1 ratio of *ssu* alleles and the dominance of *ssu* mutations is generally perceptible in growth phenotypes (e.g., Minet et al. 1979; Chattoo et al. 1979). Despite its inefficiency, the *Neurospora* heterokaryon test is very simple and would be useful in quickly identifying at least one nonsense mutant in a large array of mutants.

In a previous study of the dominance of *ssu* mutations at three loci, Griffiths (1976) concluded that none of those tested (Y319-44, the one used here, Y319-37, and Y319-45) could exert its effect in *trans*. It was therefore suggested that these *ssu* mutations act only in cellular domains controlled by their own nuclei. The reason for the disparity in Griffiths' results and our own is not certain. It is possible that our test for suppression – even weak growth in a very restricted culture volume – is more likely to yield positive evidence than that of Griffiths, who asked for continued growth of heterokaryons, established on supplemented medium, upon transfer to minimal "race tubes". Moreover, the locus studied by Griffiths, *ad-3B*, is one whose suppressed mutants (i.e., *ad*$^-$ *ssu*$^-$ homokaryons) grow with a stop-start pattern on minimal medium (Griffiths 1975). This is suggestive of weak restoration of function at best, a problem which would be exacerbated by superimposing heterokaryosis for both the suppressor and the suppressible mutation. Nevertheless, in a qualitative sense, Griffiths' results agree with ours in showing that heterokaryon tests for super-suppressor action are inefficient and are certainly not definitive.

The crossing method, in which *arg-6 ssu-1* recombinants were tested directly for growth, took advantage of the morphology of *ssu-1* strains, and of the linked *al-2* gene. We could select progeny first for the Ssu$^-$ phenotype, and absence of auxotrophs among them signified suppression. Moreover, the color marker (in this case, the Al$^+$ phenotype) identified the progeny carrying the *arg-6* mutation,

Table 5. Progeny of the hypothetical cross m^-, aux^+; $ssu^+ \times m^+$, aux^-; ssu^-, plated on minimal medium (MIN) and medium supplemented for m^- (SUP)

Genotype	Growth on		%, assuming m-aux is 10 cM
	MIN	SUP	
ssu^+ m^- aux^+	−	+	22.5
ssu^+ m^+ aux^-	−	−	22.5
ssu^+ m^- aux^-	−	−	2.5
ssu^+ m^+ aux^+	+	+	2.5
ssu^- m^- aux^+	+ or −[a]	+	22.5
ssu^- m^+ aux^-	−	−	22.5
ssu^- m^- aux^-	−	−	2.5
ssu^- m^+ aux^+	+	+	2.5

[a] Growth if m^- is suppressible; no growth if m^- is unsuppressible

even if suppressor action rendered these progeny prototrophic.

This rationale can be adapted to testing alleles of any locus for which a nearby unsuppressible marker – visible or nutritional – is available, assuming an unlinked suppressor gene is being used. The major advantages of the rationale are that tetrads need not be isolated, and that non-ideal segregation ratios can be tolerated. In the most common method of surveying for suppressible alleles in *Neurospora*, reliance is placed upon departure from a 1:1 ratio of prototrophs to auxotrophs, an excess of prototrophs being presumptive evidence of positive suppressor action (Seale 1968). Poor germination of strains carrying *arg-6*$^-$ alleles, which was not a serious problem here, would have given false-positive evidence for suppression. Poor germination of *ssu-1* alleles, as was seen here, reduced the impact of positive suppressor action on the overall prototroph:auxotroph ratio. In the actual crosses, the proportion of auxotrophs in crosses of suppressible mutants often exceeded 0.4, where only 0.25 was expected.

The "linked marker" rationale has not been widely used in *Neurospora*, despite Seale's description of such a use in his initial work (Seale 1968). This undoubletedly reflects the lower density of markers available in the past, and because in some cases (e.g. Kinsey and Hung 1981) it is possible to use a non-suppressible allele of the locus involved in place of the linked marker. Because a locus of interest is now likely to lie within 10 map units of a good visible or auxotrophic marker, we suggest the following general rationale, taking advantage of the selection one can impose against an auxotrophic marker. Mutants of locus *m* (to be tested) are mated to a single strain carrying an unlinked suppressor mutation, ssu^-, and a diagnostic auxotrophic mutation, aux^-, linked to *m* (m^-, aux^+; $ssu^+ \times m^+$, aux^-; ssu^-). The progeny are plated on minimal medium. Only suppressed mutants (m^-, aux^+, ssu^-) which arise by independent assortment, and prototrophic crossover progeny (m^+, aux^+) will grow (Table 5). The latter category will be minimized by tight linkage of *m* and *aux*, and its magnitude will be a standard percentage seen in all crosses involving m^- alleles. A control plating on a medium singly supplemented with the requirement of m^- strains will aid in assessing the significance of colonies appearing on minimal medium. In practice, even a 10 map-unit distance between *m* and *aux* will yield more than a 5-fold difference in minimal plate counts of crosses involving suppressible and unsuppressible alleles (Table 5). This is a far better distinction than the 75% *vs* 50% difference in random spore platings of m^- $ssu^+ \times m^+$ ssu^- crosses usually used to assess suppressibility. Moreover, the linked marker is useful in confirming the genetic location of each allele of the complementation group being surveyed for suppression.

The structure of the *arg-6* locus can be inferred in part from the complementation and suppression data. First, noncomplementing mutants are found which lack both enzyme activities (Wolf and Weiss, in preparation). One is a nonsense mutation, and this implies that it lacks two enzyme activities, rather than one, owing to translational polarity. The group of four A mutants lacks the kinase; none is a suppressible mutation. The B group, lacking the reductase, has a high proportion (12/28) of nonsense mutants. The simplest interpretation of these data is that the *arg-6* locus encodes a bifunctional protein, the reductase being translation-distal. Processing of this protein may then yield the separate enzymes found in isolated mitochondrion (Wolf and Weiss, unpublished observations), and as found in yeast (Minet et al. 1979).

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