FINE STRUCTURE MAPPING OF THE AM (GDH) LOCUS OF NEUROSPORA

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Manuscript received April 16, 1983
Revised copy accepted June 27, 1983

ABSTRACT

Utilizing a combination of flanking marker analysis and deletion mapping we have constructed a fine structure map of the am locus which includes 63 point mutants and ten unique deletions. Positions of point mutants can be rapidly assigned to one of 13 segments within the gene on the basis of crosses to nine deletion strains.

THE am-1 locus of Neurospora crassa codes for NADP-specific glutamate dehydrogenase (GDH). This enzyme catalyzes a reversible reaction in which ammonium ion and α-ketoglutarate are combined to form glutamate. Thus, the enzyme plays a central role in nitrogen metabolism.

The am-1 locus and its gene product have been the subject of considerable genetic and biochemical analyses. The enzyme is a hexamer which consists of six identical monomers. There are 453 amino acid residues per monomer, and the complete sequence of the monomer has been determined (Wooten et al. 1974). In addition, a number of amino acid mutational replacements have been determined (Brett et al. 1976; Seale et al. 1977; Fincham and Baron 1977; Kinsey et al. 1980). The sequence of the mutational replacements in the polypeptide chain is colinear with the sequence of the corresponding 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 been deduced by comparing the amino acid sequence of GDH from double frameshift revertants of the mutant am8 to that from the wild type (Siddig et al. 1980). This information has allowed the synthesis of a synthetic 17-base fragment that is complementary to the amino terminal coding region of the gene. The gene has recently been cloned and partially sequenced using this 17-mer as a probe (Kinnaird et al. 1982).

Because the am locus offers a particularly advantageous system for a combined genetical and molecular genetic approach to the study of recombination in a microbial eukaryote, we have constructed a detailed fine structure map of the locus which includes 63 point mutations and ten deletions. Our initial results were obtained by flanking marker analysis. However, it quickly became apparent that, in order to map the large number of mutant sites with which we were working, it would be advantageous to use deletion mapping. We, therefore, isolated a group of am deletion mutants which were subsequently utilized to complete the construction of the am locus map.

MATERIALS AND METHODS

Strains used: Mutant strains am1 through am19 and lys-1 were from the authors' stock collection. Mutant strains am199 through am148 were isolated using the direct selective procedure (KINSEY 1977), and their characterization has been described (KINSEY and HUNG 1981). Mutant strain FK059 was isolated by E. Käfer using the direct selection procedure. Isolation of the am locus deletion mutants is described in RESULTS of this communication. Stocks used for mapping by flanking marker analysis carried the markers cot-1 (allele C102t), rec-3 and either his-1 (allele K83) or ure-2 (allele 47) which were introduced through a series of crosses. The stocks used to introduce these markers were obtained originally from the Fungal Genetics Stock Center, Humboldt State University, Humboldt, California. Stocks used for flanking marker analysis were of mixed genetic background.

Chemicals and reagents: The mutagen 1,2,7,8-diepoxyoctane (DEO) was obtained from Aldrich Chemical Company. Most other chemicals were obtained from the Sigma Chemical Company.

Crosses and cross analysis: The techniques used for crosses and for flanking marker analysis were as previously described (KINSEY et al. 1980). Crosses for deletion mapping were made in the same way, however, analysis involved only the measurement of recombination frequency.

Isolation of deletion mutants: am mutants were isolated in either a lys-1 a background (series 23) or lys-1 cot-1 rec-3 a background (series 48 and 73) using the procedure of KINSEY (1977) following mutagenesis with DEO. Previous reports had indicated that DEO causes deletions in Neurospora (ONG and deSERRES 1975) and Aspergillus (HYNES 1979). The procedure for DEO mutagenesis was as follows: Conidia were treated with 100 mM DEO for 90 min at room temperature. The incubation was carried out in a large glass centrifuge tube with continuous stirring in 0.067 M phosphate buffer, pH 7.0, with conidia adjusted to a final concentration of 6 x 10^6 conidia/ml. Conidia were washed three times with ice-cold Fries basal medium after the incubation period was completed.

Rapid reversion screen: am mutants were screened for putative deletions by characterization for reversion following ultraviolet (UV) irradiation. Conidial suspensions in sterile water were adjusted to an absorbance of 0.5 at 600 nm. One-tenth of a milliliter, containing approximately 10^6 conidia, was plated on Vogels minimal salts (VOGEL 1956) containing 1.5% sorbose, 0.2% glucose, 0.2% glycerol agar plus glycine (0.02 M) and exposed to UV (1.7 x 10^3 erg/mm^2). The number of revertant colonies was compared with control plates containing either am119, which reverts with a relatively high frequency following UV irradiation, or am138, a large deletion, which is not revertable. Mutant strains that did not revert were considered to be putative deletions and further characterized by genetic analysis.

RESULTS

Flanking marker analysis

We began to map am mutants using flanking marker analysis, utilizing the conventions developed by SMYTH (1973). These conventions have been shown to give a genetic map that is colinear with the sites of altered amino acids in mutant forms of GDH (BRETT et al. 1976; KINSEY et al. 1980). Mapping is based on two criteria which can be related to the diagram in Figure 1, in which m1 represents the site of the more proximal and m2 that of the more distal of two am alleles. P,p and D,d are general terms for markers at the centromere proximal and centromere distal ends, respectively, of the am locus. The first criterion is based on prototrophic recombinants between different am alleles in which the flanking markers are also of the recombinant type (either Pd or pD). According to this criterion the more proximal am allele will have its proximal flanking marker represented less frequently and its distal marker more frequently among the prototrophic progeny (i.e., pD > Pd). The second criterion, or the polarity criterion, is based on am prototrophic recom-
binants which are not recombinant with respect to flanking markers. This criterion states that gene conversion occurs more frequently at the centromere proximal end of the gene than at the distal end; that is, a polarity of conversion exists within the gene. This criterion is empirically derived. Accordingly, the more proximal am allele is defined as that which entered the cross with the same association of flanking markers represented most frequently among the prototrophic recombinants (i.e., PD > pd).

In practice, mapping of am mutants has depended almost exclusively on the polarity criterion, since prototrophic recombinants which are also recombinant for flanking markers generally fail to reveal significant differences between recombinant classes (i.e., pD ≠ Pd) (Smyth 1973; Fincham 1974; our results).

In our experiments crosses of the general constitution ure-2 am,; rec-3; cot-1 X am, his-1; rec-3; cot-1 were made in which ure-2 is the centromere proximal flanking marker, approximately 1 map unit from am, and his-1 is the centromere distal flanking marker, approximately 1.5 map units from am. The cot-1 marker was present in these stocks and produced very compact colonies at 33°. Prototrophic am+ recombinants were counted, and the flanking marker constitution of the prototrophs was determined as previously described (Kinsey et al. 1980). The results of 90 informative crosses involving 30 previously unmapped alleles crossed to various alleles with known map positions are given in Table 1. The deduced order is shown for a cross only if the PD class was significantly (5% probability level) greater than the pd class. In addition to the data shown in Table 1, all am mutants used were crossed to themselves in opposite mating type and at least 5 X 10⁶ viable spores analyzed. No prototrophic revertants were found for any of these selfing crosses (data not shown). In addition to the informative crosses shown in Table 1, more than 100 additional crosses were made that gave significant recombination frequencies (data not shown) but for which either there was no significant bias in parental flanking marker combinations or for which flanking marker analysis was not performed. In these cases, recombination frequency, if it was very low, was taken as a presumption of closeness on the genetic map when deciding which alleles to cross to particular deletion mutants.

Isolation of deletion mutants

Early in these studies we became convinced of the need for a less equivocal and a quicker method of mapping. This was particularly so since we were
Table 1

Frequencies and distribution of flanking markers among am prototrophic recombinants from crosses of the general constitution + am, his-1 × ure-2 am, +

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<th>Parent strains</th>
<th>Frequency of am⁺ progeny per 10⁵ five spores</th>
<th>Distribution of flanking markers of the am⁺ progeny</th>
<th>Deduced order</th>
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We also found that many flanking marker crosses failed to give any significant dealing with large numbers of mutant strains and any given flanking marker cross could order only two alleles and those only with respect to one another. We also found that many flanking marker crosses failed to give any significant

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mapping data, especially when both markers were in the middle of the gene. Therefore, we have isolated and characterized deletion mutants of the \textit{am} gene and utilized these in the manner devised by \textsc{Benzzer} (1959, 1961) to map mutant sites.

Most of the deletion mutants we have mapped were found among \textsc{Deo}\textsuperscript{-}induced mutants that failed to revert following UV irradiation. The exceptions are $\Delta 73$-\textit{SG1} and $\Delta 73$-\textit{SG2}, which are spontaneous mutants, and \textit{am111}, \textit{am128} and \textit{am132}, which have been previously described (\textsc{Kinsey} and \textsc{Hung} 1981). The frequency of \textit{am} mutants following \textsc{Deo} mutagenesis was approximately $1/10^6$ surviving conidia. This figure is about tenfold higher than the frequency of spontaneous \textit{am} mutants in the strains used. One observation worth noting is that \textsc{Deo} stored at room temperature for several months ceases to be mutagenic for \textit{Neurospora} conidia while still remaining toxic. We have noted this phenomenon with several lots of \textsc{Deo}.

Of 108 \textsc{Deo}-induced mutants that we have analyzed, 59 failed to revert following UV treatment. These 59 \textit{am} mutants, as well as four spontaneous mutants with similar UV reversion characteristics, were crossed to a series of 11 point mutants that together span the \textit{am} locus (\textsc{Smyth} 1973; \textsc{Kinsey} \textit{et al.} 1980). As a control the \textsc{Deo} mutants were also crossed to \textit{am132} which deletes the entire \textit{am} locus.

The idea behind these crosses was that any substantial deletion would fail to show recombination with one or more of these point mutants. Past experience with point mutant crosses had indicated that virtually all point mutant $\times$ point mutant crosses (except selfing crosses) yield some recombinants. On the basis of the results of these screening crosses, further crosses of the putative deletions would be made to other point mutants that mapped in the indicated area. Since these crosses were intended only for rapid screening, ascospore concentrations were estimated on the basis of optical density measurements of the spore suspensions. Except as specifically noted an estimated $10^6$, \textit{etc.} were tested for each cross. It should be pointed out that some crosses that gave no recombinants in this test subsequently were shown to give a few recombinants when more spores were analyzed (compare Table 2 with Table 3). Also inherent in this scheme would be the loss of many of the possible small deletions which did not overlap one of the 11 point mutants.

Seventy-one percent (45 of 63) of the nonreverting mutants failed to show recombination with at least one of the 11 point mutants. However, only 21 of these could subsequently be demonstrated to be deletions based on the criterion that a deletion must fail to recombine with two or more mutants capable of recombination with each other. Ten of these 21 failed to recombine with any of the strains used, and presumably each deletes the entire \textit{am} locus. The results of crosses between these 21 deletion mutants and the 11 point mutants and \textit{am132} are shown in Table 2.

\textit{Crosses of confirmed deletions to each other and to relevant point mutants}

Those strains deleted for part of the \textit{am} locus were crossed to other \textit{am} strains, based on the results of flanking marker analyses or recombination frequency. The deletion mutants were also crossed to other deletion mutants
that could potentially overlap them. For example, the data in Table 2 indicate that A23-82 is a deletion in which approximately half of the gene corresponding to the amino terminus of GDH is missing, with the right end point of the deletion lying between am2 and am1. Consequently, all of the alleles mapping in the vicinity of, or in the interval between, am2 and am1 were crossed to A23-82 in order to define the right end point of the deletion. Similarly, it can be seen from Table 2 that A73-40 is a deletion in which all of the gene except that corresponding to the amino terminus of GDH is deleted, with the left end point of the deletion lying between am14 and am4. Thus, all of the alleles that mapped near or between am14 and am4 were crossed to A73-40 in order to define the left end point of this deletion. The data in Table 2 also show A73-SG2 to be a small deletion with both end points in the gene and mapping in the vicinity of the left end point of A73-40. Subsequent crosses to A73-40 and alleles mapping near the deleted region of A73-SG2 were used to show the overlapping nature of A73-40 and A73-SG2 and to define the end points of A73-SG2. Employing similar strategy with the other partial deletions shown in Table 2 and with am128 and am111 (described later), we were able to define the end point(s) and overlapping or nonoverlapping nature of these deletion mutants. In so doing we were able to assign am alleles to one of 13 regions of

### Table 2

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*The order of the tester strains is centromere am6, am14, am15 ... am11, am9. Mutant am132 is a complete deletion of the am locus.*
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**MAPPING THE NEUROSPORA AM LOCUS**
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<td>0.75</td>
<td>2.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>121</td>
<td>–</td>
<td>0/1.7 \times 10^6</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.9</td>
<td>1.6</td>
<td>1.8</td>
<td>–</td>
</tr>
</tbody>
</table>

Results are the numbers of prototrophic recombinants per 10^5 viable spores. Where no prototrophic recombinants are found, a 0 is followed by the number of viable spores tested. – = not done.
the gene by deletion analyses. The mapping crosses of am alleles crossed to deletion strains are given in Table 3, and the crosses of deletions by deletions are given in Table 4.

During this mapping process it was discovered that the previously described mutants am128 and am111 (KINSEY and HUNG 1981) are also deletion mutants. am111, which in a previous communication (KINSEY and HUNG 1981) was mistakenly reported to revert, was found to map in two nonoverlapping deletions, Δ23-81 and Δ48-2 (Table 4), and to cover the allele am17 which maps between Δ23-81 and Δ48-2 (Table 3). am128 was found to map in Δ73-40 and Δ73-SG2 (Table 4) and to cover several alleles that did not map in these deletions (Table 3). Thus, am128 and am111 are small deletions, deleting portions of the gene corresponding to the amino terminal and carboxy terminal ends of GDH, respectively. Four DEO-induced mutants—Δ23-82, Δ23-84, Δ48-35 and Δ73-38—are identical on a genetic basis; all delete approximately half of the gene corresponding to the amino terminus of GDH. Only results for Δ23-82 are listed in Tables 2 and 3. Two spontaneous deletions, Δ73-SG1 and Δ73-SG2, are also identical on a genetic basis; each deleted a small region of the gene in the vicinity of and including am4. Only results for Δ73-SG2 are listed in Tables 2 and 3.

By using a set of nine of these deletion mutants—am128, am111, Δ73-SG2, Δ73-40, Δ23-44, Δ23-82, Δ23-81, Δ48-2 and Δ39-A4—we can easily map any am allele to one of 13 discrete regions of the gene. We have used this set of deletion strains to map 7 alleles (am16, amFK059, am117, am120, am125, am129 and am136) for which we previously had no mapping information (Table 3), as well as to complete the mapping of the 49 partially mapped alleles.

DISCUSSION

Flanking marker analysis

We have utilized flanking marker analysis primarily to place mutants sites in one of four segments of the gene. This placement was subsequently corroborated in a few cases by peptide sequence information obtained from analysis of mutant GDH (KINSEY et al. 1980), in one case by DNA sequence analysis from a cloned mutant version of the am gene (J. A. RAMBOSEK and J. A. Kinsey, unpublished results), and in all cases by deletion mapping analysis.

In each case we were able to confirm independently the assignment made on the basis of conversion polarity, thus confirming the usefulness of the polarity criterion for mapping at the am locus. Additional analysis of the various classes of prototrophic recombinants, with respect to flanking markers, are also consistent with the previous observations of FINCHAM (1964, 1967) and SMYTH (1973). For example, if all of the crosses are taken together, the mean value for total prototrophs that are also recombinant for flanking markers is 22 ± 0.11%. This compares with a value of 29.5% obtained by SMYTH (1973) and once again indicates that only about 25% of conversion events at the am locus are associated with exchange of flanking DNA regions.

If the prototrophs that are recombinant for flanking markers are themselves
TABLE 4

Selected crosses between deletions

<table>
<thead>
<tr>
<th>Strains crossed</th>
<th>Prototrophs per viable spores</th>
<th>Prototrophs per 10⁵ viable spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>128 × 73-SG2</td>
<td>0/2.5 × 10⁵</td>
<td>0.0</td>
</tr>
<tr>
<td>128 × 73-40</td>
<td>0/2 × 10⁶</td>
<td>0.0</td>
</tr>
<tr>
<td>73-SG2 × 73-40</td>
<td>0/4.2 × 10⁶</td>
<td>0.0</td>
</tr>
<tr>
<td>48-2 × 111</td>
<td>0/3.1 × 10⁶</td>
<td>0.0</td>
</tr>
<tr>
<td>23-81 × 111</td>
<td>0/6.2 × 10⁶</td>
<td>0.0</td>
</tr>
<tr>
<td>48-2 × 23-81</td>
<td>29/1.2 × 10⁶</td>
<td>2.4</td>
</tr>
<tr>
<td>23-81 × 39-A4</td>
<td>0/1.6 × 10⁵</td>
<td>0.0</td>
</tr>
<tr>
<td>23-81 × 23-82</td>
<td>9/4.3 × 10⁶</td>
<td>0.21</td>
</tr>
<tr>
<td>111 × 23-82</td>
<td>0/8.4 × 10⁶</td>
<td>0.0</td>
</tr>
<tr>
<td>48-2 × 23-82</td>
<td>0/2.5 × 10⁶</td>
<td>0.0</td>
</tr>
<tr>
<td>73-SG2 × 48-2</td>
<td>2/7.6 × 10⁵</td>
<td>0.26</td>
</tr>
</tbody>
</table>

analyzed, again combining the data from all of our crosses, we find no significant difference between the two classes. This is in contrast to the results of SMYTH (1973) in which he found a slight, but significant, excess of one class over the other. It was on this basis that SMYTH decided that am₉ represents the centromere proximal end of the gene. Although we have utilized this convention throughout this study, there is no direct support for it in our data.

Deletion analysis

Our early efforts to obtain deletions were not very successful, and it was only when we started to use DEO as a mutagen (ONG and DESEMMES 1975) that we began to obtain deletions in significant numbers. DEO is a bifunctional alkylating agent that is thought to crosslink guanosine residues in adjacent positions on complementary strands (ONG and DESEMMES 1975), thus promoting double strand breaks and excision. Altogether, 21 of 108 DEO-induced mutations were shown to be deletions; this may represent only a fraction of the actual induced deletions because our method of screening would allow many small deletions to go undetected.

It is interesting to note that about half of the proven deletions cover the entire am locus; however, none of these large deletions extend into the nearest known gene, gul-1 (0.3 map units distal to am). This fact, coupled with our repeated failure to obtain am through gul-1 deletions among mutants selected simultaneously for the gul and am phenotypes, suggests to us that there is indispensable material between the two sites.

We have isolated five proven deletions not induced by DEO. Two of these were spontaneous, and both of these, Δ73-SG1 and Δ73-SG2, appear to be identical and probably represent repeat isolations of a preexisting deletion mutation present in the stock used. One of the others, Δ111, was isolated after UV mutagenesis, and two, Δ128 and Δ132, were isolated after nitrous acid mutagenesis. All of these non-DEO mutants are small internal deletions except Δ132, which removes the entire am locus and considerable material on either side but, once again, not extending to the gul-1 locus (J. A. RAMBOSEK and J. A. KINSEY, unpublished results).
For the purpose of these studies we have chosen to include mutant 23-81 as a deletion, however, its behavior is somewhat anomalous. On the basis of mutant peptide analysis one can assign certain alleles in this region to an unequivocal order (reference to Figure 2 will aid the following discussion). That order is, left to right, am17, am1, am7, am122, am3. The deletion, Δ111, covers am17 but none of the other alleles and, therefore, has a righthand end point between am17 and am1. The deletion, Δ39-A4, by the same kind of reasoning, has an end point between am1 and am7. The small deletion, Δ23-44, has its left end point between am17 and am1 and its right end point between am1 and am7. By analysis of their behavior in crosses to these three deletions, new am mutations can now be oriented on this map. The putative deletion 23-81 does not cover am17 nor does it appear to cover am1, yet it overlaps Δ111 and clearly covers am122 and am3. Moreover, it also covers am147 which is in turn covered by Δ111. Thus, 23-81 has the appearance of failing to recombine with left hand markers Δ111 and am147, as well as am105 which maps within Δ23-44 (as does am1). It then recombines with central markers am1, am7 and am11 and then fails to recombine with righthand markers am122 and am3 as well as am116, am117 and am125, all of which map to the right of Δ39-A4. It then recombines with all of the remaining markers that map to the right of Δ39-A4. We have shown 23-81 in our map as if it were a continuous deletion, however, it is possible that it represents two small neighboring deletions or that it represents an inverted region. We are currently cloning the 23-81 version of the am gene to sort out the answer to this question.

Fine structure map

The current fine structure map of the am locus based upon previous mapping experiments (Smyth 1973; Kinsey et al. 1980) as well as flanking marker analysis and deletion mapping (reported here) is shown in Figure 2. The scale of the map is based on recent nucleotide sequencing data (J. H. Kinnaird and J. R. S. Fincham, personal communication; J. A. Rambosek and J. A. Kinsey, unpublished results). Mutant sites indicated by the symbol just above the line.
are accurately placed based on sequencing of mutant peptides (Brett et al. 1976; Kinsey et al. 1980) or in the case of am126 on DNA sequencing data from a cloned am126 version of the am gene (J. A. Rambosek and J. A. Kinsey, unpublished results). Mutant sites placed just below the line are placed in the correct order, based on flanking marker analysis, but the actual distance between such sites is an estimate, based on recombination frequency. Allele designations placed well above the line are mapped only with respect to deletion end points as indicated by the vertical bars. Deleted regions are indicated by heavy bars below the line. The actual end points of the deletions are only estimates, however, the relationship of the end points to defined point mutants are accurate with respect to relative order.

Many of the mutant sites that have been placed only within a given interval as defined by deletion end points are separable by recombination and/or distinguishable on the basis of reversion frequency, complementation, cross-reacting material production, temperature sensitivity or suppressibility.

We are currently using this more detailed fine structure map to reassess the effects of rec-3 on recombination at the am locus (Smyth 1973), utilizing stocks that have been extensively backcrossed to the standard IVA/ORSa reference stocks.

We thank J. R. S. Fincham and David Stadler for reading the manuscript and making suggestions. We also thank Malcolm Luker who suggested to us that we use DE0 as a mutagen. This project was supported by grants BRSG 507 RR 05373 and GM 23967 from the National Institutes of Health.

LITERATURE CITED


MAPPING THE NEUROSPORA AM LOCUS


Corresponding editor: R. C. ULLRICH