The Use of Mitotic Crossing-over for Genetic Analysis in *Dictyostelium discoideum*: Mapping of Linkage Group II

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SUMMARY

Mitotic mapping in the cellular slime mould *Dictyostelium discoideum* was investigated by analysing the gene order and map distances of four genetic markers on linkage group II: *whi*, *acrA*, *tsgD* (previously reported) and a new spore shape marker *sprB*. The previously suggested gene order has been revised to centromere, *whi*, *acrA*, *tsgD/sprB*, on the basis of the analysis of four different diploids. One class of diploid which was previously thought to arise by mitotic crossing-over between *tsgD* and *acrA* probably arose by mutation at the *acrA* locus or by reversion of the *tsgD* locus followed by mitotic crossing-over at another interval. These and other problems associated with mitotic mapping are discussed. Evidence is presented that for a particular class of cross-over diploid (white, temperature-resistant, methanol-resistant) an origin from a single cross-over event is likely rather than a multiple cross-over origin. It is suggested that multiple mitotic cross-overs, on the same arm of a chromosome, are rare in *D. discoideum*.

INTRODUCTION

The cellular slime mould *Dictyostelium discoideum* is a haploid eukaryote which is an excellent organism for studies on differentiation (Bonner, 1967; Newell, 1971). A major drawback has been the absence of a workable system for genetic analysis. Progress has been made in the understanding of the sexual cycle in *Dictyostelium* (Clark, Francis & Eisenberg, 1973; Erdos, Raper & Vogen, 1973; Macinnes & Francis, 1974), but in *D. discoideum* genetic analysis based on meiosis is not yet feasible.

Genetic analysis in *D. discoideum* based on the model of the parasexual cycle (Pontecorvo & Käfer, 1958) is being used for genetic studies complementing biochemical analysis in this laboratory. The parasexual system involves fusion of haploid strains to form a diploid, followed by haploidization *via* transient aneuploidy (Sinha & Ashworth, 1969; Brody & Williams 1974). This process allows allocation of genetic markers to linkage groups, since chromosome loss appears to be random and mitotic crossing-over is infrequent. Since both fusion to form diploids and subsequent haploidization are rare events it has been necessary to develop selective methods. Loomis (1969) showed that diploids can be selected, at the restrictive temperature, from haploid strains bearing non-allelic recessive, growth temperature-sensitive mutations. This method was substantiated by Katz & Sussman (1972), who also showed that as in the parasexual system in fungi (Pontecorvo & Käfer, 1958) drug-resistant haploids can be selected from a diploid heterozygous for a recessive drug-resistance mutation. Katz & Sussman (1972) defined three linkage groups in *D. discoideum*,

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and we have established two more (Williams, Kessin & Newell, 1974*a*; Kessin, Williams & Newell, 1974).

Williams *et al.* (1974*a*) reported preliminary studies based on mitotic analysis where the gene order of linkage groups I and II was established. Gingold & Ashworth (1974) and Katz & Kao (1974) have also studied the gene order on linkage group II. Katz & Kao (1974) have also suggested a map of linkage group II showing the mitotic distances between three markers.

Although the methodology of mitotic mapping has been established for more than 20 years in fungi (Pontecorvo, 1953; Pontecorvo & Käfer, 1958), there may be technical problems with each different organism studied. We present a map of linkage group II of *D. discoideum* which differs from that presented by Katz & Kao (1974). The problems of mitotic mapping of *D. discoideum* are discussed, and methods are outlined which must be used to obtain reproducible map distances.

METHODS

Chemicals. Acriflavin (neutral) was obtained from Sigma. Oxoid agar No. 3, yeast extract and horse serum were purchased from Oxoid, and Bacto-peptone from Difco. Methanol and glucose (analytical reagent grade), silica gel (6–18 mesh), and other chemicals were obtained from Fisons Scientific Apparatus, Leicester.

Media and growth conditions. Amoebae and spores of *D. discoideum* were diluted in SS salt solution (Sussman, 1966). In all experiments SM medium of the following composition (g/l) was used: glucose, 10; Bacto-peptone, 10; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0; KH_2PO_4 , 2.2; K_2HPO_4 , 1.0; yeast extract, 1.0; agar, 15; pH 6.5. The medium was autoclaved at 15 lb/in² for 20 min, and approximately 40 ml dispensed into triple-vented plastic Petri dishes (Sterilin Ltd, Teddington, Middlesex).

Methanol- or acriflavin-containing medium was prepared by adding methanol to a final concentration of 2 % (v/v), or filter-sterilized acriflavin to a final concentration of 100 µg/ml, to the SM medium after autoclaving and cooling to 50 °C. In some experiments medium containing 3 or 5 % (v/v) methanol was used. Cycloheximide-containing agar plates were prepared by adding filter-sterilized cycloheximide solution (20 mg/ml) to a final concentration of 500 µg/ml, to SM agar after autoclaving. All agar plates were stored at 2 °C in the dark and used within 3 weeks of being poured.

All strains of *D. discoideum* were grown in association with *Aerobacter aerogenes* (strain 1033) at 22 °C, or at the restrictive temperature (27 °C). The growth of the *A. aerogenes* was unaffected by the presence of acriflavin (100 µg/ml), methanol (2 %, 3 % or 5 %, v/v), or cycloheximide (500 µg/ml).

Strains. Diploid strains of *Dictyostelium discoideum* DP4, DP8, DP16, DP31 and DP72, which were used to study mitotic crossing-over in linkage group II, were heterozygous (on this linkage group) for the white spore colour marker (*whi*), temperature-sensitivity for growth at 27 °C (*tsgDI2*), and acriflavin/methanol/thiabendazole resistance (*acrAr*). Strain DP72 was also heterozygous for a spore shape marker (*sprB2*) which is located on linkage group II.

The derivation and full genotype of strains DP4 and DP8 have been published previously (Williams *et al.* 1974*a*). Strain DP16 is a diploid constructed between haploid strains NP14 (Williams *et al.* 1974*a*) and NP44 [an aggregateless strain derived from strain X2 (Williams *et al.* 1974*a*) by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine]. Strain DP31 is a diploid constructed between haploid strains X9 (Williams *et al.* 1974*a*) and G2418 (Gingold,

1975). Strain DP72 was constructed between strain NP84 (a haploid, growth temperature-sensitive, axenic strain, derived from strain VI2), and a resegreated haploid growth temperature-sensitive strain, X23, originating from strain NC4, which has linkage group II of strain NP14 (Williams *et al.* 1974a). Strain VI2 was obtained from Professor K. B. Raper, University of Wisconsin, Madison, U.S.A. Strain DP72 is of interest in another context, since it is a diploid formed between strains of opposite mating type, hence the derivation of strains NP84 and DP72 will be described in detail elsewhere. For a discussion of mating types see Clark *et al.* (1973) and Erdos *et al.* (1973). Haploid strains M28 (Katz & Sussman, 1972) and NP84 were used for the estimation of the mutation frequency at the *acrA* locus. Haploid strains X9 and NP12 (Williams *et al.* 1974a) were used for the comparison of the plating efficiency on SM agar containing 0, 2, 3 and 5 % (v/v) methanol.

Maintenance of stocks. Stocks of all strains which sporulated were maintained as spores collected in horse serum and dried on to silica gel; they were stored at 4 °C. Strains being used routinely were subcultured on SM agar with *A. aerogenes* at 22 °C by cloning at weekly intervals.

Formation of diploids. Two haploid strains, each carrying recessive, non-allelic mutations to sensitivity for growth at the restrictive temperature, were incubated under conditions promoting cellular fusion. The resulting diploids were isolated by their ability to grow at the restrictive temperature (27 °C) as described by Williams, Kessin & Newell (1974b).

Selection of mitotic cross-over diploids. Resistance to acriflavin or methanol in *D. discoideum* is a recessive characteristic. However, when 5×10^3 or more drug-sensitive diploid amoebae (or spores) heterozygous for the drug-resistance marker are plated on to appropriate drug-containing plates (previously spread with *A. aerogenes*), there is growth of some resistant colonies. These are either haploid drug-resistant segregants, or diploids, homozygous for the drug-resistance marker, which arise mainly by mitotic crossing-over. In this work, diploids homozygous for *acrA* were isolated from methanol/acriflavin-sensitive diploids (heterozygous for *acrA*) by means of selection using SM agar plates containing either methanol or acriflavin. The agar plates were incubated at 22 °C, or at the restrictive temperature (27 °C) in the case of double selection (see below).

Care must be taken to isolate diploids resulting from independent cross-over events, rather than those resulting from a cross-over followed by cell multiplication before the imposition of selective conditions. Consequently, every methanol- or acriflavin-containing SM agar plate was inoculated with amoebae from a different clone of the heterozygous diploid. Clones of a standard size, containing approximately 10^7 amoebae, were always used, and between 10^3 and 5×10^4 amoebae were plated per drug-containing plate (depending on the diploid used) so as to avoid overlapping colonies and, if possible, to get one cross-over diploid on each agar plate. After the incubation at 22 °C, plates were screened to distinguish the diploids from the haploids. The distinction between haploids, which were in the majority, and diploids was made on the basis of spore size (Sinha & Ashworth, 1969).

Only one diploid was chosen for use in mapping from each initial clone. Bias was avoided by marking the bottom of each drug-containing plate into 32 radial sectors before the start of the incubation and retaining only the diploid from the sector marked with the lowest number.

All diploids chosen for mapping were checked for the other markers on linkage group II, and classified according to the chromosome interval in which the cross-over had taken place.

Isolation of diploid segregants by simultaneous double selection for crossing-over. In some

experiments selection by both methanol (2 %, v/v) and the restrictive temperature were done together. This technique eliminates all haploids and all segregant diploids except temperature-resistant (*tsgD*/+) and methanol-resistant diploids (*acrA*/*acrA*). This allows rapid screening of large numbers of independently derived diploids resulting from crossing-over in interval III (Fig. 1). In the double selection, SM agar plates containing 2 % (v/v) methanol were incubated at 27 °C after inoculation with *A. aerogenes*, and amoebal suspensions prepared from single clones of heterozygous diploids as described above, but using more amoebae (from 10^5 to 2×10^6 /plate).

*Isolation of mutants in the *acrA* locus.* Methanol-resistant mutants were obtained from two haploid strains, M28 and NP84, both sensitive to methanol and acriflavin. Ten independent clones of each haploid were chosen. Vegetative amoebae and spores from each clone, containing approximately 10^7 amoebae, were plated at 10^6 amoebae (or spores)/plate on to five SM agar plates containing 2 % (v/v) methanol and *A. aerogenes*. The plates were incubated at 22 °C. All methanol-resistant colonies arising on these plates were picked with toothpicks on to SM agar plates containing or lacking methanol, and previously spread with *A. aerogenes*. Those colonies which grew on both SM agar and SM agar + methanol were given a second test in which amoebae from the 'mutant' colony growing on SM agar were picked on to three kinds of media: SM agar, SM agar containing 2 % methanol, and SM agar containing 100 µg acriflavin/ml; all plates were previously spread with *A. aerogenes*. Only those colonies which grew on all three plates in the second test were considered to be true mutants at *acrA*.

RESULTS

Apparent map of linkage group II

Williams *et al.* (1974a), Gingold & Ashworth (1974) and Katz & Kao (1974) reported that diploids which were initially heterozygous for three markers on linkage group II

$$\left(\begin{array}{ccc} whi & tsgD & acrA \\ + & + & + \end{array} \right)$$

gave three classes of diploids which were homozygous for methanol/acriflavin resistance

$$\left(\begin{array}{c} acrA \\ acrA \end{array} \right)$$

after selection on methanol (2 %) or acriflavin (100 µg/ml) (Fig. 1). Hence the suggested gene order for this chromosome was: centromere, *whi*, *tsgD*, *acrA*, with cross-over intervals I, II and III defined in Fig. 1. In addition, Katz & Kao (1974) suggested relative mitotic map distances for intervals I (19 %), II (26 %) and III (55 %), based on the frequency of obtaining diploid segregants on methanol (3 %). We have studied the relative map distances for intervals I, II and III using four genetically different diploids (DP4, DP8, DP16 and DP72), each heterozygous for *whi*, *tsgD* and *acrA*; the results are shown in Table 1. It is important to stress that each cross-over diploid resulted from an independent cross-over event, i.e. none are of clonal origin. Considering the small numbers of diploids examined, each diploid gave a similar estimate for the mitotic distance for intervals I and II. These intervals are long and of similar length, hence *whi* is approximately equidistant between the centromere and *acrA*. In contrast, interval III is very short. These results differ markedly from those of Katz & Kao (1974) (see Discussion).

For diploid DP8, obtaining cross-over diploids was laborious because this strain produced

Fig. 1. Map of linkage group II of *D. discoideum* based on previously published results (Williams *et al.* 1974a; Gingold & Ashworth, 1974; Katz & Kao, 1974).

Table 1. Incidence of crossing-over in the three putative intervals of linkage group II

The selector used with diploids DP4 and DP72 was methanol (2 %, v/v), while either methanol or acriflavin was used for DP8 and DP16. No difference in frequency of crossing-over was observed between either selector, so the results derived using methanol and acriflavin selector have been pooled. Numbers in parentheses refer to mitotic map units as a percentage of the distance between the centromere and the *acrA* locus.

Diploid used	No. of independent cross-over diploids*			Total
	Interval I	Interval II	Interval III	
DP8	4	8	2	14
DP16	11	7	4	23†
DP72	8	23	1	32
DP4	15	10	1	27†
Total	38 (40)	48 (51)	8 (9)	96

* Intervals I, II and III are defined in Fig. 1.

† These totals include in each case a single white, temperature-resistant, acriflavin/methanol-resistant diploid. The origin of this class of diploid is considered in Fig. 5.

predominantly haploids on methanol or acriflavin (cross-over diploid:haploid ratio was approximately 1:222). In contrast, diploid DP4 gave a much higher ratio (1:3). (Diploids DP16 and DP72 gave intermediate ratios of 1:34 and 1:24, respectively.) Such different frequencies for mitotic cross-over and haploidization would seem to imply that the two events are independently controlled.

Fig. 2. Alternative origins for diploids showing an apparent cross-over at interval III. (a) Mutation at the *acrA* locus. For clarity, the allele number at the *acrA* locus is shown. (b) Reversion at the *tsgD* locus before the imposition of selective conditions which result in a mitotic cross-over event. Reversion at the *tsgD* locus is shown as *tsgD*⁺; it does not necessarily result in complete reversion to wild type.

Possible origins of temperature-resistant diploids

Cross-over at interval III. It is clear from our studies and those of Gingold & Ashworth (1974) and Katz & Kao (1974) that diploids which are suggested to result from cross-overs at interval I (white, temperature-sensitive, methanol/acriflavin-resistant) and interval II (yellow, temperature-sensitive, methanol/acriflavin-resistant) do actually result from a single mitotic cross-over event (see Fig. 1). In both cases haploid segregants from these cross-over diploids have the expected genotype (Gingold & Ashworth, 1974; Katz & Kao, 1974) and any other origin seems unlikely.

This does not seem to be the case for 'cross-overs' at interval III. Since 'cross-overs' at interval III were rare (Table 1), we examined whether the yellow, temperature-resistant, methanol-acriflavin-resistant diploids were indeed of cross-over origin. There are two other possible ways of obtaining these diploids, and these are shown in Fig. 2. The schemes suggested in Fig. 2 do not rely on *tsgD* being closer to the centromere than *acrA*, but depend on either the selection of an additional mutation to *acrA* in the heterozygous diploid, or a prior reversion at the *tsgD* locus followed by a cross-over proximal to *acrA*. Evidence in support of both such events will now be presented.

Table 2. Frequency of selection of cross-overs between *acrA* and *tsgD* using the double selection method

Diploid used	No. of independently derived clones tested	$10^{-5} \times$ no. of amoebae/plate	$10^{-6} \times$ total no. of amoebae tested	Apparent cross-overs in interval III*	
				No. obtained	$10^6 \times$ frequency
DP72	50	1	5.6	5	~ 0.9
DP72	42	5	24	27	1.1
DP72	9	20	18	18	1.0
DP4	12	1	1.2	1	~ 0.8
DP4	40	5	20	26	1.3
Total	153	—	69	77	1.1

* Interval III is defined in Fig. 1.

Table 3. The mutation frequency at the *acrA* locus

Mutants in the *acrA* locus were isolated on SM agar containing methanol (2 %, v/v), since Williams *et al.* (1974a) have shown that the *acrA* locus is the only site of methanol resistance in *D. discoideum*. Similar results to those shown below were obtained using methanol at the higher concentration (3 %, v/v) used by Katz & Kao (1974). The plating efficiency of two previously isolated strains containing mutations in *acrA* (strain X9, containing *acrA1*, and strain NP12, containing *acrA2*) was 100 % on both 2 % and 3 % methanol, whereas growth on 5 % methanol was very slow and the plating efficiency was about 50 %. Hence the *metA* locus described by Katz & Kao (1974) is almost certainly identical to the *acrA* locus.

Strain used	No. of clones tested	$10^{-6} \times$ no. of cells plated/clone	Average no. of <i>acrA</i> mutants found/clone, $\pm$ S.E.M.	Total no. of mutants found	$10^6 \times$ mutation frequency*
M28 (amoebae)	10	5	6 ± 1.1	59	1.2
M28 (spores)	10	5	9 ± 3.7	92	1.8
NP84 (spores)	10	5	8 ± 1.9	80	1.6

* Defined as the frequency of obtaining a strain mutated to resistance at the *acrA* locus, from a sensitive clone containing about 10^7 amoebae (or spores). This definition of mutation frequency (spontaneous) was chosen so as to be comparable with the results in Table 2.

*Selection of a new mutation at the *acrA* locus.* The data in Table 1 suggest that 'cross-overs' in interval III occur at a frequency of about 2×10^{-6} . This is close to our previously reported frequency of mutation to *acrA* (Williams *et al.* 1974a).

To clarify this situation we obtained a better estimate of the frequency of 'cross-overs' at interval III by selecting 'cross-over' diploids on SM agar containing methanol (2 %) at the restrictive temperature, rather than at the permissive temperature; in this way only cross-overs distal to *tsgD* but proximal to *acrA* would have been selected (see Fig. 1). Firstly it was established that the 'cross-over' frequency at interval III was independent of the number of amoebae plated in the range 10^5 to 2×10^6 amoebae/plate (Table 2). It was consequently possible to analyse many more 'cross-over' diploids with this method, and hence a more accurate estimate of the 'cross-over' frequency at interval III is 1.1×10^{-6} ; DP4 and DP72 gave very similar results and these are presented in Table 2.

The frequency of mutation to *acrA* was accurately estimated in two sensitive haploid strains under similar conditions to those used to isolate cross-over diploids. The strains used, M28 and NP84, were the strains containing the wild-type (sensitive) allele at the *acrA*

locus in diploids DP4 and DP72 used in the mapping studies. To obtain the results shown in Table 3, ten independent clones were analysed for each strain to avoid problems of errors resulting from clonally derived mutations to *acrA*. From studies with strain M28 it is clear that the mutation frequency is similar for either spores or amoebae. The frequency of isolation of mutations at *acrA* on methanol (2 %) was between 1×10^{-6} and 2×10^{-6} (Table 3), a very similar value to that obtained for 'cross-overs' at interval III (Table 2). Hence clearly most, if not all, 'cross-overs' at interval III may have resulted from the isolation of a second mutation to *acrA* at the previously sensitive locus.

*Reversion of *tsgD* to wild type, followed by a cross-over proximal to *acrA*.* While the above data suggest that most 'cross-overs' at interval III occur as a result of mutant selection, in some experiments we obtained evidence for the origin of temperature-resistant (i.e. wild-type, growth at 27 °C) diploids as a result of prior reversion at *tsgD* and then cross-over proximal to *acrA* (see Fig. 2 for the proposed mechanism and resulting diploid classes). Yellow, temperature-resistant diploids and white spored, temperature-resistant diploids are expected by this mechanism, and we have observed both in some experiments.

The temperature-sensitive mutation, *tsgD*, does revert to wild type, although usually growth is still somewhat slower at the restrictive temperature; the gradation between leakiness and reversion makes it difficult to estimate the reversion frequency accurately.

We suspected that in some cases we were studying diploids revertant to *tsgD*, because in one experiment using DP72 we obtained an unusually large number of temperature-resistant diploids after selection on methanol (2 %) at the permissive temperature; of the 32 independently derived yellow diploids, 16 were temperature-resistant. However, the yellow, temperature-resistant diploids fell into two definite classes: (i) Those occurring at a frequency of about 2×10^{-6} (2 only) grew vigorously at 27 °C, like DP72. These diploids were heterozygous for *sprB* (see below), and probably arose by selection of a new mutation to methanol resistance at *acrA* so as to give homozygosity at this locus. (ii) Those occurring much more frequently (approximately 3.2×10^{-5}), were diploids which grew less well at 27 °C; these were homozygous wild type at the *sprB* locus (see below). It is likely that an amoebae in the initial clone of DP72 used to derive the separate clones for methanol selection, reverted to wild type at the *tsgD* locus at an early stage. Hence a number of clones of DP72 would have been genetically

$$\frac{tsgD \text{ revertant}}{+}$$

at the *tsgD* locus before plating on methanol.

Similar results, i.e. two classes of yellow, temperature-resistant diploids, were obtained in an experiment involving selection on methanol (2 %) at 27 °C using the diploid DP31. In this case, occurring at a frequency of about 5×10^{-5} , were five independently derived, temperature-resistant diploids (including both white spored and yellow spored types), and four independently derived white, temperature-resistant haploids (linkage group IV segregated independently because two were non-brown and two produced brown pigment). The occurrence at high frequency of white spored, temperature-resistant haploids supports the suggestion that reversion occurred at the *tsgD* locus prior to crossing-over in this experiment (see below).

In 8 out of 13 different experiments no diploids resulting from reversion at *tsgD* were observed, although we have obtained white spored, temperature-resistant diploids from DP4 (1 only), DP16 (1 only), DP31 (1 only) and DP72 (3 only). We believe that the presence of white, temperature-resistant diploids is indicative of reversion at *tsgD*.

The growth of the temperature-resistant diploids resulting from reversion at *tsgD* and crossing-over proximal to *acrA* varied in different diploids, but it was generally somewhat poor at the restrictive temperature.

*Is tsgD further from the centromere than acrA?
Analysis of a new marker (sprB) on linkage group II*

We deduce from the evidence in the previous sections (mutation to *acrA*, reversion of *tsgD*) that if there are any cross-overs between *tsgD* and *acrA*, their frequency is indistinguishable from the spontaneous mutation frequency at the *acrA* locus. Hence it is possible that *tsgD* may be further from the centromere than *acrA*, rather than closer to the centromere as shown in Figs. 1 and 2. Studies with *sprB*, a new spore shape mutation, are consistent with this suggestion.

Strain V12 has very long and thin spores which are clearly distinguishable from the wild-type oval-elliptical spores of NC4 strain B (obtained from Professor M. Sussman in 1967), which is the parent of most of our strains (see Fig. 1c, d of Cotter & Raper, 1968). We have named the long thin spore shape locus found in strain V12, *sprB2*. (The round spore shape mutation located on linkage group I, previously designated *spr* (Williams *et al.* 1974a) has been called *sprA1*).

The diploid DP72 was formed by fusing together strain NP84 (derived from strain V12, which contains *sprB*) and strain X23 (NC4-derived strain, oval-elliptical spores). The long, thin spore shape is incompletely dominant, but spores of DP72 are still relatively long and thin. The marker *sprB* has been located on linkage group II (Williams, unpublished). Using this new linkage group II marker, we examined the spore shape of mitotic cross-over diploids selected on methanol (see Fig. 3). All 31 independently derived white and yellow temperature-sensitive diploids (Table 1, representing cross-overs in intervals I and II) produced oval-elliptical diploid spores. This is consistent with diploids resulting from mitotic cross-overs at interval I or II (Fig. 1) being homozygous wild type at the *sprB* locus. Haploid segregants of such diploids were all oval-elliptical in shape (i.e. *sprB* did not segregate). In terms of the postulated gene order shown in Fig. 1, these results show that *sprB* is distal to *tsgD*.

The temperature-resistant diploid observed in Table 1 produced spores shaped like those of DP72, and hence was still heterozygous for *sprB*; if this diploid is assumed to result from crossing-over in interval III, this indicates that *sprB* is proximal to *acrA*. No oval-elliptical, fully temperature-resistant diploids (homozygous wild type at *sprB* and *tsgD* loci) have been isolated, hence no cross-overs between *tsgD* and *sprB* have been found. This would place *sprB* very close to *tsgD* but distal to it.

If, for the sake of argument, one assumes that the gene order on linkage group II is: centromere, *whi*, *tsgD*, *sprB*, *acrA*, then the mitotic map distance between *tsgD* and *sprB* would be less than 1.3 % (i.e. $1/80 \times 100$: all 80 temperature-resistant diploids from DP72 remained heterozygous for *sprB*) of the distance between *tsgD* and *acrA*, which itself would be very small.

Taken together, the above results show that if the previously published gene order (centromere, *whi*, *tsgD*, *acrA*) is correct, the markers *tsgD*, *sprB* (established here) and *acrA* are very tightly linked.

A more plausible suggestion is that both *tsgD* and *sprB* are located further from the centromere than *acrA*, and that the apparent cross-overs between *sprB* and *acrA* result from the isolation of new mutations at the *acrA* locus. The suggested derivation of all classes of diploid obtained by selection of DP72 on methanol are summarized in Fig. 3.

Fig. 3. Phenotype, suggested genotype, and suggested origin of diploids obtained from DP72 after selection on methanol. The frequency of occurrence of the different classes of diploid is given in Tables 1 and 2 and in the text. The relative order of *sprB* and *tsgD* has not been determined.

Proposed map of linkage group II

From the foregoing data we suggest that the gene order on linkage group II is: centromere, *whi*, *acrA*, with *tsgD* and *sprB* distal to *acrA*, but their relative positions not yet determined. To order these markers a more distal selective marker is needed. The mitotic map distances between centromere and *whi*, and *whi* and *acrA* are approximately equal.

The origin of white, temperature-resistant acriflavin/methanol-resistant diploids

Mention has been made above of white, temperature-resistant, methanol/acriflavin-resistant diploids. Such diploids were postulated by Katz & Kao (1974) to arise by three cross-overs, although they concluded that there was some evidence for a more complex origin. However, a simpler mechanism, involving prior reversion at the *tsgD* locus and a single cross-over, has been suggested in Fig. 2. These different schemes are contrasted in Fig. 4, and in addition the origin of white, temperature-resistant, methanol/acriflavin-resistant haploids is shown. In the scheme of Katz & Kao (1974), white, temperature-resistant haploids can only be formed after the triple cross-over event; thus four rare events must occur simultaneously. In the alternative scheme, where *tsgD* reverted to wild type before plating on methanol, only haploidization and no cross-overs are required; this explains the relative frequency of observing white, temperature-resistant haploids in some experiments. The white, temperature-resistant diploids which grew poorly at the restric-

Fig. 4. Contrasting schemes for the origin of white, temperature-resistant, acriflavin/methanol-resistant haploids and diploids selected on methanol. For scheme (a) the gene order suggested by Katz & Kao (1974) has been employed. For scheme (b) the gene order suggested in this paper has been employed, showing *tsgD* distal to *acrA*.

tive temperatures are explained as being partial revertants in our scheme; these diploids are difficult to explain by the scheme of Katz & Kao (1974).

A prediction of the scheme of Katz & Kao (1974) is that the white, temperature-resistant diploids remain heterozygous at the *tsgD* locus, whereas the scheme suggested here results in homozygosity for the revertant at the *tsgD* locus (see Fig. 4). The different hypotheses were tested by examining whether both temperature-sensitive and temperature-resistant haploids, with respect to the *tsgD* locus, could be isolated from a white, temperature-resistant diploid obtained from DPI6. Fifty haploids were examined by segregating haploids on cycloheximide and examining growth at 27 °C. (DPI6 is heterozygous for cycloheximide resistance on linkage group I: $\frac{cycA}{+}$.) None of these haploids were temperature-sensitive at the *tsgD* locus, hence it appears that the white, temperature-resistant diploid was homo-

zygous wild type at the *tsgD* locus as predicted by our hypothesis. In fact Katz & Kao (1974) found that 90 % of their haploid segregants from a white, temperature-resistant diploid were temperature-resistant at the *tsgD* locus.

Our results therefore suggest that a single cross-over mechanism can explain the origin of white, temperature-resistant diploids and we suggest that multiple cross-overs may be rare in *D. discoideum*, as is found in *Aspergillus nidulans* (Pontecorvo & Käfer, 1958).

DISCUSSION

Although the technique of mitotic mapping has been successfully used with *D. discoideum*, this report highlights several difficulties which arise as a direct consequence of using mitotic rather than meiotic means to estimate distances between loci on a linkage group in this organism.

Firstly, since mitotic cross-overs are rare, cross-overs between closely linked markers may be as rare as either the selection of new mutations at the selective marker locus (as shown for *acrA* in this study) or reversion at other loci (as suggested in this study for *tsgD*). Further mapping studies rely, therefore, on the isolation of selective markers that show low reversion frequencies.

Secondly, mitotic mapping relies on the availability of selective markers which are located distally to the markers being mapped (Pontecorvo & Käfer, 1958). In *D. discoideum*, however, only recessive drug resistance markers are available at this stage and some of these, such as *cycA* on linkage group I, appear to be close to the centromere and therefore of little value for mapping (Williams *et al.* 1974*a*). The marker *acrA*, discussed here, is of some value since it is distal to *whi* and to some aggregateless mutations under study, but we now find that even this marker is probably proximal to *tsgD* and *sprB*.

Thirdly, Pontecorvo & Käfer (1958) also showed that unless markers are available on both arms of a chromosome, the most proximal marker on a given arm (*whi* in this paper) cannot be rigorously assigned to that arm, since the 'proximal' marker may in fact be located on the other arm. This arises because cross-overs proximal to the closest marker to the centromere would result in homozygosity for all markers on that arm, but the same result could be obtained without cross-overs by a non-disjunctional mechanism. Unfortunately, markers on both arms have not yet been found for any chromosome in *D. discoideum*. However, studies on linkage group I (Williams *et al.* 1974*a*) suggested that non-disjunctional diploids are probably rare in *D. discoideum*. Hence from such evidence we suggest that few, if any, of the white diploids obtained in the present study would have arisen by non-disjunction, and we therefore show *whi* as being on the same arm as *acrA*.

A fourth difficulty that can arise using *D. discoideum* is that, unlike in *Aspergillus nidulans* (Pontecorvo & Käfer, 1958), the positional origin of partially homozygous segregants that arise from heterozygous diploids by mitotic cross-overs is not visually obvious in clones of cells. Consequently, previous mapping studies have used all the amoebae from one or a few such clones apparently without regard to the possible common origin of any diploid segregants isolated from these clones. We have found, however, that this procedure can lead to great variation in the observed map intervals in different experiments, caused presumably by unequal multiplication of cells in different segregant classes formed early or late during growth of the clones. To circumvent this problem we have adopted the somewhat laborious procedure of retaining only one of the segregant diploids (randomly chosen) from those able to grow on drug-containing selection plates seeded with amoebae derived from the same initial clone.

In other aspects of genetic analysis with this organism, however, parasexual genetics has considerable advantages over the meiotic system. For example, locating markers to linkage groups using parasexual genetics is relatively straightforward because chromosomes assort randomly with a low frequency of crossing-over at mitosis, whereas with meiosis there is a high frequency of crossing-over. It also seems that complementation testing, which is simply conducted using parasexual genetics, will not be possible in the cellular slime moulds using meiotic methods, as in the sexual structure the diploid state is transitory (Erdos *et al.* 1973; Macinnes & Francis, 1974).

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