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Complementation tests on closely linked flagellar genes in *Chlamydomonas reinhardii*

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1. INTRODUCTION

The flagellar apparatus of the normally haploid, unicellular green alga *Chlamydomonas reinhardii* has previously been shown to be a useful system for the study of the genetic control of organelle structure and morphogenesis (Randall *et al.* 1968). For a comprehensive study it is important to know the total number of genes involved and to assign to each a detailed position on a linkage group, together with either a structural or control function.

Complementation tests have been performed on a group of closely linked genes affecting the formation of the central pair of microtubules in the flagella of *Chlamydomonas reinhardii*. The procedure adopted involved the selective isolation of diploid strains in which the mutant genes were recessive to their wild-type alleles. All seven mutations tested were found to be in the same cistron; consequently the total number of known cistrons may be less than previously believed.

The axoneme of the wild-type flagellum consists of the well-known 9+2 arrangement of microtubules (Randall, Warr, Hopkins & McVittie, 1964). Four groups of isolated mutants show a disorganization of the cental pair of tubules, designated the 9+0 arrangement, and a consequent paralysis of the organelle. A group of seven such mutations, the pf18 series, which are closely linked, and map on linkage group II, have been used in this study (Warr, McVittie, Randall & Hopkins, 1966).

Warr *et al.* (1966) demonstrated the lack of recombination between pf18 (originally isolated by Lewin) and four of their newly isolated strains, pf18A, pf18B, pf18C and pf18D. The upper limits of recombination were 0·37%, 0·44%, 0·56% and 0·32% respectively. In addition, two more alleles have been mapped at this locus pf18E and pf18F (A. McVittie, private communication). Their analysis does not distinguish between two possibilities: (1) some or all of these mutations are in the same cistron; (2) the mutations are in several closely linked cistrons.

To resolve this question use has been made of a modification of a method first developed by Ebersold (1967), which involves the formation of diploids. Similar methods were employed by Pomper & Burkholder (1949) during their work on yeast. It has previously been shown that some mutant genes are recessive to their respective wild-type alleles in *Chlamydomonas*; this conclusion has now been confirmed for the genes investigated in the present work. Thus if two mutations, in different cistrons, are introduced into the same diploid nucleus, complementation should occur and the progeny should be phenotypically wild type. Conversely, if two mutations in the same cistron are introduced, the progeny cells should be paralysed.

2. SELECTION OF DIPLOID STRAINS

The sexual cycle of *Chlamydomonas reinhardii* involves the fusion of two haploid gametes to form a diploid zygote which, after a period of 5 days maturation in the dark, can be induced to undergo meiosis by plating on fresh medium in the light. Each zygote gives rise to four or eight zoospores, which are capable of vegetative growth. Ebersold (1963) observed that a small number (4%) of newly formed zygotes may follow an alternative mode of development, when incubated on 1% agar plates in continuous light, dividing mitotically to give rise to vegetative diploid colonies. He suggested a selective method for the recovery of diploid strains, based on the inability of haploid auxotrophic mutants to grow under conditions which allow the growth of prototrophic cells.

These conditions can be achieved by plating a mating mixture of two different auxotrophs on a minimal agar medium lacking the required growth factors. Under such conditions, haploid auxotrophs would not survive. However, two types of prototrophic colony, large and small, were observed after 6 days incubation. Assuming that the mutant genes conferring auxotrophy were recessive to their wild-type alleles, mitotic division of heterozygous zygotes would lead to the formation of large (macroscopically visible) diploid prototrophic colonies. Maturing zygotes, having undergone meiotic recombination, gave rise to haploid prototrophic small colonies (visible under the dissecting microscope). Since meiotic division is delayed for at least 36 h (Ebersold, 1963) colonies formed by this mechanism would be expected to be smaller than the diploid clones.

In order to introduce the paralysed mutant genes into the diploid nucleus, it was first necessary to isolate double mutants from crosses of each of the paralysed alleles to an auxotrophic mutant (Table 1). The acetate requiring strains ac14 and ac17 were used (Ebersold, Levine, Levine & Olmsted, 1962). Mating mixtures of pairs of these double mutants, differing both in the auxotrophic gene carried and the allele at the pf18 locus, were plated on minimal medium, solidified with 1% agar. The plates were incubated at 26 °C and illuminated with 500 ft-candles for 6 days. Both large and, in some cases, small colonies were then transferred to fresh plates, by means of a small wire loop, for a further 3 days growth. The colonies were then transferred to liquid medium for motility testing. Light-microscope observations were also made at this stage.

3. EVIDENCE FOR DIPLOIDY

(1) *Mode of origin.* The failure of haploid auxotrophs to survive on minimal medium was established by separate experiments. Thus the surviving colonies must be either diploids in which the corresponding wild-type alleles were dominant, or recombinant haploids. In recombinant haploids, both motile and immotile cells would be expected in some colonies of crosses ac14, pf18C × ac17, pf18C+. Mixed small colonies only have been observed, the large colonies consisted only of motile cells. Evidence for the dominance of wild-type alleles will be outlined below.

(2) *Cell size.* Cells of presumptive diploid strains were found to be larger in the logarithmic phase of growth than haploid cultures grown under the same conditions. This is in agreement with Ebersold's findings (Ebersold, 1967).

(3) *Abnormal number of flagella.* When presumptive diploids were grown in liquid culture for 3 days and observed in the phase-contrast microscope, some cells were seen to have three flagella, instead of the normal two. The percentage of such cells in different cultures was found to be rather variable; between 2·0% and 5·0%. A small percentage had four flagella and occasional cells with five or six flagella have been observed. An abnormal number of flagella is comparatively rare in any of the haploid strains used. For example, in two counts, each of over 1000 biflagellate haploid pf18C cells, no triflagellates were seen. There were, however, in each case six cells with four flagella. In most cases the

quadriflagellate cells had two pairs of flagella at opposite ends of the cell, suggesting incomplete cell division. In multiflagellate diploids all the flagella emerge from the anterior end of the cell.

(4) *Incomplete tetrads.* When three of the diploid strains were backcrossed to wild-type 32C+ haploids, only four out of 25 surviving zygotes analysed produced complete tetrads. Incomplete tetrads are rare in haploid × haploid crosses.

(5) *Re-isolation of acetate markers.* Acetate-requiring strains have been isolated from diploid × wild-type backcrosses. These strains, in turn, were crossed to ac14 and ac17 strains. Recombination with either, but not both, of these strains, showed that both these auxotrophic markers were still present in each prototrophic diploid strain.

(6) *Mating type.* The mating type of all the large colonies was found to be mt−, showing the dominance of the minus over the plus mating type in diploids. This again is in agreement with Ebersold's observations (Ebersold, 1967).

4. TESTING FOR WILD-TYPE DOMINANCE

Crosses were made between a double mutant carrying the markers ac14, pf18C and either (1) the auxotrophic single mutant ac17, pf18C+, or (2) a double mutant ac17, pf18C. The heterozygous diploid clones isolated, as indicated above, from the cross ac14, pf18C × ac17, pf18C+ showed normal motility. Diploid colonies, homozygous for the pf18C locus isolated from the cross ac14, pf 18C × ac17, pf 18C consisted only of paralysed cells. This demonstrates the dominance of the wild-type allele.

This conclusion was tested further by the introduction of another paralysed marker, pf19B, which maps on linkage group X (Randall, 1969). From crosses of two paralysed double mutants, ac14, pf18C × ac17, pf19B, both large (diploid) and small (recombinant haploid) colonies were isolated. The large colonies contained only motile cells, demonstrating the dominance of both wild-type alleles, pf18C+ and pf19B+. The small colonies sometimes contained both the expected motile and paralysed cells, due to recombination at meiosis, as seen in the light microscope with phase-contrast illumination.

5. COMPLEMENTATION TESTING OF THE pf18 ALLELES

Pairs of the acetate-requiring double mutants of the seven pf18 alleles, pf18, p18A, pf18B, pf18C, pf18D, pf18E and pf18F, were then crossed in the manner indicated above, and the cells of the large colonies examined for motility. The results are summarized in

Table 1. *Double mutant crosses from which diploid strains were derived*

Haploid double mutants		
Double mutant 1	Double mutant 2	Diploid phenotype
ac14, pf18C	ac17, pf18C+	Motile
ac14, pf18C	ac17, pf18C	Paralysed
ac14, pf18C	ac17, pf19B	Motile
ac14, pf18C	ac17, pf18	Paralysed
ac14, pf18C	ac17, pf18B	Paralysed
ac14, pf18C	ac17, pf18D	Paralysed
ac14, pf18B	ac17, pf18D	Paralysed
ac14, pf18F	ac17, pf18D	Paralysed
ac14, pf18F	ac17, pf18	Paralysed
ac14, pf18C	ac17, pf18A	Paralysed
ac14, pf18C	ac17, pf18E	Paralysed

A summary of phenotypes of the diploid strains. The latter were obtained by crossing two haploid double mutants, each carrying one auxotrophic marker and a pf18 allele.

Table 1. It can be seen that any two pf18 alleles, when included in the same diploid nucleus, give rise to paralysed progeny. Thus all seven alleles are in the same cistron. Although immotile, several of the haploid pf18 mutants are capable of some flagellum movement. This activity is more apparent in the diploid cells.

6. DISCUSSION

It has been shown that a group of closely linked genes affecting the formation of the central pair of microtubules in the flagellum of *Chlamydomonas reinhardii* are in the same cistron.

Some 80 flagellar mutations (Randall, 1969), not all of which have been mapped, have been isolated in this organism. However, the total number of known cistrons may be considerably less than originally thought. It is important to establish the number of cistrons which control the development of this organelle (Randall, Cavalier-Smith, McVittie, Warr & Hopkins, 1968).

The procedures discussed here offer a method of studying by complementation analysis, genetic fine structure of a eucaryotic organelle in a manner analogous to that used so successfully in viral systems. It has been shown that the pf18 locus represents a single cistron. Three other well-characterized loci, the pf19, the pf20 on linkage group IV and the pf15 on linkage group III, immediately offer six, six and three alleles respectively for similar analysis. The pf18 locus was selected for initial investigation since it offered the largest number of mutants. It should later be possible to extend this approach to mutants other than those of the central pair of microtubules.

In view of the recent evidence for an abnormal protein in the pf18C mutant (Jacobs, 1969) it should be possible to investigate whether mutant protein is synthesized in motile, diploid heterozygous cells carrying the pf18C gene.

Thus the selection of diploids offers a method of finer genetic mapping and a possible means of determining the total number of genes involved in the control of a single organelle. It is also possible that this procedure may offer some insight into the mechanisms of genetic control of flagellum assembly.

SUMMARY

Complementation tests have been performed on a group of closely linked genes affecting the formation of the central pair of microtubules in the flagella of *Chlamydomonas reinhardii*. The procedure adopted involved the selective isolation of diploid strains in which the mutant genes were recessive to their wild-type alleles. The mutations tested were found to be in the same cistron.

I am at present in receipt of a Tutorial Studentship of the University of London King's College. I am indebted to a number of colleagues for suggestions made in the course of this work and particularly to my supervisor, Sir John Randall, who initiated me into this field, for valuable discussion.

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