A METHOD FOR COMPLEMENTATION ANALYSIS OF NUCLEAR AND CHLOROPLAST MUTANTS OF PHOTOSYNTHESIS IN CHLAMYDOMONAS

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ABSTRACT

The photosynthetic properties of young zygotes of *Chlamydomonas reinhardi* were analyzed. In heterozygotes for two nuclear or two chloroplast mutations affecting photosynthesis, recovery of photosynthetic activity was observed that is most likely the result of intergenic complementation.——We observed that chloramphenicol inhibited the recovery of activity in double heterozygotes for mutants lacking at least one thylakoid polypeptide of chloroplast origin, while it had no effect on wild-type homozygotes. This indicates that the recovery of activity in double heterozygotes could result from the repair of existing thylakoid membranes by *de novo* synthesis of the missing polypeptides.

A LARGE number of mutants with impaired photosynthesis have been isolated in *Chlamydomonas reinhardi*. Most of them are of nuclear origin. However, the recent discovery by WURTZ *et al.* (1979) that 5-fluorodeoxyuridine induces chloroplast gene mutations in this alga permitted the isolation of many mutants of chloroplast origin in which the photosynthetic process is impaired (SHEPPERD, BOYNTON and GILLHAM 1977, 1979; BENNOUN *et al.*, 1978). Complementation studies with photosynthesis mutants of Chlamydomonas raise some problems: (1) Because there is no natural vegetative diploid phase in the cell cycle, rare vegetative diploids, which cannot undergo meiosis, must be selected from the zygote population by using complementary arginine markers (EBBERSOLD 1963, 1967; GILLHAM 1963). The complementation test of nuclear mutants therefore requires the tedious construction of double mutant strains. (2) In either its haploid or diploid state, Chlamydomonas possesses only one chloroplast. Segregation and recombination of the chloroplast genes can be observed among the mitotic progeny of biparental diploids (GILLHAM 1963, 1969). For this reason, complementation between chloroplast mutants could not be studied at this level.

The young zygotes cannot be induced to undergo meiosis until two days after mating (SEARS 1980). These zygotes already possess a single nucleus and a single chloroplast containing the information of the two parents (CAVALIER-SMITH 1970, 1976). They are, therefore, suitable for testing complementation of nuclear and chloroplast mutations.

Mutants with impaired photosynthesis can be precisely and rapidly characterized by their kinetics of fluorescence and delayed luminescence (BENNOUN

1974; BENNOUN and CHUA 1976). Using these techniques, we investigated the phenotype of young zygotes obtained in various crosses involving photosynthesis mutants of nuclear or chloroplast origin. Results of these studies are reported here in an attempt to establish a complementation test valid for both nuclear and chloroplast mutants.

MATERIALS AND METHODS

Materials: Wild-type (WT) *Chlamydomonas reinhardi* and mutants were grown on agar plates containing Tris-acetate-phosphate medium (TAP) (GORMAN and LEVINE 1965), under continuous illumination of 200 lux of cool white fluorescent light. Gametes were obtained by growth on solid medium (TAP with 1/10 the regular amount of nitrogen) at the same light intensity for 4 to 5 days. Gametes were collected in 4 ml of phosphate buffer (10mM, pH 7) at a cell density of 10^8 cell/ml, and both mating types were mixed in a small (5 cm) petri plate. After exposure to strong light (3000 lux of cool white fluorescent light) for 4 hours, the petri plates were transferred to low light (200 lux). At this time, zygotes clump together and form a continuous layer that sticks to the bottom of the dish. Unmated cells can be removed by repeated washing of the zygote layer with phosphate buffer. In all crosses analyzed, a very high efficiency of mating was obtained. Fluorescence and luminescence measurements were performed directly on the zygote layer in the petri plate. This continuous layer was quite uniform and contained approximately the same number of cells in each cross.

Methods: Fluorescence-induction kinetics was achieved as described previously (BENNOUN 1974). Mutants lacking photosystem II reaction centers display a high fluorescence yield, with no variable part. Mutants deficient in photosystem I activity display a fluorescence rise that is not followed by the decrease observed in the wild type.

Delayed luminescence measurements were achieved as follows: a Xenon flash (General Radio Stroboslave 1539A) of 4 μsec illuminated the petri plate from below through a light pipe made from a blue Schott filter (BG38, 2×2×1 cm). A photomultiplier (EMI 8558) fitted with complementary red filters (Rubylith Ulano + Kodak Wratten 70) was placed just above the petri plate. The output of the photomultiplier was connected to a memory screen oscilloscope. The mutants, FUD17 and FUD18, which have impaired ATPase, display a very slow decay of the transmembrane electric field generated in the light across the thylakoid membrane (BENNOUN *et al.* 1978). When the mutants are given a series of saturating flashes 100 msec apart, a very high transmembrane electric field is maintained. On the other hand, in the wild type the electric field is rapidly dissipated through the active ATPase. The luminescence intensity is directly related to the intensity of the transmembrane electric field (JOLIOT and JOLIOT 1974), so that this parameter is suitable for a rapid estimation of the ATPase activity in this type of mutant.

RESULTS

Nuclear mutants: We have analyzed the fluorescence patterns of the zygotes obtained in various crosses. Figure 1 shows the results for wild-type homozygotes, mutant homozygotes F14/F14 (lacking photosystem I reaction centers) and F64/F64 (lacking photosystem II reaction centers), and F14/F64 heterozygotes. The homozygotes observed up to 48 hours after mating display the same fluorescence pattern as vegetative cells. The double heterozygotes observed at 48 hours display a phenotype very close to that of the wild type, as would be expected if complementation occurs. Figure 2 shows the fluorescence patterns of F34/F34 and F64/F64 homozygotes and F34/F64 heterozygotes. The F34 mutant displays the same phenotype as that of F64, but the two mutants are

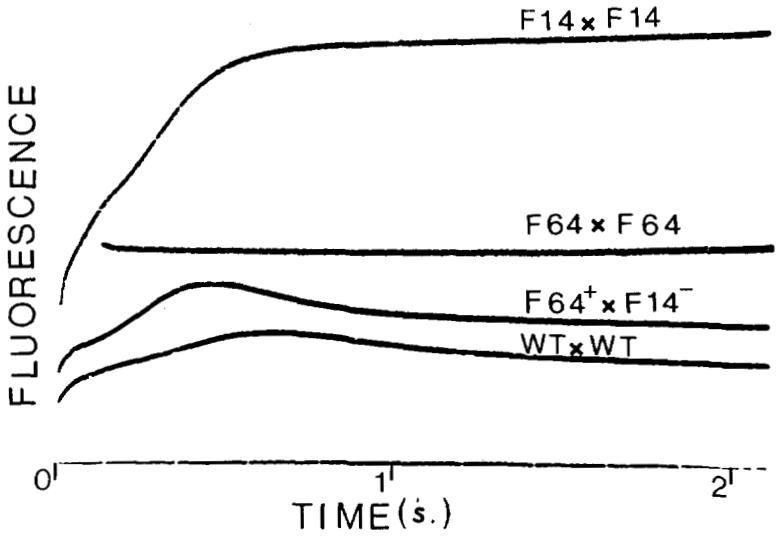

FIGURE 1.—Fluorescence induction kinetics of dark-adapted zygotes analyzed 48 hours after mating.

alleles of different genes, as shown by tetrad analysis (data not shown). The F34/F64 heterozygote shows the expected complementation. Twenty different types of crosses involving nuclear photosynthesis mutants of various phenotypes consistently showed complementation in all double heterozygotes for mutations of different genes, whereas no complementation was observed in heterozygotes

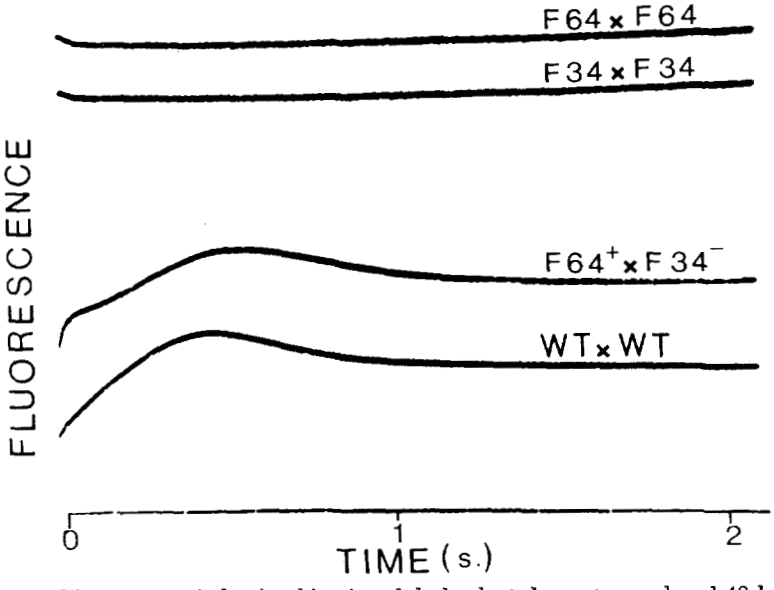

FIGURE 2.—Fluorescence induction kinetics of dark-adapted zygotes analyzed 48 hours after mating.

for mutations of the same gene. This was verified in crosses involving five mutants deficient in system I reaction centers: three alleles for one gene, F12, F14 and F20, and two alleles of another gene, F1 and M23, which were allele-tested in vegetative diploids (J. GIRARD, personal communication). Complementation can usually be detected 24 hours after mating, but the extent of complementation generally increases up to 48 hours. The amplitude of complementation can vary from one cross to another.

Since it is known that several thylakoid membrane polypeptides are synthesized in the chloroplast, we tested whether functioning of photosynthetic membranes within the young zygotes were affected by chloramphenicol. This antibiotic inhibits protein synthesis in the chloroplast, including proteins associated with the reaction centers of photosystems I and II (CHUA and GILLHAM 1977). The absence of these polypeptides from the thylakoids can easily be detected by a large increase in the fluorescence yield and an inhibition of delayed light emission. In the experiment depicted in Figure 3, wild-type zygotes treated with chloramphenicol as soon as the zygote layer is formed (about four hours after mixing the gametes) display at 48 hours a fluorescence pattern identical to the control. However, chloramphenicol prevents complementation in the F34/F64 heterozygotes. This effect is reversible, since washing out the chloramphenicol at 48 hours permitted complementation to occur in the following 24 hours. The complete inhibition of complementation by chloramphenicol was observed in double heterozygotes for mutations affecting either photosystem II or photosystem I activity. In addition to their lacking thylakoid polypeptides synthesized in the cytoplasm, these mutants are deficient in at least one thylakoid polypeptide synthetized inside the chloroplast. Most photosynthesis mutants lack a set of polypeptides corresponding to a structural complex of the thylakoid (ATPase, System I and II complexes). Each complex is made of polypeptides synthetized both in the cytoplasm and in the chloroplast.

Chloroplast mutants: We investigated the phenotype of zygotes obtained with photosynthesis mutants of chloroplast origin. Figure 4 shows the fluorescence

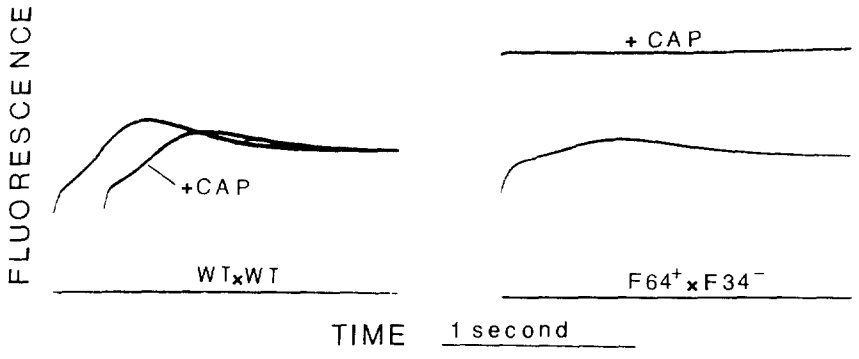

FIGURE 3.—Fluorescence induction kinetics of dark-adapted zygotes analyzed 48 hours after mating. (Chloramphenicol, when present, is added 4 hours after mating at a concentration of 100 μg/ml.)

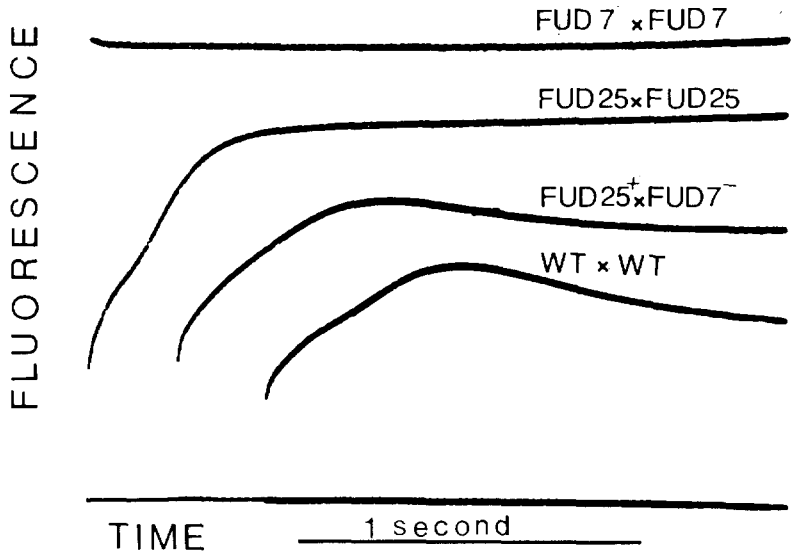

FIGURE 4.—Fluorescence induction kinetics of dark-adapted zygotes analzed 24 hours after mating.

patterns of FUD7/FUD7 (lacking photosystem II reaction centers) and FUD25/FUD25 homozygotes (deficient in photosystem I activity), and of FUD7/FUD25 heterozygotes. The homozygotes display the same features as vegetative cells, whereas the heterozygotes display a pattern close to that of the wild type. This was observed in many other crosses involving different types of mutants. For instance, Figure 5 shows the delayed luminescence pattern of wild-type homozygotes, of two mutant homozygotes lacking the ATPase CFI (FUD17 and FUD18) and of the heterozygotes FUD17/FUD18. The characterization of this type of mutant is performed by recording delayed luminescence, which shows a higher intensity when ATPase is inactive (see METHODS). The heterozygotes display a reduced luminescence yield as compared to the mutant homozygotes, indicating a recovery of activity. We have used this method to analyze several mutants described by SHEPHERD, BOYNTON and GILLHAM (1979), which have a phenotype similar to that of FUD17 and FUD18 (deficient in the ATPase CFI). Recombination analysis showed that the mutants *ac-u-b-1-5* and *ac-u-b-1-10* are alleles of the same locus, whereas *ac-u-a-1-15* belongs to a different one. We observed a mutant phenotype in *ac-u-b-1-5/ac-u-b-1-10* zygotes, whereas a recovery of activity was observed in *ac-u-a-1-15/ac-u-b-1-10* zygotes. Thus, both recombination and complementation analysis give consistent results. The recovery of activity in heterozygotes for two photosynthesis mutants of chloroplast origin is generally detectable eight hours after mating, and reaches its maximum 24 hours after mating. The extent of recovery can vary from one cross to another. Figure 6 shows that chloramphenicol also inhibits the complementation in heterozygotes of the chloroplast mutants FUD17/FUD18, but does not alter the luminescence pattern of wild-type homozygotes. These two mutants lack at least two

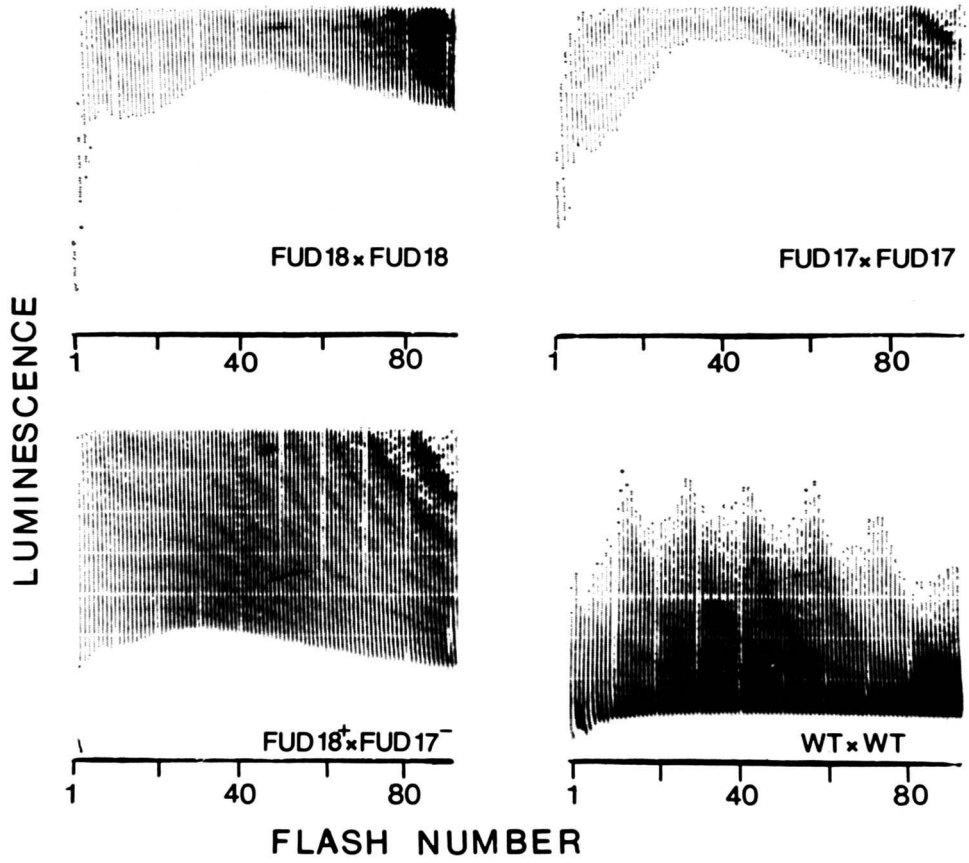

FIGURE 5.—Delayed luminescence emitted by 24-hour-old zygotes during a series of flashes 100 msec apart. The bottom of each vertical line of the oscilloscope tracing is a measurement of the intensity of luminescence 100msec after each flash. The intensity of luminescence is related to the activity of the ATPase (see METHODS for details).

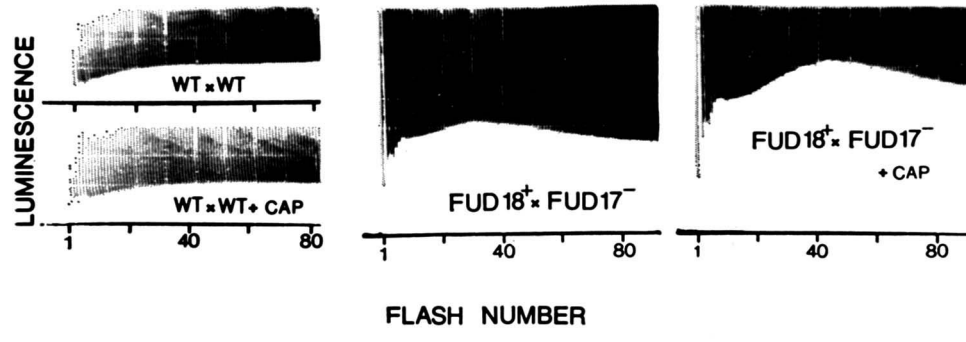

FIGURE 6.—Delayed luminescence emitted by 24-hour-old zygotes as depicted in Figure 5. Chloramphenicol when present is added 4 hours after mating at a concentration of 100 μg/ml.)

polypeptides synthesized inside the chloroplast, the α and β subunits of the ATPase CF1 (BENNOUN *et al.* 1978).

DISCUSSION

The study of the phenotype of young zygotes obtained from either nuclear or chloroplast mutants shows that they displayed photosynthetic characteristics identical to those of the vegetative cells. Crosses involving nuclear photosynthesis mutants clearly show that complementation occurs in the young zygotes up to 48 hours after mating. At this stage, meiosis has not yet started; therefore, our results cannot be accounted for by recombination. The complementation test is both fast and sensitive and is generally useful for geneticists working on Chlamydomonas. It is likely that the recovery of activity in heterozygotes for two chloroplast mutations affecting photosynthesis also results from intergenic complementation.

Our system is similar to that developed for mitochondria genes in yeast by SLONIMSKI *et al.* and FOURY and TZAGOLOFF, both in 1978. As in the yeast system, biochemical complementation (*i.e.*, exchange of wildtype chloroplast proteins of the two strains) cannot account for our results since restoration is sensitive to an organelle protein-synthesis inhibitor and occurs when both parents lack the same set of polypeptides, *e.g.*, ATPase mutants in Chlamydomonas. Interallelic complementation (*i.e.*, complementation of heteroalleles) is also not likely to occur in either organelle system, as restoration is observed in crosses involving mutants having completely different phenotypes and, therefore, thought to be nonallelic. An example of this is the complementation observed in a cross of a mutant deficient in photosystem I activity with a mutant deficient in system II reaction centers in Chlamydomonas (Figure 4). In the case of yeast, there are good reasons to believe that the recovery of respiration in the zygotes is due to intergenic complementation, rather than to recombination. In the case of Chlamydomonas, the possibility that recombination can account for some of our data is not ruled out yet, since recombination analysis of chloroplast photosynthesis mutants is at a very early stage. However, the fact that restoration of photosynthetic activity is observed in populations of zygotes, more than 90% of which will transmit only chloroplast mutations from the maternal parent, favors the hypothesis that we are dealing with complementation.

We have observed that chloramphenicol inhibits the recovery of activity in double heterozygotes of both nuclear and chloroplast mutants, all of which lack at least one thylakoid polypeptide of chloroplast origin. These mutants lack polypeptides associated with photosystem I, photosystem II or ATPase. We do not know which proteins must be synthetized on 70 S ribosomes in order to recover photosynthetic activity during complementation. However, it is reasonable to assume that these polypeptides are the thylakoid polypeptides of chloroplast origin, which are missing in the mutant. If this is the case, then there is no pool of these proteins in the young zygotes. In contrast, chloramphenicol has

essentially no effect on the photosynthetic ability of wild-type homozygotes up to 48 hours, as shown by their fluorescence and luminescence patterns.

Taken together, these arguments suggest that there is neither important turnover of thylakoid polypeptides of chloroplast origin in the photosynthetic membranes of young zygotes nor synthesis of new photosynthetic membranes. Thus, the complementation we observe is likely to involve the repair of existing membranes. One must, therefore, be cautious in the interpretation of a dominance test: in a cross of a mutant with wild type, the heterozygotes may always contain half of their thylakoid membranes, which are functional.

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