Microinjection of Cytoplasm as a Test of Complementation in Paramecium

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ABSTRACT Mutants in *Paramecium tetraurelia*, unable to generate action potentials, have been isolated as cells which show no backward swimming in response to ionic stimulation. These "pawn" mutants belong to at least three complementation groups designated *pwA*, *pwB*, and *pwC*. We have found that microinjection of cytoplasm from a wild-type donor into a pawn recipient of any of the three complementation groups restores the ability of the pawn to generate action potentials and hence swim backward. In addition, the cytoplasm from a pawn cannot restore a recipient of the same complementation group, but that from a pawn of a different group can. Electrophysiological analysis has demonstrated that the restoration of backward swimming is not due to a simple addition of ions but represents a profound change in the excitable membrane of the recipient pawn cells. Using known pawn mutants and those which had previously been unclassified, we have been able to establish a perfect concordance of genetic complementation and complementation by cytoplasmic transfer through microinjection. This method has been used to classify pawn mutants that are sterile or hard-to-mate and to examine the ability of cytoplasms from different species of ciliated protozoa to restore the ability to swim backward in the pawn mutants of *P. tetraurelia*. A cell homogenate has also been fractionated by centrifugation to further purify the active components.

These results demonstrate that transfer of cytoplasm between cells by microinjection can be a valid and systematic method to classify mutants. This test is simpler to perform than the genetic complementation test and can be used under favorable conditions in mutants that are sterile and in cells of different species.

Complementation usually refers to the ability of a double heterozygote to give a normal phenotype. Although the standard complementation tests put the genes into the same cytoplasm, it is the gene products that complement. Complementation of biological functions can therefore be demonstrated by combining the two gene products without direct gene transfer. The clearest demonstration is the physical complementation of gene products in bacteriophage morphogenesis in vitro (42). In more complex biological systems, transfer of gene products without gene transfer has been demonstrated by fusion of cells with preloaded membrane vesicles (11, 32, 40, 41) or erythrocyte ghosts (12, 27, 36) and by microinjection of wild-type cytoplasm or cytoplasmic fractions (6, 19, 39).

Because of their large size and lack of cell wall, ciliated protozoa are especially amenable to a transfer of cytoplasm through microinjection. Kappa particles (14, 21), mitochondria (3, 22), immature trichocyts (2, 4), general cytoplasm (13, 20, 23), and "immaturin" (15) have been transferred through microinjection in a variety of cell-biological studies in *Paramecium*.

Mutants in *Paramecium* have been selected for their abnormal response to various ionic stimulations (24). Normally, such a stimulus triggers a Ca-action potential, causing a transient increase in intracellular Ca^{++} concentration. The increase in Ca^{++}-concentration in turn causes the cilia to reverse their beat, resulting in backward swimming. Mutants unable to swim backward have been isolated and the defects in most cases have been traced to Ca-channel function (25, 26, 30, 34) or to the ciliary components (axoneme) that respond to the increase in intracellular Ca^{++} concentration. Mutants missing their Ca-channel function in *P. tetraurelia* are called pawns. Standard genetic crosses of these recessive mutants separate them into at least three complementation groups: *pwA*, *pwB*, and *pwC* (8). Berger (5) found that, during a pawn-wild-type conjugation,

the pawn partners often gain the ability to swim backward before the scheduled nuclear transfer and fertilization, suggesting the transfer of a cytoplasmic substance which restores part of the wild-type function in the pawns. This restoration has been more directly demonstrated by microinjecting the cytoplasm or a cytoplasmic fraction of a wild-type into a "CNR" mutant of *P. caudatum* (the equivalent of a pawn in *P. tetraurelia*; Hiwatashi and Haga, manuscript in preparation) (17). Using the pawn mutants, we show here that microinjection of cytoplasm containing the product, but not the nucleus containing the gene into a recipient, can be a valid, systematic complementation test. This test is sometimes much simpler to perform than the genetic test. It does not depend on conjugation or fertilization and can therefore be used, under favorable conditions, in mutants that are mating-incompetent or sterile and in cells of different species.

MATERIALS AND METHODS

Cell Culture and Strains

Paramecium tetraurelia were grown in Cerophyl medium buffered with sodium phosphates and bacterized with *Enterobacter aerogenes* (38). The pawn-mutant strains used in this study are listed in Tables I and II. Wild-type refers to stock 51S.

P. caudatum was obtained from Carolina Biological Supply Co. (Burlington, N.C.). *Tetrahymena thermophila* stock AE1 was a gift of E. Orias (Department of Biological Sciences, University of California Santa Barbara, Calif.). Cells were grown at 23° or 35°C as indicated.

Microinjection and Behavioral Testing

Microinjection was performed by a modified method of Koizumi (21). ~15 to 20 pl of cytoplasm or crude cytoplasmic fraction (see below) was withdrawn directly from a donor cell or from a drop of the fraction and injected with a micropipette (inside tip Diam ~5 μm) into a recipient under a compound microscope. The two micronuclei and the macronucleus are attached to the cell matrix at fixed positions. Thus, they do not enter the micropipette when the cytoplasm is withdrawn unless special microsurgery is performed. There is also little probability that part of the cell cortex is grafted by the procedure (17). The recipient cells were first placed in exhausted culture medium containing at 0.15% (wt/vol) methyl cellulose and 3 mg/ml bovine serum albumin. Exhausted culture medium was prepared by filtering a stationary-phase culture of paramecium through a Millipore filter (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.). The recipient was temporarily held nearly stationary for injection by depleting the fluid in the hanging drop in which it had been isolated. The injected cells were then incubated in 3.5 mM KCl, 0.5 mM KOH, 1 mM $CaCl_2$, 1 mM HEPES, 10^{-5} M EDTA, pH 7.4 or in culture medium exhausted of its bacteria and then periodically withdrawn through a micropipette and transferred into a solution containing 20 mM KCl in Dryl's solution (9). The duration of backward swimming induced by this transfer was then measured. The injected cells were kept in the exhausted medium for up to 2 d during which there is no growth, although those committed to cytokinesis would divide once. In cases of successful restoration, both daughters of these rare dividers swim backward for similar durations.

Electrophysiological Recording Techniques

The methods of capturing, rinsing, immobilizing, penetrating, and recording from paramecia were basically those described by Naitoh and Eckert (29). The voltage clamp was similar to that of Machemer (28).

Preparation of Crude Cytoplasmic Fraction

Paramecia (wild-type or pawns) were grown in the improved Cerophyl medium of Hansma (16) and harvested at early stationary phase (1). The cells were then washed twice with Dryl's solution (38) and once with 20 mM Tris pH 7.5, 300 μM P-tosyl-L-argininemethylester, 300 μM phenylmethylsulfonylfluoride and 0.11 U/ml aprotinin. Protease inhibitors were purchased from Sigma Chemical Co. (St. Louis, Mo.). The cell pellet was homogenized by forcing the cells ~10 times through a 10-ml syringe. The resulting cell homogenate was centrifuged at 4°C, 27,000 *g* for 30 min and the supernatant (S_1) further centrifuged at 100,000 *g*, 4°C in a Spinco type 65 rotor (Palo Alto, Calif.). The pellet from this 100,000

TABLE I

Restoration of the Ability to Swim Backward by Microinjections

Donor → Recipient (genotypes)		Number of cells tested	Number of restored recipients	Duration of backward swimming of recipients
				mean SD, seconds
Uninjected 51S	+	36	—	41.4 ± 6.5
51S → 51S	+ → +	12	—	40.0 ± 10.0
51S → d4-500	+ → *pwA*	29	25	21.3 ± 9.6‡
51S → d4-95	+ → *pwB*	17	17	49.9 ± 13.9
51S → d4-580*	+ → *pwC*	6	6	40.8 ± 8.1
uninjected d4-500	pwA	40	—	0.0 ± 0.0
d4-500 → d4-500	*pwA* → *pwA*	47	0	0.0 ± 0.0
d4-94 → d4-500	*pwA* → *pwA*	5	0	0.0 ± 0.0
d4-132* → d4-500	*pwA* → *pwA*	11	0	0.0 ± 0.0
uninjected d4-95	pwB	40	—	0.0 ± 0.0
d4-95 → d4-95	*pwB* → *pwB*	17	0	0.0 ± 0.0
d4-501 → d4-95	*pwB* → *pwB*	11	0	0.0 ± 0.0
uninjected d4-580*	pwC	30	—	3.4 ± 2.7
d4-580* → d4-580*	*pwC* → *pwC*	11	0	3.0 ± 3.6
d4-131* → d4-580*	*pwC* → *pwC*	5	0	7.6 ± 3.0
d4-500 → d4-95	*pwA* → *pwB*	8	8	57.5 ± 18.3
d4-500 → d4-580*	*pwA* → *pwC*	8	8	41.5 ± 11.1
d4-95 → d4-500	*pwB* → *pwA*	12	12	39.0 ± 12.7
d4-95 → d4-580*	*pwB* → *pwC*	6	6	36.0 ± 6.6
d4-580* → d4-500	*pwC* → *pwA*	6	6	35.0 ± 12.4
d4-580* → d4-95	*pwC* → *pwB*	6	6	35.7 ± 12.0

· Duration of backward swimming tested as described in the text.

* Indicates cells grown at 35°C before use as a donor or recipient. All cells are homozygous.

‡ The four cases of unsuccessful restorations are excluded from the calculations.

TABLE II

Complementation by Microinjection and by Genetics in Previously Unclassified Mutants, in Sterile Mutants, and Between Species

Donor → Recipient	Cells restored / Cells tested	Duration of backing	Fraction injected	Cytoplasmic complementation	Genetic* complementation
		sec			
d4-500 → 2-12	3/3	38.7 ± 5.9	C	+	+
d4-500 → 3-37	3/3	35.0 ± 2.8	C	+	+
d4-500 → 1-34	3/3	53.3 ± 23.6	C	+	+
d4-500 → 1-39‡	4/4	47.8 ± 15.7	C	+	+
d4-95 → 2-12	6/6	0.0 ± 0.0	S_1	−	−
d4-95 → 3-37	5/5	0.0 ± 0.0	S_1	−	−
d4-95 → 1-34	5/5	0.0 ± 0.0	S_1	−	−
d4-95 → 1-39‡	3/3	43.3 ± 7.6	C	+	+
d4-580‡ → 2-12	5/5	23.4 ± 3.2	P_2	+	+
d4-580‡ → 3-37	5/5	36.5 ± 12.9	P_2	+	+
d4-580‡ → 1-34	5/5	28.7 ± 3.8	P_2	+	+
d4-580‡ → 1-39‡	6/6	1.8 ± 2.1	C	−	−
d4-500 → 1-26	3/3	42.7 ± 8.7	C	+	NP*
d4-500 → 1-2	3/3	41.0 ± 20.8	C	+	NP
d4-500 → 6-1	3/3	36.8 ± 5.4	C	+	NP
d4-500 → d4-553	6/6	17.8 ± 8.6	S_1	+	NP
d4-95 → 1-26	7/7	0.0 ± 0.0	S_1	−	NP*
d4-95 → 1-2	9/9	0.0 ± 0.0	S_1	−	NP
d4-95 → 6-1	9/9	0.0 ± 0.0	S_1	−	NP
d4-95 → d4-553	8/8	0.0 ± 0.0	S_1	−	NP
d4-580‡ → 1-26	5/5	30.3 ± 9.5	P_2	+	NP
d4-580‡ → 1-2	5/5	44.0 ± 7.6	S_1	+	NP
d4-580‡ → 6-1	5/5	24.2 ± 7.6	S_1	+	NP
d4-580‡ → d4-553	5/5	47.6 ± 5.3	S_1	+	NP
uninjected *P. caudatum*	34	28.0 ± 2.6			
P. caudatum → d4-500	18/22	17.2 ± 9.1§	C	+	NP
P. caudatum → d4-95	3/3	58.3 ± 2.9	C	+	NP
P. caudatum → d4-580‡	5/5	50.8 ± 8.7	C	+	NP
uninjected *Tetrahymena*	20	19.5 ± 2.2			
Tetrahymena → d4-500	40/40	0.0 ± 0.0	C	−	NP

C refers to the injection of a cytoplasm taken directly from the donor cell. Preparation of fractions S_1 and P_2 is described in Materials and Methods.

* Genetic analysis was done as described in the text and in reference 38 by crossing the donor stock to the recipient line with a body-deformation marker and scoring the F_1 phenotype and F_2 segregation. *NP* are cases where crosses are not possible.

‡ Indicates cells grown at 35°C before use as a donor or recipient.

§ The four cases of unsuccessful restoration are excluded from the calculations.

g spin (P_2), the original supernatant (S_1), and the cell homogenate from wild-type or pawn cells were all active in curing pawn cells as described in the test. These fractions were used in place of the cytoplasm taken directly from a donor cell because it allowed for the injection of a large number of cells more quickly and easily.

Mutagenesis, Selection, and Genetic Analysis

Mutagenesis and selection of the new pawn mutations described here was done essentially by the method described in reference 24. Cells were treated with diepoxyoctane (18, 31) at 4 μg/ml for 1 h. X-ray-induced pawns were isolated from cells irradiated with 8 kRad. The methods of obtaining F_1's from conjugation and F_2's from autogamy were those of Sonneborn (38). The behavioral phenotype of cells was determined by standard criteria (24).

RESULTS

Restoration of Membrane Excitability by Microinjection

Wild-type cytoplasm restores the ability to swim backward in the pawns. The cytoplasm from a pawn of the same complementation group cannot restore a recipient but that from a different pawn group can (Fig. 1). The ability to swim backward is quantified by transferring a paramecium from a solution of 4 mM K^+ to one with 20 mM K^+ and timing the duration of backward swimming. A series of experiments on pawn mutants with different degrees of leakiness shows that their remnant Ca^{++} inward current is directly proportional to the duration of backward swimming in this test (Haga and Satow, unpublished observation). Normal paramecia swim backward for ~40 s as do wild types injected with wild-type cytoplasm. Typical pawns do not swim backward in this test (Fig. 2, Table 1). Injection of wild-type cytoplasm or cytoplasmic fraction into a pawn of any of the three complementation groups restores its ability to swim backward for tens of seconds (Fig. 2, Table 1). Cytoplasmic transfer restores the ability of a pawn cell to swim backward within 2 h. The restoration is maximal by 8 h and lasts at least 2 d (Fig. 2).

Electrophysiology of Restored Pawns

The restoration is not due to a simple addition of ions in the cytoplasm but represents a profound change in the membrane excitability of the recipient. Action potentials can be recorded from wild-type paramecia through an intracellular electrode by conventional electrophysiological techniques. The pawn mutants, uninjected or injected with cytoplasm from another pawn mutant of the same complementation group, have no action potentials (25, 30, 34) (Fig. 3 *B*), even when 1 nA of depolarizing current is injected to trigger the action potential.

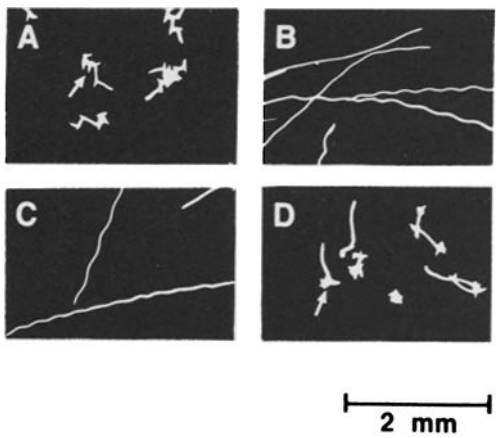

FIGURE 1 Dark-field photographs of the behavioral response of pawn mutants of *P. tetraurelia* after injection with cytoplasm from a different cell. Dark-field photographs are made (7) by putting the cells in a solution of 4 mM $BaCl_2$, 0.75 mM $CaCl_2$, 0.25 mM $Ca(OH)_2$, 1 mM HEPES, 10^{-5} M EDTA. This transfer causes action potentials, and each action potential corresponds to an avoiding reaction (transient reversal of ciliary beat direction) in the wild-type paramecium (*A*) (25). Pawn mutants (d4-500, *pwA*) fail to generate action potentials and have no avoiding reactions (*B*) (25). In a control experiment (*C*) where pawns (d4-500, *pwA*) received cytoplasm from different pawns of the same complementation group (d4-94, *pwA*), they fail to swim backward ~11 h after the injection. However, pawns (d4-500, *pwA*) microinjected with cytoplasm of pawns of a different complementation group (d4-95, *pwB*) can generate the action potentials and avoiding reactions ~11 h after the injection (*D*). Avoiding reactions interrupt the forward swimming and appear as kinks in the track photograph (arrows in *A* and *D*). Duration of tracks, 5 s.

Action potentials can be recorded from the restored recipient and correlated with the return of the ability to swim backward (Fig. 3 *D*). Membrane excitability can be further examined by use of the voltage-clamp technique (30, 33, 34). A step membrane depolarization by the clamp induces a transient Ca-inward current followed by a sustained K-outward current in normal paramecia (10, 35). Such a depolarization does not induce the transient Ca-inward current in a pawn, uninjected, or injected with cytoplasm from another pawn mutant of the same complementation group (Fig. 3 *A*). The same depolarization induces a transient inward current in pawns that have received cytoplasm from other pawns of a different complementation group (Fig. 3 *C*). Although the restored current may be smaller (~5-nA peak) than that of the normal paramecia (~7 nA), the time-course of the rise and fall of the inward current as indicated by the current peak time (~2 msec after the step depolarization) and the voltage at which the maximal inward current is observed (~30 mV more depolarized than the resting level) indicate that the Ca-channels in the restored specimens are normal in their opening and closing kinetics and their voltage sensitivity (30, 34).

Concordance of Genetic Complementation with Cytoplasmic Complementation

There is perfect concordance of genetic complementation and complementation by cytoplasmic transfer through microinjection (Fig. 2, Table I). Stock d4-94, d4-132, and d4-500 are three independently derived pawn mutants previously assigned to the same complementation group, *pwA*, based on their failure to complement in genetic tests (8, 34). Injection of cytoplasm from d4-94 to d4-500 or from d4-132 to d4-500 does not restore backward swimming in the recipient. The same is true of injection between d4-95 and d4-501 which, by genetic analyses, have been assigned to the *pwB* complementation group (8). d4-580 and d4-131 belong to the *pwC* group (8) and both are heat-sensitive, i.e., they are capable of backward swimming when grown at a permissive temperature (23°C) but become very deficient when grown at a restrictive temperature (35°C) (7). When transferred into the standard K^+-test solution, they swim backward for ~3 s (d4-580) or ~7 s (d4-131), after being grown at 35°C. Wild-type paramecia grown at the same temperature respond for ~40 s. Transfer of d4-131 cytoplasm to d4-580 or of d4-580 cytoplasm to d4-580 does not significantly enhance the ability for backward swimming in the recipients.

However, like the cytoplasm of wild-type, the cytoplasm from d4-500 (*pwA*) injected into d4-95 (*pwB*) or d4-580 (*pwC*) restores backward swimming to nearly the untreated wild-type level (~40 s) (Fig. 2, Table I). Injected cytoplasm of d4-95 (*pwB*) cures d4-500 (*pwA*) and d4-580 (*pwC*) to the similar extent, as does injected cytoplasm of d4-580 (*pwC*) to d4-500 (*pwA*) or d4-95 (*pwB*). Control injections of buffer, the cytoplasm from a sister cell of the same pawn clone, or the cytoplasm from a pawn of the same complementation group (Figs. 1 and 3; Table I), do not restore the ability to swim backward in the recipient. Injection of cytoplasm from one

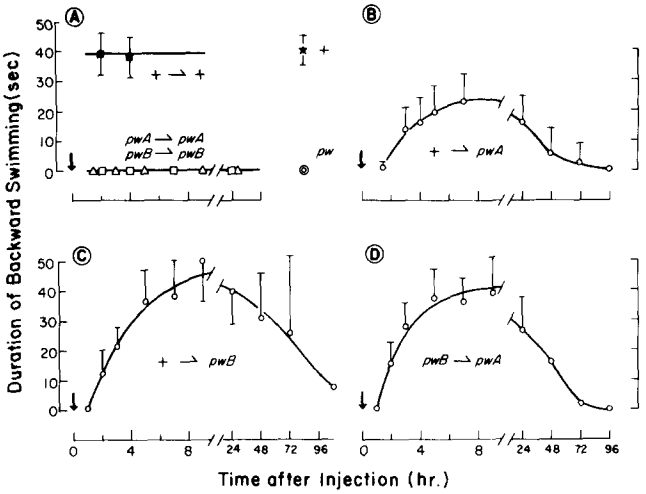

FIGURE 2 Time-course of the restoration of backing after microinjection of the cytoplasm. The recipients are injected at time 0 (arrows), periodically withdrawn from the incubation solution through a small pipette and transferred into a solution containing 20 mM KCl in Dryl's solution. The duration of backward swimming induced by this transfer is measured and plotted over time. Untreated wild type (★) backs up for ~40 s (mean ± SD, n = 10) shown in the center of this figure. Untreated pawns d4-500 (*pwA*) or d4-95, (*pwB*) (◎) have no backward swimming in this test. *A*: Wild types after injection with wild-type cytoplasm swim backward as before (n = 12, ■). Pawns injected with cytoplasm of sister cells from the same stock (*pwA* → *pwA*, n = 47, □; *pwB* → *pwB*, n = 17, △) do not swim backwards. *B* shows that injection of wild-type cytoplasm restores the ability to swim backward in the recipient pawn (*pwA*) cells (n = 29). *C* shows a similar restoration of *pwB* with wild-type cytoplasm, n = 17. *D* shows that the cytoplasm of *pwB* can also restore the ability to swim backward in *pwA* (n = 12). Note that the time-course of gain and loss of the ability of backward swimming is similar in all cases where restoration is successful. This ability peaks by 8 h after injection and lasts for over 2 d.

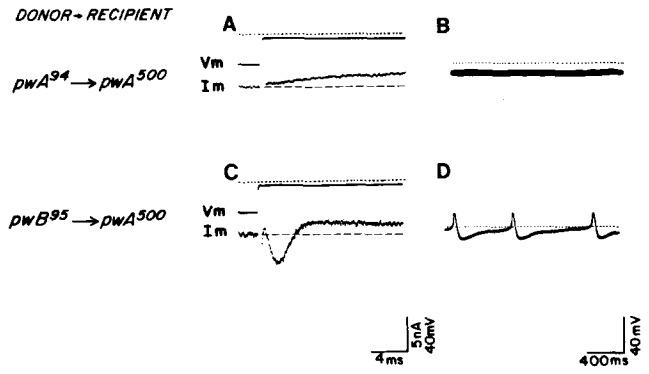

FIGURE 3 Restoration of membrane excitability in pawns by microinjection of cytoplasm. Wild-type paramecia are excitable, i.e., they can generate a Ca-action potential and a step depolarization controlled by a voltage clamp induces a transient Ca-inward current. Typical pawn mutants have no action potential or action current. Recovery of the Ca-current in *pwA* (d4 500) ~8 h after receiving cytoplasm of *pwB* (d4-95) is shown in (*C*). The ability to generate spontaneous action potentials in a Ba-solution is also restored (spikes in *D*). On the other hand, the injection of the cytoplasm from one *pwA* (d4-94) to another *pwA* (d4-500) does not restore the Ca-inward current (*A*) or the action potential in the Ba-solution ~8 h after the injection (*B*). (*A*) and (*C*) are experiments under voltage clamp. The cells are bathed in the standard solution of 4 mM K^+ (see Materials and Methods). Dotted lines are the reference levels for the clamped membrane potential (the *Vm* trace) and the interrupted lines are the zero level for the membrane current (the *Im* trace). Currents below the zero line are inward. (*B*) and (*D*) are recordings of free-running membrane potentials of cells bathed in the solution of 4 mM Ba^{++} (see legend of Fig. 1). Dotted lines are the reference levels for the membrane potential. See Fig. 1 for the corresponding behavior.

wild-type to another does not enhance or impede the backing response of the recipient.

To test further the concordance of standard genetic complementation and complementation by cytoplasmic transfer through microinjection, we classified four mutants by microinjection before performing the genetic analysis. Cytoplasm or cytoplasmic fractions from tester stocks of the three complementation groups was injected into four independent pawn-mutant lines recently isolated in mutagenesis experiments using diepoxyoctane (lines 2–12, 3–37, 1–34) and nitrosoguanidine (1–39) as mutagens (18, 24, 31) (Table II). Cytoplasm or crude cytoplasmic fractions from d4-500 (*pwA*) restores backing ability to all four mutants. Cytoplasm of d4-95 restores the backing ability in line 1–39 but not line 2–12, line 3–37 or line 1–34. Cytoplasm of d4-580 (*pwC*) restores the backing ability in all lines except 1–39. Thus, by microinjection line 2–12, line 3–37, and line 1–34 can be designated *pwB* and line 1–39 *pwC*. Standard genetic analyses confirm these designations (Table II).

Complementation Tests Where Genetics Failed

The above results confirm the validity of using microinjections of tester cytoplasm to classify the genetic defects in the pawn mutants. We now use this method to examine mutants which we cannot analyze successfully by genetic crosses.

Some pawn mutants grow very poorly and are difficult to make mating-reactive. Others fail to form conjugating pairs with any partner or, through the paroral union, form only loose pairs which soon dissolve. Still others form tight pairs but the descendants of the two exconjugants retain their two different original parental phenotypes, indicating that cross-fertilization has not been consummated. These difficulties in mating are presumably due either to the pleiotropic effect of the specific pawn mutations or to other mutations induced by the mutagen treatment. (Because the doses of mutagen used are usually calibrated to give 50% exautogamous death after treatment, it is likely that some mutants have more than one induced defect.)

Four mutant lines (line 1–26, diepoxyoctane induced; line 1–2 and 6–1, x-ray induced; and d4-553, nitrosoguanidine induced [8]) were examined by the injection of cytoplasms or cytoplasmic fractions from the three testers d4-500 (*pwA*), d4-95 (*pwB*), and d4-580 (*pwC*). The lines to be tested are all typical pawns, nonleaky in phenotype and cannot be crossed. Cytoplasm or cytoplasmic fractions of d4-500 or d4-580 restores the ability to swim backward in all four lines 1–26, 1–2, 6–1, and d4–553 but cytoplasm of d4-95 does not (Table II). These results put these lines into the *pwB* complementation group. Shusterman (37) found that *pwB* mutants are also K^+-resistant, i.e., they survive and grow in culture medium with 35 mM KCl added which kills wild-type, *pwA*, and *pwC*. The mutant lines in question as well as 2–12, 3–37, 1–34 and 1–39 were subjected to the K^+-resistance test. All except 1–39 grow in the K^+-enriched medium.

Another situation where standard genetic study cannot be performed is when two different species are involved. With microinjection, however, it is still possible to determine whether cytoplasm from one species contains a gene product that complements mutants of a different species. Cytoplasm of wild-type *P. caudatum* injected into d4-500 (*pwA*), d4-95 (*pwB*) or d4-580 (*pwC*) restores their ability to swim backward with a similar time-course and to a similar extent as does *P. tetraurelia* cytoplasm (Table II). Cytoplasm from wild-type *Tetrahymena*, another holotrichous ciliate, fails to restore the backing ability of d4-500 (*pwA*) in 40 separate injections.

DISCUSSION

The nature of the gene products in the cytoplasm that can restore the backing ability in pawns is now under intensive investigation. Crude fractions can be prepared from wild-type cells that are active in restoring some degree of wild-type behavior to pawn mutants (see Materials and Methods). The presence of the active factors in the microsomal pellet (P_2) suggests that the activity is associated with a macromolecular aggregate. Preliminary experiments indicate that restoration of backward swimming by these fractions can occur even in the absence of protein synthesis. The activity is apparently not sensitive to endogenous or exogenous RNase's but is destroyed by trypsin. It is, therefore, likely that the activity represents membrane proteins existing in a complex in the cytoplasm. If so, the time required for restoration may represent the time for these proteins to be assembled into the proper surface sites. Further fractionation of P_2 is in progress with the aim of isolating the gene products that control the generation of action potentials. The advantage offered by the paramecium system in this case is that these molecules can be isolated in an active form as demanded by the assay.

The fact that the pawns can be temporarily "cured" by a wild-type transfusion indicates either that the mutant gene product in the pawn recipient does not interfere with the function of the injected normal product even when the former is in great excess or that the mutant does not have the gene product in question. That complementation occurs among

pawns of different groups indicates that the "curing" of each defect is independent of the other, i.e., the presence or absence of one mutant product does not interfere with the curing process by the wild-type product of another gene. In addition, it appears that the pawn-gene products of the two *Paramecium* species are similar but different from those of *Tetrahymena*.

Mutants of the three pawn groups may not be "cured" to the same extent. Pawn A's sometimes fail to respond to the injection or respond to a lesser degree (Tables I and II, Fig. 2). In *P. caudatum*, only the "*cnr C*" mutation is cured by the injection of wild-type cytoplasm whereas "*cnr A*" and "*cnr B*" are not (17).

As described here, the method of cytoplasm microinjection used to classify mutants is superior to the standard genetic crosses because it is rapid, straightforward, and does not depend on the mating and fertilization process of the cells. A cross from two parent paramecia stocks to autogamous F_2's takes ~2 wk to perform and includes such maneuvers as obtaining mating-reactive cells of opposite mating types, isolating mating pairs, cloning exconjugants, daily isolation of F_1's, cytological examination for the occurrence of autogamy, cloning F_2's, and phenotypic scoring. On the other hand, microinjection of 10 paramecia can be done in 1 h. Less than 8 h total are needed for injection and testing of behavioral restoration.

This method has been used systematically in the pawn system because (*a*) paramecia are large cells making microinjection relatively easy, (*b*) the mutants respond to the injected gene-product, and (*c*) the phenotype (backing) can be readily and repeatedly tested. Other mutants in paramecium appear not to be cured by injection of wild-type cytoplasm. In *P. tetraurelia*, a mutation in an axonemal component (unpublished observations) and some trichocyst mutants cannot be cured by this technique. However, other trichocyst mutants have been shown to be cured by the injection of wild-type cytoplasm (2, 4, 13). Thus, for this method to be used in other systems, especially other animal cells in culture, the choice of a responsive system and a proper test for the restored phenotype is important. As microinjection becomes a routine cell-biological technique for many cell types (4, 6, 19, 39), its use in circumventing standard genetics may become important.

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