RESTORATION OF MEMBRANE EXCITABILITY IN A BEHAVIORAL MUTANT OF *PARAMECIUM CAUDATUM* DURING CONJUGATION AND BY MICROINJECTION OF WILD-TYPE CYTOPLASM

KOICHI HIWATASHI, NOBUYUKI HAGA, and MIHOKO TAKAHASHI. From the Biological Institute, Tohoku University, Sendai 980, and the Institute of Biological Sciences, University of Tsukuba, Ibaraki 300–31, Japan

ABSTRACT

When cells of the behavioral mutant *cnrC* of *Paramecium caudatum* were mated with the wild type, phenotypic change from CNR (no backward swimming) to wild type in the *cnrC* mate occurred immediately after the formation of tight pairs. No change of phenotype occurred when cells of *cnrA* or *cnrB* were mated with wild type. Phenotypic change from CNR to wild type in cells of *cnrC* was also induced by microinjection of wild-type cytoplasm. Microinjection of wild-type cytoplasm induced no change in cells of *cnrA* or *cnrB*.

Phenotypic change in the *cnrC* mate during conjugation can be explained by cytoplasmic exchange during conjugation, though transfer of membrane sites for excitability through membrane fluidity cannot be ruled out.

KEY WORDS *Paramecium* · behavior · mutants · conjugation · microinjection

When paramecia encounter physical or chemical stimuli, they show a reversal of the direction of the effective stroke of the cilia. This ciliary reversal is caused by the Ca^{2+} action potential across the membrane (6, 7). Behavioral mutants of *Paramecium* that are unable to show ciliary reversal and, thus, are incapable of swimming backward in the face of a stimulus were first found in *P. tetraurelia* (14, 15, 19) and later in *P. caudatum* (21, 22). These recessive mutations are called 'Pawn' in the former species and 'CNR' in the latter. They have a defect in membrane excitability (16, 22). In *P. caudatum*, CNR mutants are divided into three complementation groups and, thus, are controlled by three genes at different loci: *cnrA*, *cnrB*, and *cnrC*.

In the cell-to-cell union of conjugation between the wild type and *cnrA* or *cnrB*, no change of phenotype in the CNR mates occurs throughout the period of conjugation. In conjugation between the wild type and *cnrC*, however, a change in phenotype from CNR to wild type occurs as early as 1 h after the formation of tight conjugating union (21). A similar change from mutant to wild-type phenotype during conjugation has been reported in the Pawn (*pwA*) of *P. tetraurelia* by Berger (3). He interpreted the phenomenon to be the result of extensive cytoplasmic exchange between mates during conjugation.

In this paper, we report in some detail the change of the CNR phenotype during conjugation and also the change induced by microinjection of cytoplasm from wild-type cells. Implications of the latter method for future analysis of membrane excitation are also discussed.

MATERIALS AND METHODS

P. caudatum, syngen 3, was grown in fresh lettuce juice medium (10). A day before inoculation with paramecia, the medium was bacterized with a strain of *Klebsiella pneumoniae* (formerly *Aerobacter aerogenes*). The wild-type strains used were Kyky-1 (mating type V) and d119a (mating type VI). The CNR mutants used were three of *cnrA/cnrA*, four of *cnrB/cnrB*, and six of *cnrC/cnrC*, and the double CNR mutants used were one each of *cnrA/cnrA–cnrC/cnrC* and *cnrB/cnrB–cnrC/cnrC*. For identification of behavior, cells previously adapted to adaptation medium (1 mM KCl, 1 mM $CaCl_2$ in 1 mM Tris-HCl, pH 7.2) were gently released in a small volume of test solution containing 20 mM KCl, 1 mM $CaCl_2$ in 1 mM Tris-HCl, pH 7.2. In some experi-

J. CELL BIOLOGY © The Rockefeller University Press · 0021-9525/80/02/0476/05 $1.00
Volume 84 February 1980 476–480

ments, 25 mM KCl in Dryl's solution (4) was used instead of the test solution described above.

Microinjection of cytoplasm ($5–10 \times 10^3$ μm^3) was performed with a Chambers micromanipulator with a single needle after the method of Koizumi (12). Before microinjection the cells to be injected were placed in immobilization solution (Dryl's solution containing 0.5% (wt/vol) methylcellulose). Care was taken not to draw the macronucleus, which is easily observable under the microscope, into the needle. Because the needle is filled with liquid paraffin when it penetrates the donor plasma membrane and cortex, it is unlikely that the microinjection method transplants intact pieces of plasma membrane or of cortex. Even if intact pieces were transplanted, their amount was estimated to be less than 1/500 of the total volume of injected materials.

RESULTS

Behavior of Conjugating Pairs between Wild-type and CNR Mutants

When wild-type conjugating pairs were transferred into the test solution, which contains a high concentration of K^+ and a low concentration of Ca^{2+}, they swim backward rapidly for a short time, reversing the direction of their ciliary beats. This reaction to the test solution is totally lacking in conjugating pairs of CNR mutants. When heterotypic pairs of wild-type and CNR cells are transferred into the test solution, they whirl or swim slowly backward in large spirals. This is the result of the reversing of the cilia of the wild-type cell but not of the cilia of its CNR mate. Wild-type cells of one mating type were mixed with CNR cells of the complementary mating type, and, 2 h later, when tight pairs (paroral union) had formed, more than 200 heterotypic pairs were identified by their behavior in the test solution. These were isolated, pooled, and kept in the adaptation medium. Every 2 h thereafter, samples of about 30 pairs were tested for their swimming behavior in the test solution.

When cells of *cnrA* or *cnrB* were paired with wild-type cells, no change of phenotype was observed through the entire period of conjugation. When cells of *cnrC* were mated with wild-type cells, however, a phenotypic change of the CNR mate to wild type occurred as early as 2 h after the formation of tight pairs. Subsequently, heterotypic pairs of *cnrC* and wild type behave like wild-type pairs. The time course of phenotypic change is shown in Fig. 1. Three different strains of *cnrC* were tested, and, in every case, the behavioral phenotype of all *cnrC* mates changed to wild type within 5 h after the formation of tight pairs. The expression of wild-type phenotype in the CNR mate continued even after the separation of conjugating pairs, and, thus, all exconjugants showed wild-type phenotype immediately after separation of pairs. From 10 to 15 hours after the separation, some exconjugants returned to the CNR phenotype, but the number of exconjugant cells returning to the CNR phenotype never exceeded 20%. This suggests that most exconjugant cells expressed the phenotype of F_1 progeny without returning to the parental phenotype. To confirm that the exconjugant cell population contains 50% cells of CNR parentage, they were treated with antiserum homologous to the wild-type strain but heterologous to the CNR strain. Exactly 50% of the cells were immobilized by the antiserum treatment, which proves that the heterotypic pairs studied consisted of a CNR and a wild-type cell and were not contaminated with selfing wild-type pairs.

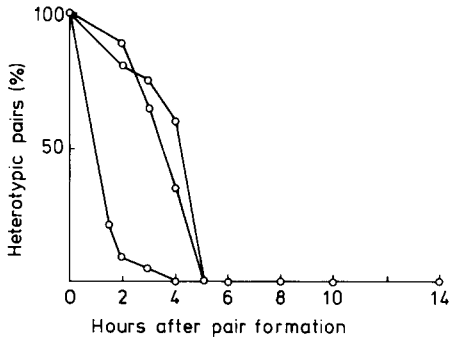

FIGURE 1 Phenotypic change from CNR to wild type during conjugation between *cnrC* and wild-type cells. Abscissa: time in hours after the formation of tight conjugating pairs (paroral union); ordinate: percent of pairs showing heterotypic pair phenotype when tested in a high K^+ solution. Lines show phenotypic changes in three different *cnrC* strains, 16D108, 16D102, and 16D104, paired with wild-type strain d119a.

A similar experiment was performed with heterotypic conjugating pairs consisting of wild-type and double CNR mutant cells. When the CNR cell was a double homozygote of *cnrA* and *cnrC* or *cnrB* and *cnrC*, no change of CNR phenotype was observed during conjugation. Thus, expression of either *cnrA* or *cnrB* during conjugation is epistatic to *cnrC*.

Behavior of Conjugating Pairs Consisting of Different CNR Mutants

To determine whether the phenotypic change from *cnrC* to wild type also occurs when *cnrC* cells

are mated with *cnrA* or *cnrB* cells, which should contain normal products of the *cnrC* locus, the behavior of *cnrA–cnrC* and *cnrB–cnrC* pairs was also examined during conjugation. Both types of pairs showed the CNR phenotype when tested immediately after the formation of tight pairs. In about 1 h, however, a few pairs began to swim backward in large spirals or whirl, which is the typical characteristic of heterotypic pairs. This suggests that the *cnrC* mate reversed its ciliary beat. In 4 h, more than 60% of the pairs showed the heterotypic pair characteristic (Fig. 2). When *cnrA* cells were mated with *cnrB*, no change of phenotype occurred throughout conjugation.

Microinjection of Wild-type Cytoplasm into CNR Cells

In conjugation between the behavioral mutant Pawn (*pwA*) and wild type, Berger (3) reported that extensive cytoplasmic exchange often occurs, leading to the phenotypic change of Pawn to wild type. Inasmuch as formation of a cytoplasmic continuity between two conjugating cells through openings of 0.2–0.5 μm has been reported in *P. caudatum* (23), the same interpretation as Berger's can be given to the change of CNR phenotype to wild-type phenotype during conjugation. If the phenotypic change of the *cnrC* during conjugation is induced by cytoplasmic exchange between mates, microinjection of wild-type cytoplasm into cells of *cnrC* may be expected to produce change of the *cnrC* phenotype to wild-type. To verify this prediction, 5,000–10,000 μm^3 of cytoplasm from wild-type cells was injected into cells of *cnrA*, *cnrB*, and *cnrC*. When cytoplasm of wild-type cells was injected into cells of *cnrA* or *cnrB*, no change of phenotype occurred, but when injected into cells of *cnrC*, a change of the CNR to wild type was observed in nearly 50% of the injected cells (Table I). The phenotypic change began to appear about 3 h after injection in a small number of cells. About 5 h after injection, many injected cells showed backward swimming when tested in the high K^+ solution. The injected cells continued to express the changed phenotype as long as 24 h after injection (Fig. 3) and then gradually returned to the CNR phenotype. As the control, cytoplasm of *cnrC* was injected into cells of the same *cnrC*. No change of the CNR phenotype was observed (Table I). The duration of the backward swimming

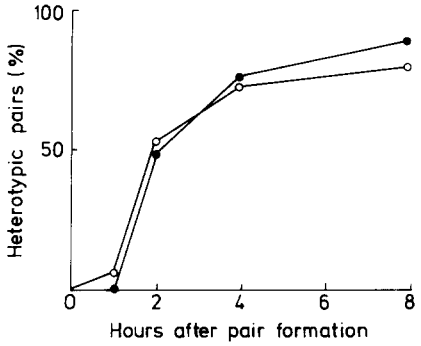

FIGURE 2 Phenotypic change from CNR to wild type during conjugation between cells of *cnrC* and *cnrA* or *cnrB*. Abscissa and ordinate are the same as in Fig. 1. Circle, pairs between cells of *cnrA* (16A101) and *cnrC* (16D202). Solid circle, pairs between cells of *cnrB* (16B102) and *cnrC* (16D203).

TABLE I

*The Effect of Cytoplasm from Cells of Various Genotypes Injected into Cells of CNR Mutants**

Donor cell	Recipient cell	No. of cells injected‡	No. of cells surviving injection	No. of cells showing ciliary reversal§	Percent of surviving cells showing reversal
Wild type	*cnrA*	61	43	0	0
Wild type	*cnrB*	82	47	0	0
Wild type	*cnrC*	45	38	17¶	44.7
cnrA	*cnrC*	30	17	8	47.0
cnrB	*cnrC*	27	19	11	57.9
cnrC	*cnrC*	53	43	0	0

* Injected cells were kept in adaptation medium that contains 1 mM KCl and 1 mM $CaCl_2$ in 1 mM Tris-HCl, pH 7.2, and the test for ciliary reversal was made by temporarily transferring cells into test medium that contains 20 mM KCl and 1 mM $CaCl_2$ in 1 mM Tris-HCl, pH 7.2, at various times from 2 to 72 hours after injection. Temperature, 25 ± 0.5°C.

‡ Volume of injected cytoplasm was 5,000–10,000 μm^3.

§ Total number of cells showing ciliary reversal when tested.

¶ Mean duration of ciliary reversal was 20.0 ± 4.4 s, whereas that of wild-type cells was 60.2 ± 9.9 s.

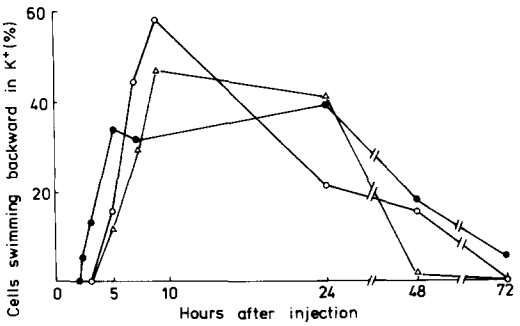

FIGURE 3 Change to, and retention of, wild-type phenotype in *cnrC* cells injected with wild-type, *cnrA*, or *cnrB* cytoplasm. Ordinate: percent of injected cells that show backward swimming in a high K^+ solution. Recipient: *cnrC* (16D313). Donors: solid circle, wild type (Kyky-1); circle, *cnrB* (16B101); triangle, *cnrA* (16A101).

was measured every time the injected *cnrC* cells showed wild-type phenotype in the test solution and was compared with that of the wild-type cells that had been used as the donor of cytoplasm. Mean duration of backward swimming of the injected and phenotypically changed cells was 20.0 ± 4.4 s, whereas that of the wild-type cells used as the donor was 60.2 ± 9.9 s at 25°C.

Microinjection of Cytoplasm from cnrA or cnrB into cnrC Cells

As described, when *cnrA* or *cnrB* cells were mated with *cnrC*, the presumed *cnrC* mates showed wild-type phenotype, suggesting that the phenotype of *cnrC* cells would change to wild type if they were injected with cytoplasm from *cnrA* or *cnrB*. Cytoplasm from *cnrA* or *cnrB* was injected into cells of *cnrC*. Almost the same results were obtained as when cytoplasm of wild-type cells was injected (Table I, Fig. 3), clearly showing that cells of *cnrA* and *cnrB* contain materials capable of repairing the defect caused by the *cnrC* allele.

DISCUSSION

The CNR mutants in *P. caudatum* are known to have defects in their membranes because Triton-extracted models behave almost like the models of the wild type (21). Two alternative mechanisms for the phenotypic change of the CNR mates to wild-type phenotype during conjugation with wild-type cells can be postulated: membrane-membrane interaction by the cell contact of conjugation, and exchange of cytoplasm through the contact region. Membrane-membrane interaction has been reported in fused cells between cell lines of mouse and human origin (8) in which specific surface antigens spread and intermix within minutes after membrane fusion. In *Paramecium*, membrane fusion in the region where cytoplasmic connnections between mates are formed has often been reported (20, 23), and, in *P. caudatum*, this occurs immediately after the formation of tight pairs (paroral union).[1] Thus, transfer of normal membrane sites through membrane fluidity cannot be ruled out. In this interpretation, however, normal membrane sites of wild-type cilia have to be transferred through the fused portions to the ciliary membrane of the mutant because membrane excitability in *Paramecium* is known to be located in the ciliary membrane (5, 18). In *Tetrahymena*, it has been reported that ciliary membranes are less fluid than other membranes isolated from the same cells (17). The lipid composition of cilia has been compared with that of deciliated cells or whole cells in *Paramecium* (1, 11), and the fatty acid composition of the ciliary lipids suggests that the ciliary surface membrane in *Paramecium* is more fluid than the membrane of the deciliated cell body because the former contains larger amounts of unsaturated fatty acids than the latter, though comparison of the sterol content has not been reported. However, to know exactly whether this fluidity of the ciliary membrane makes for rapid intermixing of ciliary membrane components for excitability, further study, probably with membrane labeling, will be necessary.

The other interpretation, i.e., that the phenotypic change is caused by repair of the mutational lesion by wild-type cytoplasm migrating through the cytoplasmic connection, is supported by the results of the injection of cytoplasm. In *P. tetraurelia*, Berger (3) reported that, by the end of conjugation, 73% of recipient cells showed at least 30% of the label concentration of the donor when [^{3}H]leucine-labeled cells were mated to nonlabeled cells. In *P. caudatum*, the amount of cytoplasm exchanged during conjugation is unknown, but, inasmuch as injection of 5,000 μm^3 of cytoplasm, which is about 1.6% of total cell volume, was effective, a small amount of cytoplasmic exchange may be enough to change the mutant phenotype. Why about 50% of the injected cells did not change their phenotype is unknown. One of the possible causes may be leakage of the injected cytoplasm

[1] T. Watanabe, Tohoku University. Personal communication.

after injection.

Why cells of *cnrA* and *cnrB* do not change their phenotype during conjugation with wild type or following injection of wild-type cytoplasm is still unknown. The fact that conjugation between *cnrA* or *cnrB* and *cnrC* induces phenotypic change in the latter rules out the possibility that cytoplasmic exchange does not occur between *cnrA* or *cnrB* and wild type. Presumably, the amount of normal gene product introduced into cells of *cnrA* or *cnrB*, either by cytoplasmic exchange during conjugation or by injection, is insufficient to change the phenotype. Alternatively, the mutational defect in *cnrC* may be easily repaired by a small amount of normal gene product, but those of *cnrA* and *cnrB* may not be. Another possibility is that the normal functions of the ion-gating system require some diffusible factor(s), which may be the *cnrC* product, in addition to the structural elements, which may be the products of *cnrA* and *cnrB*.

In *Paramecium* genetics, microinjection has proven to be a powerful tool to identify specific gene products (9, 13) or to determine effective sites of mutations (2). We expect that the same technique will make possible the identification of molecules involved in membrane excitation in *Paramecium*.

We thank Dr. James D. Berger for his critical reading of the manuscript. This work was supported in part by Grant-in-Aid for Special Project Research, 'Mechanisms of animal behavior,' to K. Hiwatashi and M. Takahashi from the Ministry of Education, Science and Culture, Japan.

Received for publication 2 September 1979, and in revised form 2 November 1979.

REFERENCES

1. ANDREWS, D., and D. L. NELSON. 1979. Biochemical studies of the excitable membrane of *Paramecium tetraurelia*. II. Phospholipids of ciliary and other membranes. *Biochim. Biophys. Acta.* **550**:174–187.
2. AUFDERHEIDE, K. J. 1978. The effective sites of some mutations affecting exocytosis in *Paramecium tetraurelia*. *Mol. Gen. Genet.* **165**:199–205.
3. BERGER, J. D. 1976. Gene expression and phenotypic change in *Paramecium tetraurelia* exconjugants. *Genet. Res.* **27**:123–134.
4. DRYL, S. 1959. Antigenic transformation in *Paramecium aurelia* after homologous antiserum treatment during autogamy and conjugation. *J. Protozool.* **6**:s25 (Abstr.).
5. DUNLAP, K. 1977. Localization of calcium channels in *Paramecium caudatum*. *J. Physiol. (Lond.).* **271**:119–133.
6. ECKERT, R. 1972. Bioelectric control of ciliary activity. *Science (Wash. D. C.).* **176**:437–481.
7. ECKERT, R., and Y. NAITOH. 1972. Bioelectric control of locomotion in the ciliates. *J. Protozool.* **19**:237–243.
8. FRYE, L. D., and M. EDIDIN. 1970. The rapid intermixing of cell surface antigens after formation of mouse-human heterokaryons. *J. Cell Sci.* **7**: 319–335.
9. GAROFALO, R. S., J. K. C. KNOWLES, and B. H. SATIR. 1978. Restoration of secretory capacity in a non-discharge *Paramecium* mutant by microinjection of wild type cytoplasmic factor(s). *J. Cell Biol.* **79**(2, Pt. 2): 245 *a* (Abstr.).
10. HIWATASHI, K. 1968. Determination and inheritance of mating type in *Paramecium caudatum*. *Genetics.* **58**:373–386.
11. KANESHIRO, E. S., L. S. BEISCHEL, S. J. MERKEL, and D. E. RHOADS. 1979. The fatty acid composition of *Paramecium aurelia* cells and cilia: changes with culture age. *J. Protozool.* **26**:147–158.
12. KOIZUMI, S. 1974. Microinjection and transfer of cytoplasm in *Paramecium*. *Exp. Cell Res.* **88**:74–78.
13. KOIZUMI, S., and S. KOBAYASHI. 1976. A study on the mate-killer toxin by microinjection in *Paramecium*. *Genet. Res.* **27**:179–185.
14. KUNG, C. 1971. Genic mutants with altered system of excitation in *Paramecium aurelia*. I. Phenotypes of the behavioral mutants. *Z. Vgl. Physiol.* **71**:142–164.
15. KUNG, C. 1971. Genic mutants with altered system of excitation in *Paramecium aurelia*. II. Mutagenesis, screening and genetic analysis of the mutants. *Genetics.* **69**:29–45.
16. KUNG, C., and Y. NAITOH. 1973. Calcium-induced ciliary reversal in the extracted models of "Pawn," a behavioral mutant of *Paramecium*. *Science (Wash. D. C.).* **179**:195–196.
17. NOZAWA, Y., H. IIDA, H. FUKUSHIMA, K. OHKI, and S. OHNISHI. 1974. Studies on *Tetrahymena* membranes: temperature induced alteration in fatty acid composition of various membrane fractions in *Tetrahymena pyriformis* and its effect on membrane fluidity as inferred by spin-label study. *Biochim. Biophys. Acta.* **367**:134–147.
18. OGURA, A., and K. TAKAHASHI. 1976. Artificial deciliation causes loss of calcium-dependent responses in *Paramecium*. *Nature (Lond.).* **264**: 170–172.
19. SCHEIN, S. J. 1976. Nonbehavioral selection for pawns, mutants of *Paramecium aurelia* with decreased excitability. *Genetics.* **84**:453–468.
20. SCHNEIDER, L. 1963. Elektronenmikroskopische Untersuchungen der Konjugation von *Paramecium*. I. Die Auflösung und Neubildung der Zellmembran bei den Konjuganten. *Protoplasma.* **56**:109–140.
21. TAKAHASHI, M. 1979. Behavioral mutants in *Paramecium caudatum*. *Genetics.* **91**:393–408.
22. TAKAHASHI, M., and Y. NAITOH. 1978. Behavioral mutants of *Paramecium caudatum* with the defective membrane electrogenesis. *Nature (Lond.).* **271**:656–569.
23. VIVIER, E., and J. ANDRÉ. 1961. Données structurales et ultrastructurales nouvelles sur la conjugaison de *Paramecium caudatum*. *J. Protozool.* **8**:416–426.