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

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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.

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. For identification of behavior, cells previously adapted to adaptation medium (1 mM KCl, 1 mM CaCl₂ in 1 mM Tris-HCl, pH 7.2) were gently released in a small volume of test solution containing 20 mM KCl, 1 mM CaCl₂ in 1 mM Tris-HCl, pH 7.2. In some experi-
ments. 25 mM KCl in Dryl's solution (4) was used instead of the test solution described above.

Microinjection of cytoplasm (5–10 x 10^6 pin') 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 Ca²⁺, 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 F₁ progeny without returning to the parental phenotype. To confirm that the exconjugant cell population contains 50% cells of CNR parentage, they were treated with antisera homologous to the wild-type strain but heterologous to the CNR strain. Exactly 50% of the cells were immobilized by the antisera 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.

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 μm³ 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](https://example.com/figure2.png)

**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**

| 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 CaCl₂ 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 CaCl₂ 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 μm³.

§ 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 1, 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 connections 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 [3H]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 µm³ 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.

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