BEHAVIORAL MUTANTS IN *PARAMECIUM CAUDATUM*

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ABSTRACT

Mutants of *Paramecium caudatum* with abnormal swimming behavior or responses to cations were obtained by mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine. Some of the mutants, like pawn in *P. tetraurelia*, cannot swim backward and are called CNR. Seven independently obtained CNR mutants belonged to three complementation groups, designated as *cnrA*, *cnrB* and *cnrC*. Some characteristics of double homo- and heterozygotes were compared with single homo- and heterozygotes. Other behavioral mutants shown to have a genic basis included K^+-sensitive, temperature-shock behavioral and slow swimmer. All those mutants except for slow swimmer had lesions in the membrane because Triton-extracted models of them show almost the same swimming behavior as wild type.

PARAMECIUM is one of the classic materials for the study of behavior in unicellular organisms (JENNINGS 1906). The behavior of Paramecium is controlled by the direction, frequency and three-dimensional features of the beating of cilia that cover the whole surface of the cell. The direction and frequency of the ciliary beat are correlated with changes in electrical potential across the cell membrane (NAITOH and ECKERT 1974). Since the pioneer work by KAMADA (1934), *P. caudatum* has been used in many studies that are important for understanding the physiological mechanisms of behavior (KINOSITA, DRYL and NAITOH 1964a,b; KINOSITA, MURAKAMI and YASUDA 1965; NAITOH and ECKERT 1968a,b, 1969).

KUNG (1971a,b) initiated a new approach to understanding the mechanisms of behavior in Paramecium using *P. tetraurelia* (formerly *P. aurelia* syngen 4, SONNEBORN 1975), long the favorite material of protozoan genetics. KUNG and his colleagues have screened over 350 lines of behavioral mutants that map at over 20 genetic loci. Most of them produce defects in membrane functions. The work was recently reviewed by KUNG *et al.* (1975). Further extensive electrophysiological analyses of the class of behavioral mutants called pawn have recently been reported (SCHEIN, BENNETT and KATZ 1976; SATOW and KUNG 1976; OERTEL, SCHEIN and KUNG 1977).

Many techniques, such as nickel paralysis (KUZNICKI 1963), demembranated modelling (NAITOH and KANEKO 1972), deciliation and reciliation procedures (DUNLAP 1976; OGURA and TAKAHASHI 1976) and all the electrophysiological

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methods (NAITOH and ECKERT 1974) including voltage clamping, (ECKERT, NAITOH and MACHEMER 1976) were first developed in the study of *P. caudatum* and many data have been accumulated. Because of its large size, *P. caudatum* can withstand the trauma of electrode penetration better than *P. tetraurelia.* Therefore, *P. caudatum* remains the favorite subject for electrophysiological research. Though *P. caudatum* has less advantage for genetic study than *P. tetraurelia* because of frequently encountered high mortality after conjugation and the lack of autogamy, accumulated physiological and cell-biological results in wild type can fully and directly be applied to the behavioral mutants if they are obtained in *P. caudatum*. However, there has been no attempt to generate such mutants in this species. The work reported in this paper fills this vacuum.

The genetics of *P. caudatum* has also suffered from a lack of convenient genetic markers. The behavioral mutants I have isolated will be useful as markers to advance the genetics of this species as counterparts have in the genetics of *P. tetraurelia.*

In the present article, I will report the mutagenesis, screening, phenotypic and genotypic analyses of behavioral mutants in *P. caudatum.*

MATERIALS AND METHODS

Cell strains: All strains used belong to *P. caudatum*, syngen 3. Those used for mutagenesis and for cross breeding analyses are listed in Table 1.

Culture methods: The culture medium used was either Cerophyl medium (SONNEBORN 1970) or fresh lettuce juice medium (HIWATASHI 1968). Cerophyl grass powder (Cerophyl Laboratories, Inc.), 2.5 gm in 1 l of double-distilled water, was boiled for five min, filtered through a sheet of filter paper (#40) after cooling, supplemented by 0.3 gm Na_2HPO_4 and autoclaved for 15 to 20 min. For fresh lettuce juice medium, fresh lettuce leaves were washed and immersed in boiling water for a few seconds; the juice was then obtained by homogenizing in a Waring blendor and by filtering through cheesecloth. One kg of lettuce leaves make 2 l of juice. The juice was sterilized at 100° for 15 min on three successive days. The juice was diluted 1:40 with sterilized Dryl's solution (DRYL 1959). A day before inoculation with paramecia, both media were bacterized with a strain of *Klebsiella pneumoniae* (formerly *Aerobacter aero-*

TABLE 1

Stocks or strains of P. caudatum

Stocks or strains	Mating type	Source
For mutagenesis		
Kok-1	V	Natural stock
Kyky-1	V	Derived from crosses between natural stocks, Ksy-1 and Ky
dK20a	VI	A progeny from selfing of natural stock Ky
d^m-3	VI	A progeny from the cross between natural stocks, Kok-1 and Koj
Kyk201	VI	Derived from crosses between natural stocks, Ksy-1 and Ky
MK124	VI	Derived from crosses among natural stocks, Kok-1, Koj, Ky and Ksy-1
For crosses		
Kyky-1	V	Derived from crosses between natural stocks, Ksy-1 and Ky
dKK14a	V	Derived from crosses between natural stocks, Ksy-1 and Ky
d^m-11	V	Derived from crosses between natural stocks, Kok-1 and Koj

genes). The Cerophyl medium was mainly used for culturing single cells (including exconjugants) and the lettuce juice medium was used for mass culture and for inducing mating reactivity.

Mutagenesis: Slow centrifugation of log phase cells yielded 1 ml containing 60,000 cells to which 1 ml of freshly prepared 100 μg per ml N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, Wako Chemicals) and 8 ml of Dryl's solution were added. The final concentration of MNNG was 10 μg per ml. Cells were treated in this solution for 40 minutes in the dark and then washed three times with Dryl's solution. The washed cells were transferred into 450 ml Cerophyl culture medium, which was immediately divided into about 30 test tube cultures. After about 12 hr, approximately 500 single cells from each tube were isolated into fresh culture medium in disposable trays and grown for several days until the cultures cleared, that is in the stationary phase. When the cultures cleared, selfing pairs appeared. Portions containing many selfing pairs from 30 to 50 cultures were pooled. They were left without adding fresh culture medium for a few days. After this period of starvation, cells were transferred into a large volume of fresh culture medium and allowed to go through five to ten fissions. The cultures were then ready to be screened for mutants. In earlier experiments, cross conjugation was used instead of selfing, because selfing strains with a high postconjugational survival were not yet established. When cross conjugation was used, two strains of complementary mating types were mutagenized and then mixed to induce mating. The rest of the process of handling mutagenized cultures was almost the same as when selfing pairs were used. Crossing decreases the possibility of obtaining recessive homozygotes, but nevertheless it proved to be useful. Probably, recessive homozygotes were recovered from rare instances of self-fertilization (cytogamy).

Crosses: Cross-breeding analysis was made difficult by poor survival after conjugation, especially in the F_2 generations. In all crosses and selfings, pairs and exconjugants were isolated into exhausted culture medium made from the Cerophyl culture or from lettuce juice culture after TAKAHASHI and HIWATASHI (1970) and then transferred into fresh culture medium about on day later. In *P. caudatum*, this process is necessary to avoid the occurrence of macronuclear regeneration (MIKAMI and HIWATASHI 1975).

Selection of behavioral mutants: When paramecia are placed in the path of a galvanic current, they swim toward the cathode. The pattern of swimming to the cathode changes as the current increases; first straight forward, then forward in large spirals, and finally backward swimming frequently interrupted by transient forward swimming. This change of pattern is clear especially when the cells were cultured in lettuce juice medium. Cells that have an altered electrical response may show different galvanotactic swimming patterns. Every cell that showed an abnormal response or swimming behavior in various strengths of electrical field was isolated and grown to establish a clone. Galvanotactic screening was made with a plastic vessel (135 mm long, 18 mm wide) containing 4 mM KCl and 1 mM $CaCl_2$ in 1 mM Tris-HCl (pH 7.2). Though the method was effective, I have not tested its efficiency.

I also used the same method as that of CHANG and KUNG (1973), chemotactic inhibition of geotaxis, with a slight modification. CHANG and KUNG used a high concentration of Na^+ and a relatively low concentration of Ca^{2+} to fill a column, while I used a high concentration of K^+ (16 mM) instead of Na^+, because the cells cultured in lettuce juice culture medium were rather insensitive to Na^+.

Besides these screening methods, a solution of tetraethylammonium chloride (TEA) was used for selecting mutants. Wild-type cells in 4 mM TEA solution show slow dispersion with repeated avoiding reactions (backward swimming with ciliary reversal). Cells that showed rapid dispersion or no avoiding reaction were picked up.

RESULTS

Altogether more than 650,000 cells were mutagenized in eight independent experiments and 23 different clones that showed abnormal swimming behavior or response were obtained (Tables 2 and 3). Among those 23 abnormal clones,

TABLE 2

Mutagenesis and behavioral mutants screened

Muta-genesis	Mutagenized strains	No. of cells treated	Conjugation used for recovering mutants	No. of clones selfed / Total clones isolated	No. of mutants obtained	Strains of mutants
1	dK20a and Kok-1*	65,000	cross	—	1	10A
2	d^m-3 and Kok-1*	57,000	cross	—	2	9G, 9Z
3	Kok203 and Kok-1*	40,000	cross	—	3	11B, 11C, 11F
4	Kyky-1	35,000	cross†	—	1	12B
5	MK124	300,000	cross†	—	2	15A, 15C
6	Kyk201	55,000	selfing	335/8,000	4	13A, 13B, 13C, 13D
7	Kyk201	50,000	selfing	1,700/4,300	1	14A
8	MK124	60,000	selfing	4,414/12,000‡	9	16A, 16B, 16C, 16D, 16E, 16F, 16H, 16I, 16J
Total		650,000			23	

* Both strains were mutagenized and then crossed.
† Mutagenized strains were crossed to nonmutagenized strains.
‡ Survival after selfing of nonmutagenized MK124 was more than 80%; after mutagenesis 22.6%.

TABLE 3

Strains and phenotypes of the behavioral mutants in P. caudatum

Class of mutant	Strains	Behavioral phenotypes
CNR (caudatum nonreversal)	10A*, 13B*, 14A*, 16A*, 16B*, 16C*, 16D*	Cannot swim backward
FR (frequently reverse)	11B, 12B	Frequent spontaneous avoiding reaction
K^+ sensitive	13D*	Long backward swimming in K^+ solution
Ba^{2+} sensitive	13C	Long backward swimming in Ba^{2+} solution
Zigzag swimmer	9G	Frequent stops during forward swimming
Spinner	15A	Spin swimming instead of backward swimming
Temperature-shock behavioral	13A*	Repeated backward swimming frequently when temperature was raised rapidly
Fast swimmer	9Z, 11F, 15C, 16F, 16H	Swim faster than wild-type
Slow swimmer	11C*, 16E	Swim slower than wild-type
TEA insensitive	16I, 16J	Show no or weak avoiding reaction to TEA solution

* Strains on which genetic analysis has been performed.

ten were confirmed to be true genic mutants by genetic analysis, four were presumed to be genic mutants by indirect evidence, and nine were not diagnosed as to whether they were genic or somatic mutants.

Character of the behavioral mutants: Responses of the ten true and three putative genic mutants to three different cationic solutions are shown in Table 4. As shown in Tables 3 and 4, 10A, 13B, 14A, 16A, 16B, 16C and 16D are called 'CNR' which means *P. caudatum* nonreversal. Their characteristics in general are very much like "pawn" in *P. tetraurelia* reported by KUNG (1971a,b). When they were transferred into test solutions, such as high K^+, Na^+ or Ba^{2+} solutions, they failed to swim backward (Table 4), though 14A was leaky and responded to K^+ and Ba^{2+} solution for a few seconds. 16D was also slightly leaky and responded to K^+ solution by whirling, although it showed no backward swimming. 10A and 13B were tested for their behavior in galvanotaxis. They showed a smooth and fast swimming to the cathode in a strong electric field.

KUNG and NAITOH (1973) reported that the ATP-Mg reactivated Triton-extracted models of "pawns" in *P. tetraurelia* show backward swimming in the presence of Ca^{2+} above 10^{-6} M, and concluded that the pawn phenotype is due to an impairment in the membrane and not in the calcium-sensitive motile system.

TABLE 4

Mean duration (seconds) of ciliary reversal in various mutants of P. caudatum *in a variety of test solutions**

Strains	20 mM KCl 1 mM $CaCl_2$†	20 mM NaCl 1 mM $CaCl_2$†	4 mM $BaCl_2$ 1 mM $CaCl_2$†
Wild type			
Kyky-1	55.6 ± 7.9‡	< 2	12.3 ± 2.4
Kyk201	43.6 ± 4.8	< 2	12.3 ± 4.9
Mutant			
10A (CNR)	0	0	0
13B (CNR)	0	0	0
14A (CNR)	8.9 ± 3.0	0	6.0 ± 1.4
16A (CNR)	0	0	0
16B (CNR)	0	0	0
16C (CNR)	0	0	0
16D (CNR)	0§	0	0
13D (K^+ sensitive)	255.6 ± 31.0	3.6 ± 1.4	34.2 ± 7.0
13C (Ba^{2+} sensitive)	65.9 ± 7.8	3.1 ± 1.6	102.0 ± 17.5
13CD (double mutant derived from 13C × 13D)	132.9 ± 14.0	13.5 ± 3.0	86.8 ± 7.7
13A (Temp.-shock behavioral)	70.1 ± 12.8	7.0 ± 3.7	22.9 ± 11.6
11B (FR)	78.6 ± 3.1	< 2	28.9 ± 2.7
9Z (fast)	31.7 ± 2.5	< 2	5.8 ± 1.5
11C (slow)	26.3 ± 6.1	< 2	35.1 ± 12.7

* Adaptation medium; 1 mM KCl and 1 mM $CaCl_2$ in 1 mM Tris-HCl buffer (pH 7.0).
† In 1 mM Tris-HCl buffer (pH 7.0).
‡ Mean ± confidence limit.
§ Whirling in seconds but no backward swimming.

Triton-extracted models of CNR mutants were prepared according to NAITOH and KANEKO (1972). The models were reactivated to swim in the solution containing 4 mM ATP, 4 mM $MgCl_2$, 3 mM EGTA and 50 mM KCl in 10 mM Tris-maleate buffer (pH 7.0). When about 50 models each from the CNR mutants were transferred into the above medium, they swam forward, but when they were transferred into the solution containing 5×10^{-5} M (0.05 mM) $CaCl_2$ instead of 3 mM EGTA, every model prepared from the seven different CNR mutants showed backward swimming almost in the same way as wild type. The results shows that all CNRs have some defect in the membrane and not in the motile system.

Genetic analysis of CNR, F_1 and F_2: When CNR mutants were crossed to wild-type stocks, F_1 survival was first checked using a variety of different wild-type strains. A combination of mutant and wild-type strains producing relatively high F_1 survival was selected to get F_1 data. Since very few F_1 clones produced enough F_2 progeny, they had to be selected for production of sufficient F_2 progeny. Natural selfing was mostly used to produce F_2 progeny because clones of mating type VI in *P. caudatum* easily self naturally (MYOHARA and HIWATASHI 1975). When natural selfing failed to occur because all surviving F_1 progeny were mating type V (*e.g.*, 10A × Kyky-1), mating-reactive F_1 progeny were mixed with mating-reactive CNR of mating type VI, and F_1 selfing pairs behaving as wild-type pairs were selected on the basis of their reaction to a high K^+ solution (20 mM KCl). In *P. caudatum*, mating mixtures produce not only cross pairs but also selfing pairs (HIWATASHI 1951). In a high K^+ solution, F_1 selfing pairs with wild-type phenotype swim straight backward, while CNR selfing pairs swim forward and cross pairs show spiral and rhythmic backward swimming. In the cross 13B × Kyky-1, sibling crosses of F_1 were used to obtain the F_2 because survival of the natural selfing progeny was very poor.

Table 5 gives the results of crosses between seven different CNR clones and wild-type strains. All F_1 progeny showed normal behavior, and F_2 progeny obtained by selfing conjugation or sibling crosses of F_1 gave segregation of CNR to wild type with ratios not significantly different from 1:3 in every cross. The results show that the phenotypes of all seven CNR mutants are controlled by recessive alleles.

Complementation and number of loci in CNR: Using the F_2 CNR segregants, interstrain crosses were carried out to determine the number of loci controlling the CNR phenotype. In this complementation experiment between different CNR strains, isolation of conjugating pairs was not performed. About 20,000 mating reactive cells of an F_2 CNR clone were mixed with nearly the same number of mating-reactive cells of another F_2 CNR expressing the complementary mating type. After confirming that large mating clumps were at the bottom of the test tube used for mixing, the upper two-thirds of the medium was discarded and the lower portion, which contained mainly large clumps, was examined four hours later for conjugating pairs. If it contained many pairs, it was kept without supplying fresh culture medium for two days, letting the cells go through conjugation and postconjugation processes normally. Then, the cells

TABLE 5

Phenotypic segregation of F_1 and F_2 progenies from crosses between various CNRs and wild-type strains

Crosses	Number of F_1 (all wild type)	Survival percent* of F_1	Segregation in F_2 (theoretical ratio) CNR	wild-type	Chi-square and *P*-value	Survival percent* of F_2
10A × Kyky-1	3	4.3	1	4	$\chi^2 = 0.37$†	11.9
			(1.3	: 3.7)	$p = 0.5–0.7$	
13B × Kyky-1	85	60.7	4	15	$\chi^2 = 0.01$†	22.6
			(4.8	: 14.2)	$p = 0.9–0.95$	
14A × Kyky-1	45	64.0	18	34	$\chi^2 = 1.15$	41.6
			(13	: 39)	$p = 0.2–0.3$	
16A × d^m-11	25	32.9	15	39	$\chi^2 = 0.11$	42.9
			(13.5	: 40.5)	$p = 0.7–0.8$	
16B × dKK14a	16	23.9	2	3	$\chi^2 = 0.04$†	4.2
			(1.3	: 3.7)	$p = 0.8–0.9$	
16C × dKK14a	12	40.0	4	13	$\chi^2 = 0.08$†	13.9
			(4.3	: 12.7)	$p = 0.8–0.9$	
16D × dKK14a	32	75.2	11	24	$\chi^2 = 0.37$	27.3
			(8.8	: 26.2)	$p = 0.5–0.7$	

* Percent of synclones, including those in which only one clone of a synclone survived.
† For the calculation of χ^2, YATES' correction was applied.

were grown for two days in fresh culture medium, during which time they underwent an average of about four fissions. At least two tube cultures were made for each combination of clones. Phenotypic diagnosis was made with the test solution containing 20 mM KCl in Dryl's solution (containing 1.5 mM $CaCl_2$). If a sample from the test tube contained cells showing clear backward swimming in the test solution, the combination of clones was judged to contain CNR genes that complement each other. If at least two independent tests yielded no cells showing backward swimming, the two clones in the combination were judged to carry CNR genes at the same locus. As shown in Table 6, F_2 segregants from seven parental CNR strains were divided into three complementation groups; those from 10A, 13B, 16A and 16C, from 16B and 14A, and from 16D. The results show that CNR mutants obtained in these experiments were classified into three groups and are consistent with the interpretation that they are conrolled by at least three genes at different loci. The locus controlling the group of 10A, 13B, 16A and 16C is designated *cnrA*, that of 16B and 14A *cnrB*, that of 16D *cnrC*.

An F_2 segregant, 16D106, of the cross between 16D and a wild-type complemented not only with clones of *cnrA* and *cnrB*, but also with sister segregants from the same CNR parent, 16D. Why this one of 11 F_2 segregants of 16D behaved exceptionally is unknown. Though some F_1 were obtained when 16D106 was crossed to wild-type, no selfing progeny have been obtained. Whether this exceptional segregant suggests a fourth locus or not will be discussed later.

Double homozygotes and heterozygotes of CNR: To understand the interactions between genes at different CNR loci, double homozygotes were looked

TABLE 6

*Results of crosses between different strains of CNR mutants**

	Parental CNR	F_2 segregant	10A	16C	14A	16B	16D		
			10A-212	16C-103†	14A-101	16B-101	16D-102	16D-107	16D-108
cnrA	10A	10A-204		—		+			
	13B	13B-101	—	—	+	+	+	+	
	16A	16A-104		—		+	+		
		-117	—	—	+	+	+		
	16C	16C-104	—	—	+	+		+	
cnrB	14A	14A-111	+		—		+		
		-115	+	+		—			+
	16B	16B-102	+	+	—	—			
cnrC	16D	16D-101			+	+	—		
		-104	+	+		+		—	—
		-115	+	+	+	+		—	—

* — means no wild-type progeny in at least two separate complementation tests; + means having wild-type progeny.

† Cells genotypically mating type VI but phenotypically mating type V were used (see HIWATASHI and MYOHARA 1975).

for among progeny from crosses between various combination of double heterozygotes. Wild-type clones of complementary mating types obtained when the complementation tests were done were crossed because they should be double heterozygotes at two different CNR loci. Progeny of the crosses were tested with a high K^+ solution and those expressing the CNR phenotype were selected. The latter clones were crossed to the two parental CNR strains that were used in the cross for the complementation experiment. If progenies of the crosses to both parental CNRs yielded no wild-type recombinants, the clone was identified as a double homozygote.

When clones of the double heterozygotes *cnrA/+;cnrB/+* were crossed, all progeny with the CNR phenotype (62 clones) were *cnrB/cnrB* for some unknown reason. No double homozygotes and no *cnrA/cnrA* clones were recovered. When heterozygous clones of *cnrA/+;cnrC/+* were crossed, 11 clones showing the CNR phenotype were isolated, but only one of them proved to be the double homozygote, *cnrA/cnrA;cnrC/cnrC*. When clones of *cnrB/+;cnrC/+* were crossed, 74 clones of CNR were isolated and 25 of them proved to be the double homozygote, *cnrB/cnrB;cnrC/cnrC*. Deficiencies in the expected *cnrA/cnrA;cnrB/cnrB* and *cnrB/cnrB* classes in the first set of crosses, and the excess of *cnrB/cnrB;cnrC/cnrC* class in the third cross were probably due to the method of selecting CNR segregants, because isolation of conjugating pairs was not performed in those crosses and selection might occur before isolation of the CNR. Double homozygotes thus obtained were compared with single homozygotes and wild type for reactions to K^+ and Ba^{2+}. Qualitative results are shown in Table 7. Although a clear difference was observed between wild type and CNR mutants, no clear difference in the reaction to K^+ was observed between single and double

TABLE 7

Sensitivities to K^+ and Ba^{2+} of single and double homozygotes of various CNRs

Strains	Reaction to K+* 7.5 mM	15 mM	30 mM	Reaction to Ba2+† Time in minutes of treatment 15	30	60
Wild type (MK 124)	short B	long B	long B	repeated B	+++	+++
cnrA (13B–102)	N	W	W	N	±	±
(10A–201)	N	S	W	N	S	±
(16A–107)	N	S	W	N	±	+
cnrB (16B–101)	S	S	W	S	±	++
cnrC (16D–101)	S	W	short B	S	±	+
(16D–111)	N	S	W	S	S	±
cnrA-cnrC (F3–2)	N	S	S	N	S	±
cnrB-cnrC (L1–13)	N	S	W	N	S	+
(L3–9)	N	S	S	N	S	±

* In 0.5 mM Ca^{2+} and 1 mM Tris-HCl (pH 7.2–7.4).
† 15 mM Ba^{2+}, 10 mM K^+, 0.5 mM Ca^{2+} and 1 mM Tris-HCl (pH 7.2–7.4).
N, normal swimming; B, backward swimming; S, slow swimming; W, whirling; ±, less than 1%; +, 10–20%; ++, about 50%; and +++, more than 80% lethal in about 100 cells.

homozygotes of CNR. Though not shown in Table 7, many F_2 segregants of *cnrC* tend to become sensitive to K^+ when they are grown for a long period. In this respect, the *cnrC* trait is different from both *cnrA* and *cnrB*. Some difference of sensitivity to Ba^{2+} was observed between loci and between single and double homozygotes. Clones of *cnrB/cnrB* appear more sensitive and clones of double homozygotes more resistant to Ba^{2+} than other single homozygote clones, though some clonal differences among clones of the same CNR genotype were also observed. SCHEIN (1976) reported that not only rapid phenotypic separation of pawns from wild type, but also separation of different pawn genes is made possible by the response to Ba^{2+}. The present results suggest the same possibility.

Since no clear difference of the reaction to K^+ was observed between single and double homozygotes of CNR, single and double heterozygotes were compared. Cells in the stationary phase (one day after final feeding) were washed in a solution consisting of 1 mM KCl, 1 mM $CaCl_2$ and 1 mM Tris-HCl (pH 7.2–7.4) and then equilibrated for 30 to 60 min in this solution. Equilibrated cells were transferred into the test solution consisting of 20 mM KCl, 1 mM $CaCl_2$ and 1 mM Tris-HCl (pH 7.2–7.4). Average duration of backward swimming in the test solution was obtained, with 12 independent measurements each of 25 single and 20 double heterozygotes. Though considerable intra- and interclonal variation was observed, statistically significant differences were confirmed not only between wild type and single heterozygotes clones, but also between those of single and double heterozygotes (Table 8). Mean duration of backward swimming in single heterozygotes was about 10% shorter than that in wild type and about 20% longer than that in double heterozygotes. The difference between single and double heterozygotes suggests that the mutant genes are not com-

TABLE 8

Responses to high K^+ solution of single and double CNR heterozygotes

	n	$\bar{x}$	u^2
Wild type	9	64.53*	79.19
cnrA/+	9	55.19	57.23
cnrB/+	9	51.73	10.18
cnrC/+	7	65.03	39.02
Total single heterozygotes	25	56.70	62.58
cnrA/+;*cnrB*/+	11	49.86	26.25
cnrA/+;*cnrC*/+	5	48.22	29.54
cnrB/+;*cnrC*/+	4	48.17	15.51
Total double heterozygotes	20	48.11	20.61

* Duration of backward swimming in sec. in 20 mM K^+, 1 mM Ca^{2+} when transferred from the adaptation medium (1 mM K^+, 1 mM Ca^{2+} in 1 mM Tris-HCl, pH 7.2).

n; Number of different clones tested. Wild type *versus* single heterozygotes; $u_1^2/u_2^2 = 1.27$ ($F_{0.025} = 2.78$), $t = 2.47$, significant at $p < 0.05$ level. Single heterozygotes *versus* double heterozygotes; $u_1^2/u_2^2 = 3.04$ ($F_{0.025} = 2.47$), by COCHRAN's approximation $t = 4.57$ [$t'_{(0.05)} = 2.08$] significant at $p < 0.05$ level.

pletely recessive and that effects of different loci upon the CNR phenotype are additive at least between *cnrA* or *cnrB* and *cnrC*.

Expression of the CNR phenotype during conjugation: In *P. tetraurelia*, BERGER (1976) reported that in conjugation between wild type and pawn extensive cytoplasmic exchange often occurs during conjugation and leads to immediate change of phenotype in the pawn mate to wild type without the usual phenotypic lag. In the conjugation between wild type and *cnrA* or *cnrB*, no change of phenotype in the CNR mates was observed during the periods of conjugation and early postconjugation. In the conjugation between wild type and *cnrC*, change of phenotype from CNR to wild type occurred in all conjugating pairs as early as one hour after the formation of true conjugating pairs (paroral union) (HIWATASHI and TAKAHASHI, unpublished). When either *cnrA/cnrA*;*cnrC/cnrC* or *cnrB/cnrB*;*cnrC/cnrC*, was crossed to wild-type strains, the result was the same as in the case when the single homozygote *cnrA/cnrA* or *cnrB/cnrB* was crossed to wild-type strains. In this respect, features of *cnrA* and *cnrB* during conjugation are epistatic to those of *cnrC*.

Other behavioral mutants: Among 16 other strains that showed abnormal swimming behavior or responses to physical or chemical stimuli, only three, 11C, 13A and 13D, have been genetically analyzed and confirmed to be true genic mutants. Results of genetic analyses of 11C and 13D have been reported (TAKAHASHI and NAITOH 1978). The 11C trait, slow swimmer, is controlled by a recessive allele, *sl* and the 13D trait, K^+ sensitive, is controlled by a dominant allele, *Ks*. The K^+ sensitive mutant 13D showed migration to the anode

with backward swimming in a weak electric field. The trait of 13A, temperature-shock behavior, is its extraordinarily high sensitivity to rapid elevation of environmental temperature. Cells of 13A respond to rapid elevation of temperature, of 10° or more, with repeated backward swimming. This repetition of backward swimming often continues for more than five minutes. The response to the temperature shift of this mutant differs from that of other so-called temperature sensitive mutants in Paramecium, where the lag between temperature shift and phenotypic response is measured in hours. Such response has never been observed in wild-type strains. This trait is controlled by a recessive allele (*tsb*). A detailed description of the character and the result of genetic analysis of this mutant will be presented elsewhere.

Genetic analyses of the other 13 mutants have not been performed, and we do not know whether they are genic or nongenic variants. However, they reproduce true to type for many fissions, and some of their characters can be described. 11B and 12B are called FR (frequently reverse) because their frequency of spontaneous avoiding reaction is noticeably higher than that of wild-type strains. Not only frequency, but also duration of backward swimming in K^+ and Ba^{2+} solutions by 11B is significantly different from wild type (Table 4). The Ba^{2+} sensitive strain 13C is characterized by its extraordinarily high sensitivity to Ba^{2+}. When cells of 13C were transferred to a solution containing 4 mM $BaCl_2$ and 1 mM $CaCl_2$, they immediately swim backward for more than 100 seconds and then show a "barium dance" (repetition of forward and backward swimming, DRYL 1961) with a short forward and a very long backward swimming. Attempts to obtain an F_1 by crossing to wild type have so far failed, but when 13C was crossed to the K^+ sensitive mutant 13D, F_1 clones with high sensitivities to both K^+ and Ba^{2+} were obtained (13CD in Table 4). This suggests the possibility that the 13C trait is also controlled by a dominant allele. The zigzag swimmer 9G trait is transient; it also frequently stops forward swimming somewhat like the "Atalanta" mutant in *P. tetraurelia* (KUNG *et al.* 1975). The mutational lesion of Atalanta is known not to be in the membrane, but in the motile system of cilia. Unlike the case of Atalanta, however, Triton-extracted models of 9G behave almost like wild type either in the presence of Ca^{2+} or in its absence. This shows that zigzag swimming of 9G is due to some membrane lesion. The spinner 15A trait is spinning for a few seconds instead of the usual backward swimming when cells meet a solid obstacle. Probably this is the same kind of mutant as spinner in *P. tetraurelia* (KUNG *et al.* 1975; SCHEIN 1976). Wild-type cells show repeated avoiding reactions in the solution consisting of 4 mM TEA, 1 mM $CaCl_2$ and 1 mM Tris-HCl (pH 7.2). The TEA-insensitive mutants, 16I and 16J, show no avoiding reaction to the above solution and are probably the same kind of mutant as TEA^+-insensitive in *P. tetraurelia.*

DISCUSSION

By treating exponentially growing populations of *P. caudatum*, syngen 3, with the mutagen MNNG and then inducing selfing or cross conjugation, 23 clones with significant behavioral alterations were isolated by galvanotactic,

geotactic and chemical (TEA) screening methods. While many mutants or variants with subtle behavioral differences were also found, they were discarded because mutants without clear-cut and dependable phenotypes are often difficult for genetic analysis. Among the 23 clones with clear behavioral alterations, ten have been genetically analyzed and shown to be true genic mutants. Seven of them are CNR mutants that did not respond to mechanical or cationic stimuli with backward swimming. In every examined character, the CNR mutants closely resemble pawn in *P. tetraurelia*. KUNG and his colleagues have isolated over 60 independent pawn mutants including some temperature-sensitive pawns, which were assigned to three unlinked loci, *pwA*, *pwB* and *pwC* (KUNG 1971b; KUNG *et al.* 1975). SCHEIN (1976) also isolated seven pawn mutants falling into the three complementation groups of KUNG (1971b). In the present study, seven independent CNR mutants were isolated and assigned to three different loci, *cnrA*, *cnrB* and *cnrC*. As mentioned in the RESULTS, an F_2 segregant of the cross between the *cnrC* mutant and wild type complemented not only with clones of *cnrA* and *cnrB*, but also with sister segregants of *cnrC*. The fact that only one of 11 F_2 segregants of the *cnrC* original mutant belongs to a different group suggests that an additional CNR gene is contained in the original mutant. Another possibility is that the abnormal segregant might be a heterokaryon whose macronuclear genotype is *cnrC/cnrC* but whose micronuclear genotype is *cnrC/+*, because such a clone would complement all tested strains. This possibility can be tested by cross-breeding analysis of the abnormal segregant.

The ease of obtaining the double homozygotes, *cnrA/cnrA;cnrC/cnrC* and *cnrB/cnrB;cnrC/cnrC* suggests that *cnrC* is unlinked to *cnrA* or *cnrB*. Whether *cnrA* is linked to *cnrB* is not known, although I failed to obtain the double homozygote.

Extensive electrophysiological studies have been done with pawns in *P. tetraurelia*. They have made clear that the defect of these mutants is a malfunctioning of the Ca channels (KUNG and ECKERT 1972; SCHEIN, BENNETT and KATZ 1976; OERTEL, SCHEIN and KUNG 1977). Only one CNR mutant of *P. caudatum*, 13B (cnrA/cnrA), has so far been analysed electrophysiologically. It fails to produce the regenerative calcium action potential in response to a depolarizing current (TAKAHASHI and NAITOH 1978). In all of the seven independent CNR mutations, ATP-reactivated Triton-extracted models act like wild-type in that they reverse their orientation of ciliary beat when the Ca^{2+} concentration in the reactivation medium is raised above 5×10^{-6} M. This indicates that the mechanism of ciliary reversal is normal, but that the calcium gating system in the membrane of CNR has some defect. In this respect, the CNRs are essentially the same kind of mutant as pawn in *P. tetraurelia* (KUNG and NAITOH 1973). Some differences in phenotype among three different pawn loci are reported (CHANG and KUNG 1973; CHANG *et al.* 1974; SCHEIN 1976; SCHEIN *et al.* 1976). Among the three loci, the expression of *pwA* is often leaky, while that of *pwB* is rather stable. Temperature-sensitive pawns have been isolated of *pwA* and *pwC*, but not of *pwB*. Mutants of *pwB* are more resistant of barium toxicity than *pwA* mutants, and defective anomalous rectification is observed

only in *pwB* mutants. From these phenotypic differences between *pwA* and *pwB*, SCHEIN proposed a hypothesis that *pwA* mutants are defective in the depolarization-sensitive "gate" function and *pwB* mutants in the "pore" function of the Ca^{2+} channels (SCHEIN 1976; SCHEIN, BENNETT and KATZ 1976). In the CNR mutants reported here, some differences in phenotype among three different loci were also observed. In the tests of reactivity to a high K^+ solution, the expressions of *cnrA* and *cnrB* are stable, but that of *cnrC* tends to be leaky when clones are aged. Mutants of *cnrB* are less resistant to barium toxicity than those of *cnrA* and *cnrC*. Expression of the mutant phenotype during conjugation with wild-type cells is stable in *cnrA* and *cnrB*, but unstable in *cnrC*. It is suggestive that both in pawns in *P. tetraurelia* and in CNRs in *P. caudatum*, three different mutant loci with differentiatable phenotypes have been discovered. In the present study, however, it is difficult to find a one-to-one correspondence between the pawns and the CNRs. It is noteworthy that every mutant of *pwC* so far isolated is either conditional or "partial" (KUNG *et al.* 1975; SCHEIN 1976), while all mutants of three CNR loci so far obtained are neither conditional (TAKAHASHI and OHWADA, unpublished) nor "partial" in the same sense as SCHEIN (1976).

It is worthy of mention that in the CNR 13B analyzed electrophysiologically, the mechanoreceptor potential remains normal irrespective of an almost complete lack of the regenerative action potential that usually follows mechanical stimulation of the anterior membrane (TAKAHASHI and NAITOH 1978). This makes possible an analysis of the mechanoreceptor system independently of the voltage sensitive calcium gating system.

The K^+ sensitive mutant 13D overreacts to K^+ with prolonged backward swimming and shows a more marked increase in K^+ permeability when tested electrophysiologically (TAKAHASHI and NAITOH 1978). Paranoiac in *P. tetraurelia* is known to overreact to Na^+ with prolonged backward movement (KUNG 1971a). SATOW, HANSMA and KUNG (1976) reported that both the active and passive electrophysiological properties of the membrane in the paranoiac, *PaA*, appear normal in K^+ solution. In this respect, the K^+-sensitive mutant in *P. caudatum* is qualitatively different from *PaA* in *P. tetraurelia*. No mutant of *P. caudatum* that overreacts to Na^+ has been discovered. This is probably because a Na^+ solution has not been used for screening mutants in the present study.

Recessive mutants overreacting to Ba^{2+} that fall into three unlinked loci have been isolated in *P. tetraurelia* (KUNG, personal communication). Genetic analysis of the Ba^{2+} sensitive mutant in *P. caudatum*, 13C has largely failed due to poor survival after conjugation, but indirect evidence (see RESULTS) suggests that the Ba^{2+}-sensitive phenotype 13C is controlled by a dominant gene.

Slow swimmer 11C is the mutant with a probable defect in the motile system instead of a lesion in membrane regulation. In the unicellular alga, *Chlamydomonas reinhardi*, a variety of motility mutants having defects in the flagellar axonemal system has been reported (RANDALL *et al.* 1964; WARR *et al.* 1966; MCVITTIE 1972; GOODENOUGH and ST. CLAIR 1975, and others). Whether any

fine structural abnormality can be found in the motile system of 11C remains for future study.

JENNINGS (1906) reported that paramecia moving toward a hotter or colder region show a more frequent avoiding reaction than in an adapted temperature, and TAWADA and MIYAMOTO (1973) also noted that a sudden temperature change increases the frequency of change in the swimming direction. Such change of swimming direction is usually so subtle that only close observation can detect it. In agreement with OLIPHANT (1938), wild-type cells do not show any remarkable increase of avoiding reaction upon sudden transfer to higher temperatures. The temperature shock behavioral mutant 13A responded to rapid elevation of temperature with frequent avoiding reactions, which continued for an extraordinarily long time. Although the cause of this altered excitability is still unknown, there is a possibility that the mutant has some abnormality in the thermoreception system. Since little is known about the thermoreception mechanism in unicellular eukaryotes, this mutant may be useful for analysis of that mechanism.

Artificial induction of Paramecium conjugation among cells of a single mating type by certain chemical solutions is called the chemical induction of conjugation (MIYAKE 1968; HIWATASHI 1969). CRONKITE (1976) reported that conjugation of pawn in *P. tetraurelia* can be induced by the ordinary mating reaction, but cannot be induced chemically and suggested that fertilization events in Paramecium cannot proceed under conditions unless an increase in internal Ca^{2+} concentration can occur. Preliminary experiments showed that most CNR mutants were not only capable of mating-type induced conjugation, but also of chemically induced conjugation, providing that they are in highly mating-reactive condition. Since the conditions necessary for chemical induction of conjugation parallel those that stimulate ciliary reversal in Paramecium (low concentration of Ca^{2+} relative to some other cation like K^+ or Na^+), this line of work is promising for understanding the control of mating behavior with the help of ion physiology. Extensive analysis of mating behavior and conjugation processes with the behavioral mutants reported in this paper is under way and will be reported later.

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