GENETIC ANALYSES OF "PARANOIAC" MUTANTS OF *PARAMECIUM TETRAURELIA*

JUDITH VAN HOUTEN*, SHENG-YUNG CHANG AND CHING KUNG

Laboratory of Molecular Biology and Department of Genetics, University of Wisconsin, Madison, Wisconsin 53706

Manuscript received October 18, 1976
Revised copy received December 28, 1976

ABSTRACT

Six mutants of *Paramecium tetraurelia* with curious "Paranoiac" phenotypes have been isolated and examined. Instead of the normal transient avoiding reactions in Na+ solutions, these mutants show "violent avoidances"—backing continuously for 10 to over 60 sec. This behavior corresponds to prolonged membrane excitation.—Genetic analyses establish five genic loci at which mutations give the "Paranoiac" phenotype. Close linkage between two of these genes occurs. Allelic variants are found for two of the genes. In one case, the two alleles determine very different behavioral phenotypes ("Paranoiac" and "fast-2"). These results show that the mechanism(s) which shuts off excitation in the wild-type membrane is (are) complex, but in the future may be fruitfully pursued in mutants which are defective.

SWIMMING and avoiding reactions are the main components of the behavior of Paramecium. JENNINGS (1906) first described the avoiding reaction as transient backing away from a stimulus, turning, and renewed forward swimming in a random direction.

Direction of ciliary beat, hence, direction of swimming, is correlated with the membrane potential in Paramecium. Forward and backward swimming are associated with the resting potential and active depolarization of the membrane respectively (KINOSITA, MURAKAMI and YASUDA 1965). Active depolarization (the Ca action potential) causes a transient increase in internal concentration of Ca^{++}, which reverses the beating direction of cilia, hence causing backward swimming (NAITOH and ECKERT 1969; NAITOH, ECKERT and FRIEDMAN 1972). Observation of behavior, therefore, is a convenient way to monitor the activity of the membrane.

We have succeeded in finding mutants of *P. tetraurelia* that are altered in their avoiding reactions (KUNG 1971a; KUNG *et al.* 1975). In most of these mutants, the behavioral changes are due to altered membrane electric properties. One type of such membrane mutant is called "Paranoiac." These mutants show avoiding reactions, but often the backward swimming is not transient but prolonged. Electrophysiological studies have shown that, instead of showing the transient active depolarization characteristic of wild-type paramecia during the avoiding

* Department of Biological Sciences, University of California, Santa Barbara, Santa Barbara, California 93106.

reaction, Paranoiacs' membranes often remain depolarized for up to a minute or more (SATOW and KUNG 1974).

We present here the genetic analyses of all the "Paranoiacs" of *P. tetraurelia* isolated to date.

MATERIALS AND METHODS

Strains: All strains belong to *Paramecium tetraurelia* (previously *P. aurelia*, species 4; see SONNEBORN 1975). The subjects of this paper are six lines of mutants having the "Paranoiac" behavioral phenotype. They were isolated from mutagenized populations through one of two screening procedures (see below). Mutants previously reported were also used in the breeding analyses. They are d4–90, the "Paranoiac A" mutant studied by KUNG (1971b); d4–94, "pawn A"; d4–95, "pawn B"; d4–91, "fast-2"; and d4–93, a behaviorally normal, body-deformation mutant used as a genetic marker.

Cultures: Paramecia were cultured in Cerophyl media inoculated with *Aerobacter aerogenes* 24 hours before use (SONNEBORN 1970). Clones were often incubated at 28° to speed up growth. All other experimental procedures were performed at room temperature (23 ± 1°).

Behavioral tests: To determine the behavioral phenotype of a clone, we first observed the behavior of the cells in the culture medium. Wild-type paramecia swim forward, often stop, or give relatively slow and transient avoiding reactions (JENNINGS 1906). "Paranoiac" mutants exhibit all this behavior as well as spontaneous backward swimming for up to 60 sec. The "Paranoiac" behavior could be enhanced by jarring the culture vessel or passing the culture through a pipette.

We could also classify a given clone by the immediate reaction of its members when transferred from culture medium to a test solution. Transfers were made with a micropipet and the behavior was monitored with a stereo-microscope. Wild-type paramecia gave repeated avoiding reactions when transferred to a Na-test solution (20 mM NaCl, 0.3 mM $CaCl_2$, 1 mM Tris, pH 7.2 adjusted with HCl). "Paranoiacs" swam backward continuously for over 15 sec.

Other behavioral mutants ("pawns" and "fast-2") were used in various crosses. Their diagnostic features have been given elsewhere (KUNG 1971b; CHANG and KUNG 1976).

Mutagenesis: We induced mutations by adding N-methyl-N'-nitro-N-nitrosoguanidine to cultures about 20 fissions after the last autogamy (SONNEBORN 1970; KUNG 1971b). After this treatment, autogamy was again induced and 5 to 10 fissions were allowed for phenotypic lag before we screened the population for "Paranoiac" mutants.

Screening methods

A. *Column method*: Procedures exploiting the behavioral differences between wild-type and mutant paramecia in salt-solution columns have been developed to isolate mutants lacking avoiding reaction in that salt solution (KUNG 1971a; CHANG and KUNG 1973, 1976). A modified column method was used here to isolate the "Paranoiac" mutants (VAN HOUTEN, CHANG and KUNG 1973). Paramecia from mutagenized populations were injected into the top of a glass column filled with a solution of high Ca++ concentration (8 mM $Cacl_2$, 2 mM KOH, 1 mM citric acid, 1mM Tris, 0.5% sucrose pH 7.2 or 10 mM $CaCl_2$, 1 mM Tris, 0.5% sucrose, pH 7.2). After 8–10 minutes, an aliquot was drawn from the bottom of the column. Cells in this aliquot were cloned individually and further scrutinized for behavioral abnormalities.

B. *Galvanotaxis method*: This method of screening for galvanotactic variants has been briefly described elsewhere (VAN HOUTEN, CHANG and KUNG 1973). We set up an electric field in a trough of Dryl's solution (1 mM Na_2HPO_4, 1 mM NaH_2PO_2, 2 mM Na citrate, 1.5 mM $CaCl_2$, pH 7) (DRYL 1959). Wild-type cells migrated towards the cathode. Damaged animals remained at the bottom of the trough. Animals not migrating or swimming backwards towards the anode were pumped out and examined. Paranoiacs swam backward to the anode (VAN HOUTEN, unpublished observations).

The efficiency of these screening methods has not been established. However, six lines of Paranoiacs were isolated using these methods. They are designated lines I–VI in various crosses described below.

Crosses: We carried out crosses as described by SONNEBORN (1970) and KUNG (1971b). Lines I through VI were crossed to stock d4–93 individually. (The body deformation introduced into the lines through d4–93 did not interfere with the expression of the behavioral phenotypes). The behavior of genuine F_1 was noted. A clone was considered a genuine F_1 if body deformation (a recessive trait) failed to appear. Since "Paranoiac" mutant alleles were often dominant (below), complementation tests could not be carried out. Genic relations were ascertained from the segregation ratios observed in the F_2 generation. In all crosses, F_2 individuals were derived by autogamy of the F_1 generation. At least 48 cells descended from each member of the original mating pair were isolated, cloned and scored for body shape and behavior. (Data showing more than 96 segregants in the F_2 generation were the sums of two or more crosses.) The deformed: normal ratio should approach 1:1 if 100% autogamy was induced. F_2 clones of Paranoiacs with body deformation were saved and were used in crosses with other Paranoiacs in order to determine their genic relations.

Photography: We made dark-field macrophotographs of the cells' movements using the method of CHANG and KUNG (1973). The solution in which the movement was registered was the Na-test solution above, filtered through 0.45 micron Millipore filters to remove dust.

RESULTS

Mutant phenotypes: The behavioral phenotypes of mutants I, II, IV, V, and VI are indistinguishable. They all occasionally swim backward for long periods of time in culture media. They are also stimulated to swim backward immediately upon transfer to solutions of high Na^+ concentration. Line III does not display as distinct a phenotype. It spontaneously swims backward in culture media but the backing is shorter and less frequent than that of the other Paranoiacs. It is not stimulated to swim backward in Na^+.

Backward swimming can be distinguished from forward swimming in tracks of the cells' movement registered in dark-field macrophotographs. The wild-type tracks (Figure 1, top) are not smooth, but jagged, indicating frequent transient avoiding reactions (arrows) in Na^+ solutions that interrupt smooth forward swimming. However, the tracks of typical Paranoiacs, represented by line IV (Figure 1, bottom) are the tight, helical paths characteristic of continuous backward swimming (KUNG 1971a). Such trajectories clearly differ from the loose helical paths traced by forward swimming specimens (Figure 1, top).

Nature of the Paranoiac mutants: We first asked the question whether all of the Paranoiacs are due to single-gene mutations and, if so, whether the Paranoiac allele is dominant. This was done by crossing the Paranoiacs in question to the behaviorally normal strain d4-93. The body deformation introduced through d4-93 provides a marker for these and other crosses (below). The results of these crosses are summarized in Table 1. The phenotypes of the F_1 progeny from different crosses are different. F_1 individuals from I, V or VI parentage are indistinguishable from the Paranoiac parents, whereas F_1 individuals from II or III parentage are like the wild type. The F_1 progeny from IV, however, display only a partial Paranoiac phenotype; *i.e.*, their backward swimming is less frequent and shorter in duration than that of typical Paranoiacs.

Segregation in the autogamous F_2 progeny from these crosses approaches the 1:1 expectation in most cases for both the behavioral and the body-shape phenotype. No linkage between the Paranoiac mutations and the body-deformation mutations was observed. The normal segregations of the body-deformation

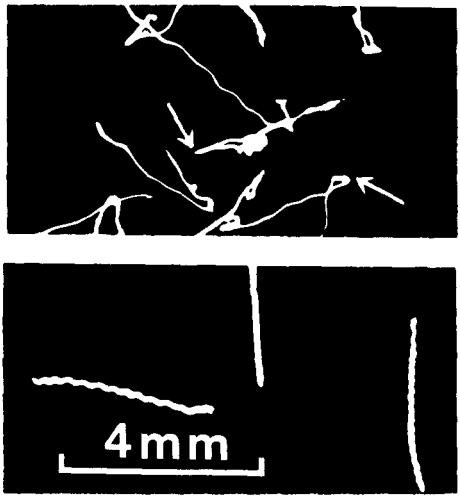

FIGURE 1.—Behavioral response to the Na+ solution of two strains of *P. tetraurelia*. These are dark-field macrophotographs registering the trajectories traversed by the paramecia during the exposure time, 2 to 5 sec after they are put into the solution. Top: Wild type, showing transient avoiding reactions (arrows) which interrupt the loose helical paths of forward swimming. Exposure time, 3 sec. Bottom: "Paranoiac" mutant (line IV, d4–150) showing continuous backward swimming which registers as tight helices. Exposure time, 5 sec.

marker indicate that the crosses are real and autogamy complete. The normal segregations of the behavioral phenotypes indicate that lines I through VI are homozygotes each carrying a single mutation responsible for the Paranoiac phenotype.

Number of genes for the Paranoiac phenotype: To determine the number of genes involved and their linkage relations, we crossed line II through VI with

TABLE 1

*F_1 phenotypes and autogamous F_2 segregations of crosses between the Paranoiac mutants and d4–93**

Lines	F_1	F_2			
		Normal	Paranoiac	Deformed	Paranoiac & deformed
I	full Paranoic	26	39†	23	31†
II	normal	32	31	27	22
III	normal	19	21	22	15
IV	partial Paranoiac‡	24	20	24	26
V	full Paranoiac	39	48	45	53
VI	full Paranoiac	38	58	38	37

* d4–93 is normal in behavior but is deformed in shape. The deformation serves as a genic marker.

‡ Partial Paranoiacs were clones that showed prolonged backward swimming but less frequently and for shorter durations than full Paranoiacs. They were not strongly stimulated to swim backward in high concentrations of Na+.

† Note the preponderance of Paranoiacs. Macronuclear regeneration, although not examined for cytologically, may be the cause in this case.

one another and individually to the Paranoiac A strain previously studied (KUNG 1971b). Line I was crossed only to Paranoiac A (below). The results of these crosses are given in Table 2. Paranoiacs of two unlinked loci crossed to each other are expected to give a segregation of autogamous F_2 progeny in a ratio of 3 Paranoiac:1 normal if the double mutant gives a paranoiac phenotype. Most Paranoiacs when intercrossed yield an F_2 segregation ratio that conforms to this pattern, indicating that they carry mutations of unlinked loci.

Three crosses do not conform to the above pattern. Two of them (I × Paranoiac A, and VI × V) give no normal segregants, indicating that the pairs of Paranoiac parents carry mutations of the same genes or very closely linked genes. The third cross, namely line IV × Paranoiac A, gives an F_2 segregation of 714 Paranoiac: 11 normal. This result indicates that the genes are closely linked, recombination being 3%. The validity of this cross is insured since the marker segregates 1:1 (349 body-deformed:376 normal).

Paranoiacs vs. *other behavioral mutants*: The Paranoiacs were crossed to the two classes of behavioral mutants—pawns and fast-2. The results of these crosses are given in Table 3. Most crosses segregated 2:1:1 in the F_2 for pawn:Paranoiac: normal or fast-2:Paranoiac:normal, as expected for unlinked genes, one of which is epistatic to the other (KUNG 1971b). There are three exceptions to the 2:1:1 ratio. The first two exceptions appear to be trivial. They are the crosses of line IV × pawn A and line IV × pawn B. The latter cross suffered from high inviability; the reason for the large number of deaths is unknown. This high inviability and the previously observed unexplained preponderance of F_2 Paranoiacs (KUNG 1971b) may account for the shortage of F_2 pawns in these crosses. The third exception to the 2:1:1 ratio is the most interesting one. When line II is crossed to fast-2, the F_1 individuals are normal phenotypically, but no behaviorally normal F_2 individuals segregated from the cross. Instead, 202 fast-2 and 241 Paranoiacs were observed, approaching a 1:1 ratio. Thus, Paranoiac line II and fast-2 appear to be due to allelic mutations. It is interesting that these two allelic variants have drastically different phenotypes.

TABLE 2

Segregation of autogomous F_2's from crosses between different lines of Paranoiacs

Lines	Paranoiac A*		II*		III*		IV*		V*	
	Pa:+§	bd:+§	Pa:+	bd:+	Pa:+	bd:+	Pa:+	bd:+	Pa:+	bd:+
I	139:0	66:73								
II	69:24	39:54								
III	133:52	91:94	132:50	97:85						
IV	714:11‡	349:376	216:57	141:132	71:25	52:44				
V	130:39	82:87	127:43	86:84	139:38	86:89	73:20	42:51		
VI	224:40†	123:148	76:14†	50:41	109:28	65:72	——	——	236:0	114:122

* These lines are derived from previous crosses and carry the body-deformation markers.
§ Pa means Paranoiac; bd means body deformed; + means normal in the respective phenotype.
† Note the preponderance of Paranoiacs.
‡ Highly significantly different from the 3:1 ratio of Pa:+ expected for nonlinkage of Paranoiac loci. Thus, the genes are linked, with 3% recombination being observed.

TABLE 3

Segregation of autogamous F_2 progeny from crosses between the Paranoiac mutants and d4-91, d4-94 and d4-95

Lines	Fast-2* fna‡:Pa:+	Fast-2* bd:+	Pawn A* pw:Pa:+	Pawn A* bd:+	Pawn B* pw:Pa:+	Pawn B* bd:+
I	———†	——	34:25:21	39:41	49:34:24	54:53
II	202:241:0	214:229	65:22:31	65:53	55:31:25	46:65
III	48:26:16	49:41	42:32:15	35:54	77:41:59	93:84
IV	40:18:27	36:49	31:37:17	46:41	31:31:12	35:39
V	41:33:32	52:54	49:22:22	42:51	43:25:27	45:50
VI	50:37:25	47:65	———†	——	67:37:31	62:73

* These lines are derived from previous crosses and carry the body-formation markers.
‡ fna means fast-2; Pa means Paranoiac; pw means pawn; bd means body deformation and + means wild type.
† These crosses were not carried out. The findings (Table 2) that line I is probably allelic to Paranoiac A (d4–90) and that line VI is probably allelic to line V make these crosses unnecessary.

DISCUSSION AND CONCLUSION

Six Paranoiac mutants isolated through the two different screening methods have been cloned and genetically analyzed. Genetic analyses show that at least five genes are involved. Not all the Paranoiac × normal or the Paranoiac × Paranoiac crosses yielded the expected 1:1 or 3:1 segregations. A preponderance of Paranoiacs in the F_2 generation are sometimes encountered (crosses marked † in Table 1 and 2), as first noticed by KUNG (1971b) in his study on what is now d4-90, Paranoiac A. This preponderence remains unexplained.

Five of the six lines are now assigned the standard d4 designation (*d*erived stocks of *P. tetraurelia*). Table 4 summarizes these designations and the genic symbols for the Paranoiac mutations known to date.

Since the mutations are dominant in both lines V and VI, we cannot perform complementation tests between the two. Either the mutations formed in the two lines are allelic, or they involve two genes too closely linked to be resolved by the V × VI cross in Table 2. The result of this cross shows that the linkage would have to be closer than 0.8 map units.

TABLE 4

Stocks, genotype, phenotype and genic symbols of Paranoiac mutants in P. tetraurelia

Line	Stocks	Genotype	Notes
—	d4–90	*PaA/PaA*	*PaA* co-dominant with + allele (see KUNG 1971a,b)
I	d4–578	*PaA1/PaA1*	allelic or closely linked to d4–90, *PaA1* dominant over +
III	d4–147	*paB/paB*	leaky Paranoiac phenotype
IV	d4–150	*PaC/PaC*	*PaC*, locus closely linked to *PaA*
V	d4–565	*PaD/PaD*	*PaD* dominant over +
II	d4–149	fna^P/fna^P	allelic to d4–91 which shows "fast-2" phenotype (KUNG 1971a,b)

The close linkage of *PaC* and *PaA* is interesting. Linkage remains a novelty in Paramecium, presumably because of the large number of chromosomes (n = 45) (SONNEBORN 1974). It is intriguing that the linkage revealed in this study is between two genes in which mutations can give an identical phenotype. This fact may hint at the function grouping of related cistrons in Paramecium. Functional organization has rarely been observed in the chromosomes of eukaryotes.

Even more surprising is the discovery that line II (now d4–149) having a full Paranoiac phenotype is allelic to d4–91 having the fast-2 phenotype. Fast-2 not only has no prolonged backward swimming in Na^+ solution, it does not even have the normal transient avoiding reaction of wild type, but simply swims rapidly forward in Na^+ (KUNG 1971a). This defect in fast-2 has now been traced to a change in the relative K^+ permeability of the membrane (SATOW and KUNG 1976). How the same ion channels can be modified by two allelic mutations to give two such drastically different phenotypes needs to be understood.

SATOW, HANSMA and KUNG (1976) showed that during prolonged ciliary reversal, the membrane of paranoiac A (d4–90) is suspended at an excited, depolarized state. At this state the membrane is highly conductive, allowing fluxes of Na^+, K^+ and presumably Ca^{++} along their electrochemical gradients. Similar electrophysiological (SATOW and KUNG, personal communication) and biochemical (HANSMA and KUNG 1976) defects are found in some of the other Paranoiacs. It is not yet understood how the mutations hamper the mechanism(s) responsible for membrane repolarization. The fact that at least five gene products are involved in the normal function of this mechanism illustrates its complexity. The Paranoiacs, like other membrane mutants, now provide contrast and control for experiments on various membrane components important in excitation.

This work was supported by Public Health Service grant GM 22714–02 and National Science Foundation grant BMS 75–10433.

LITERATURE CITED

CHANG, S. Y. and C. KUNG, 1973 Temperature-sensitive pawns: conditional behavioral mutants of *Paramecium aurelia*. Science **180:** 1197–1199. ——, 1976 Selection and analysis of a mutant *Paramecium tetraurelia* lacking response to tetraethylammonium. Genet. Res. **27:** 97–107.

DRYL, S., 1959 Antigenic transformation in *Paramecium aurelia* after homologous antiserum treatment during autogamy and conjugation. J. Protozool. **6:** Suppl: 25.

HANSMA, H. G. and C. KUNG, 1976 Defective ion regulation in a class of membrane-excitation mutants in *Paramecium*. Biochim. Biophys. Acta **436:** 128–139.

JENNINGS, H. S., 1906 *Behavior of the Lower Organisms*. Indiana University Press, Bloomington, Indiana.

KINOSITA, H., A. MURAKAMI and M. YASUDA, 1965 Interval between membrane potential change and ciliary reversal in *Paramecium* immersed in a Ba-Ca mixture. Journal of the Faculty of Sciences, Imperial University of Tokyo IV **10:** 421–425.

KUNG, C., 1971a Genic mutations with altered systems of excitation in *Paramecium aurelia*. I. Phenotypes of the behavioral mutants. Z. verg. Physiol. **71:** 142–164. ——, 1971b Genic mutations with altered system of excitation in *Paramecium aurelia*. II. Mutagenesis, screening and genetic analysis of mutants. Genetics **69:** 29–45.

KUNG, C., S. Y. CHANG, Y. SATOW, J. VAN HOUTEN and H. HANSMA, 1975 Genetic dissection of behavior in *Paramecium*. Science **188**: 898–904.

NAITOH, Y. and R. ECKERT, 1969 Ionic mechanisms controlling behavioral responses of *Paramecium* to mechanical stimulations. Science **164**: 963–965.

NAITOH, Y., R. ECKERT and K. FRIEDMAN, 1972 A regenerative calcium response in *Paramecium*. J. Exp. Biol. **56**: 667–681.

SATOW, Y., H. HANSMA and C. KUNG, 1976 The effect of sodium on "paranoiac"—a membrane mutant of *Paramecium*. Comp. Biochem. Physiol. **54A**: 323–329.

SATOW, Y. and C. KUNG, 1974 Genetic dissection of active electrogenesis in *Paramecium aurelia*. Nature **247**: 69–71. ——, 1976 A mutant of *Paramecium* with increased resting potassium permeability. J. Neurobiol. **7**: 325–338.

SONNEBORN, T. M., 1970 Methods in *Paramecium* research. pp. 243–339. In: *Methods of Cell Physiology*, Vol. 4. Edited by D. M. PRESCOTT. Academic Press, New York. ——, 1974 *Paramecium aurelia*. pp. 469–592. In: *Handbook of Genetics*, Vol. 2. Edited by R. C. KING. Plenum Press, New York. ——, 1975 Species designations and descriptions of the 14 syngens of *Paramecium aurelia*. Trans. Amer. Micro. Soc. **94**: 155–178.

VAN HOUTEN, J., S. Y. CHANG and C. KUNG, 1973 Isolating new types of behavioral mutants in *Paramecium aurelia*. Genetics **74**: S282.

Corresponding editor: S. L. ALLEN