

## An extensive behavioural and genetic analysis of the Pawn mutants in *Paramecium aurelia*

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### SUMMARY

Pawns are behavioural mutants which show impairment in membrane excitability and are, therefore, devoid of normal avoiding reactions. We obtained 103 lines of Pawns through mutagenesis and screening. Among those that yielded to breeding studies, 59 lines belonged to the *pwA* complementation group and 38 lines belonged to the *pwB* group. No other genic loci were found in this extensive analysis. Mutants of the *pwA* locus showed various degrees of phenotypic leakiness. Based on the pheno- and genotypic differences, we estimated that at least 45 independent mutational events were represented in these 103 Pawn lines.

### 1. INTRODUCTION

Various stimuli elicit 'avoiding reactions' in ciliated protozoa. An avoiding reaction, as seen in paramecia, involves an interruption of forward swimming by transient reversal of ciliary beat, resulting in a short backward movement or sudden stoppage before resumption of forward movement in a new direction (Earnings, 1906). The locomotor behaviour of the cells corresponds to the electric potential across the membrane. While the normal ciliary beats (hence, forward swimming) are correlated with the resting potential, the reversal of the beats (backward swimming) is the result of active membrane depolarization (Kinosita, Murakami & Yasuda, 1965). Recent work shows that the increase in internal  $Ca^{2+}$  concentration, resulting from  $Ca^{2+}$  influx in the form of the action current, causes the reversal of the ciliary beating direction (Eckert & Naitoh, 1972).

Pawns are behavioural mutants of *Paramecium aurelia* that are unable to perform the avoiding reaction upon stimulation (Kung, 1971*a*). The fact that they are unable to reverse their ciliary beat is not due to a genetic lesion on the ciliary apparatus (Kung & Naitoh, 1973). This behavioural deficit is the result of the loss of membrane excitability by the mutational impairment of the mechanism responsible for the voltage-sensitive Ca-conductance increase upon depolarization. This has been demonstrated by direct intracellular recording during the application of electric and chemical stimulations (Kung & Eckert, 1972; Satow & Kung, 1973). This dramatic phenotype can result from mutations at either one of two unlinked genic loci (Kung, 1971*b*). A third locus was recently identified, on which a mutation results in a heat-sensitive Pawn that is close to normal when grown at

23 °C, but behaves as Pawn when grown at 35 °C (Chang & Kung, 1973*a, b*; Satow, Chang & Kung, 1973).

We have obtained a large number of Pawn lines through mutant screening. This paper describes our studies of these Pawns, including observation of behaviour and extensive genetic analyses on a scale unprecedented in ciliate genetics.

## 2. MATERIAL AND METHODS

All strains belonged to syngen 4 of *P. aurelia*. Besides the Pawn lines to be tested, the stocks used were: 51s non-kappa-bearing wild-type; d4-93 behaviourally normal but body-deformed mutant, genotype *bd bd*; d4-94 Pawn, genotype *pwA pwA*, and d4-95 Pawn, genotype *pwB pwB* (Kung, 1971*b*; Chang & Kung, 1973*b*). Stocks were kept and clones were cultured in Cerophyl medium bacterized with *Aerobacter aerogenes* (Sonneborn, 1970).

Mutations were induced with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. After induction of autogamy and a phenomic lag of 5–10 fissions, the mutagenized populations were used to screen for Pawn mutants (Sonneborn, 1970; Kung, 1971*b*).

The Pawns (with one exception) were selected with the screening method of Kung (1971*a*) or that method as modified by Chang & Kung (1973*a*). Basically, the mutagenized exautogamous population was injected into the bottom of a column filled with a solution known to elicit strong and repeated avoiding reactions that confine the normal animals in the vicinity of the bottom of the column. Pawns, unable to avoid, rose to the top of the column by negative geotaxis and were thus selected from the top fractions of the solution in the column. The recent modification involved stabilizing the column by increasing the density of the bottom layer with sucrose.

The diagnostic behavioural tests involved collecting some 20 animals from a clone with a micropipette and transferring them to a test solution for behavioural observation. The Na-test solution consisted of 20 mM-NaCl, 0.3 mM-CaCl<sub>2</sub>, 1 mM Tris pH 7.2, and the Ba-test solution was 8 mM-BaCl<sub>2</sub>, 1 mM-CaCl<sub>2</sub>, 1 mM Tris pH 7.2. Wild-type animals generated strong and repeated avoiding reactions against these solutions. Typical Pawns would not avoid these solutions but simply swam forward in their usual left-handed helical paths.

In this study the  $F_1$  was derived from conjugations of parents and the  $F_2$  from autogamy of  $F_1$ . In autogamy, the two identical haploid gametic nuclei, derived from the same meiotic product, fuse to restore diploidy. This process results in homozygosity at all loci. (See Beale (1954) and Sonneborn (1970) for detailed descriptions of conjugation, autogamy and other techniques for the genetic manipulation of *P. aurelia*.)

## 3. RESULTS

### (i) *The mutant lines and their behaviour*

A total of 117 lines of Pawns were isolated in various mutagenesis experiments starting from 51s populations. All but one were obtained by screening methods

based on the chemotactic interference, Line 11-2-10, was obtained from a device which eventually (Van Houten, Chang & Kung, 1973).

Fourteen lines out of the total 117 were studied in detail. The remaining 103 lines are listed in Table 1.

Column A of Table 1 lists the lines which were selected for mutagenesis experiments performed on animals in the same experimental treatment, long before the first mutagenesis treatment. In these different populations are almost always only a minute possibility that spores are present before mutagen treatment. Lines 11-2-10 and 11-2-11 may be the mitotic copies of the same line, as proven otherwise by phenotypic differences.

Column B lists the 103 lines of clones in mass screening experiments.

Typical Pawn behaviour in culture was found in 89 of the 117 lines. Degrees of leakiness of the basic Pawn culture medium. A typical Pawn (AR). 'Leaky' in column C means observed at the wall of the culture line to the Na-test solution. A typical solution with forward movement and forward movement in the test solution very weak avoiding reaction. Column test solution. Again a typical non-forward movement (FM). Various reaction to this solution: 'leaky' slightly leaky which reacts to the obvious avoidance; 'leaky 2' mutant transient slowing in the forward very slow backward swimming; 'leaky 4' mutants react to the Ba-representative leaky mutants were Na- and Ba-solutions, they became avoidance.

Note that even the leakiest mutant the wild-type, which has spontaneously and repeatedly avoids both test solutions.

based on the chemotactic interference of geotaxis, as described above. The exception, Line 11-2-10, was obtained from a trough designed for galvanotactic migration, a device which eventually evolved to a system for other screening purposes (Van Houten, Chang & Kung, 1973).

Fourteen lines out of the total 117 lines grew slowly and poorly and were lost before they could be studied in detail. The origins and characteristics of the remaining 103 lines are listed in Table 1.

Column A of Table 1 lists the mutagenized, exautogamous populations from which the lines were selected. Different populations are either from different mutagenesis experiments performed at different times or from different groups of animals in the same experiment, separated immediately after nitrosoguanidine treatment, long before the first post-treatment fission occurred. Thus, lines from different populations are almost certainly mutants of different origins. There is only a minute possibility that spontaneous Pawn mutations might have occurred before mutagen treatment. Lines from the same population could theoretically be the mitotic copies of the same original mutants and were assumed to be unless proven otherwise by phenotypic or genotypic analyses.

Column B lists the 103 lines studied. The hyphenated digits serve to identify clones in mass screening experiments and have no biological meaning.

Typical Pawn behaviour in culture medium and in reactions to the Na- or Ba-test solution were found in 89 of the 103 lines. The remaining lines showed various degrees of leakiness of the basic Pawn behaviour. Column C gives the behaviour in culture medium. A typical Pawn showed no spontaneous avoiding reactions (no AR). 'Leaky' in column C means that weak avoiding reactions were sometimes observed at the wall of the culture vessels. Column D gives the reaction of each line to the Na-test solution. A typical Pawn reaction is simply to disperse into the solution with forward movement (FM). 'Leaky' in this column D means that forward movement in the test solution was interrupted by whirling, slowing or very weak avoiding reaction. Column E lists the reaction of the lines to the Ba-test solution. Again a typical non-leaky Pawn reaction consists of nothing but forward movement (FM). Various degrees of leaky expressions were found in reaction to this solution: 'leaky 1' marks the class of Pawn which is only very slightly leaky which reacts to the Ba-solution by slow forward movement without obvious avoidance; 'leaky 2' mutants including d4-94 are those that show some transient slowing in the forward movement; 'leaky 3' mutants show whirling or very slow backward swimming after a short distance of forward movement; 'leaky 4' mutants react to the Ba-solution by obvious backward movement. When representative leaky mutants were grown at 35° C overnight before test with the Na- and Ba-solutions, they became much less leaky and showed no sign of avoidance.

Note that even the leakiest mutants studied here are still clearly different from the wild-type, which has spontaneous avoiding reactions in culture and violently and repeatedly avoids both test solutions.

(ii) *Generating marked tester strains for genetic analyses*

The strategy of our breeding analyses is to cross each of the 103 lines to Pawn mutants of previously identified mutations (*pwA* or *pwB*). Such crosses should yield information on dominance, complementations, linkages and genic additive effects. Since the partners of the crosses have the same or very similar phenotypes,

Table 1. *Characteristics of mutant Pawn lines*

Population (A)	Line no. (B)	Behaviour in culture medium (C)	Reaction to the Na solution (D)	Reaction to the Ba solution (E)	Complementation with $l_{13}$ ( <i>pwA</i> ) (F)	Complementation with $l_{40}$ ( <i>pwB</i> ) (G)	Independent stocks (H)
I	11-4-20*	No AR	FM	Leaky 2	?	?	S
II	3-4-36	No AR	FM	FM	+	-	S
III	2-2-1	No AR	FM	Leaky 1	-	+	S
	4-1-34*	No AR	FM	FM	?	?	S
IV	4-2-34	No AR	FM	Leaky 2	-	+	S
	4-2-35	Leaky	Leaky	Leaky 3	-	+	S
V	6-1-23	No AR	FM	Leaky 2	-	+	S
	6-3-47	No AR	FM	Leaky 2	-	+	S
	3-4-29	No AR	FM	FM	+	-	S
VI	10-5-9†	No AR	FM	Leaky 2	-	+	S
	14-5-43†	No AR	FM	Leaky 1	-	+	S
	14-4-4†	No AR	FM	FM	+	-	S
	14-4-3*	No AR	FM	Leaky 2	?	?	S
	14-4-29*	No AR	FM	FM	?	?	S
	10-5-27*	No AR	FM	FM	?	?	S
VII	11-1-12†	No AR	FM	Leaky 2	-	+	S
	15-1-13	No AR	FM	Leaky 1	-	+	S
	15-2-15	No AR	FM	FM	+	-	S
	16-2-21	No AR	FM	FM	+	-	S
VIII	13-1-4	No AR	FM	Leaky 1	-	+	S
	15-3-18	No AR	FM	Leaky 2	-	+	S
	15-3-38	No AR	FM	Leaky 2	-	+	S
	13-1-34†	No AR	FM	FM	+	-	S
IX	15-4-24†	No AR	FM	Leaky 2	-	+	S
	16-4-40	No AR	FM	Leaky 1	-	+	S
	16-5-17†	No AR	FM	FM	+	-	S
X	3-1-3	No AR	FM	Leaky 2	-	+	S
XI	4-1-45	Leaky	FM	Leaky 4	-	+	S
	4-1-46	Leaky	FM	Leaky 4	-	+	S
XII	6-3-16*	No AR	FM	Leaky 1	+	?	S
	6-4-8†	No AR	FM	FM	+	-	S
XIII	6-5-41†	No AR	FM	Leaky 2	-	+	S
XIV	5-2-2	No AR	FM	Leaky 2	-	+	S
XV	5-2-32	No AR	FM	Leaky 2	-	+	S
	5-2-18†	No AR	FM	FM	+	-	S
XVI	7-3-6	Leaky	Leaky	Leaky 4	-	+	S
	7-3-36	No AR	FM	Leaky 2	-	+	S
XVII	8-1-40	No AR	FM	FM	+	-	S
	8-2-33	No AR	FM	FM	+	-	S
XVIII	5-1-38	No AR	FM	FM	+	-	S
	5-1-40*	No AR	FM	FM	?	?	S

Pawn mu

Population (A)	Line no. (B)	Behaviour in culture medium (C)	Re to sol (D)
XIX	3-1-33	No AR	I
XX	3-5-23	No AR	F
XXI	7-1-21	No AR	F
XXII	5-1-27	No AR	F
XXIII	1-4-7	No AR	F
XXIV	11-2-10	No AR	F
	5-1-36†	No AR	FM
	5-2-30†	Leaky	FM
	12-3-9†	No AR	FM

\* At least five separate attempts were made to find the lines.

† Lines with same characteristics derived from: VI: 14-5-9 (S), 14-5-1, 14-5-6, 14-5-24. VII: 14-8-1-5, 14-4-7, 14-4-21, 14-4-36, 14-4-41, 14-11-5-7, 11-5-28, 14-2-20, 16-1-48. VIII: 12-3-27, 12-4-39, 13-2-33, 13-2-39, 13-3-48, 16-3-33, 16-3-48, 16-4-45, 16-4-12 (S), 16-16-12-3. IX: 6-4-8 (S), 6-3-18, 6-3-36, 6-4-2, 6-4-26, 6-5-43, 8-1-7. XV: 5-2-18 (S), 5-2-31, 5-2-40. ‡ These lines were of dubious origin. Line 12-3-9 was from VI.

it is important to be sure that fertilization assurance is especially important in crosses involving both recessive and do not must be genetically marked. In our study

We crossed the Pawn d4-94 (*pwA pwB*) to the body-deformation mutant d4-93 double mutants showing both the Pawn were chosen as tester stocks for the analysis.

The results of these two preparatory crosses the  $F_1$  was wild-type and the autogamous double deformed: double mutant = 24:29:25:1 chosen as the marked tester. In d4-95: autogamous  $F_2$  segregated into wild-type = 20:22:25:28. The double mutant clone used below.

(iii) *Crosses of representative*

Pawn lines were selected among the 103 populations or different phenotypic leakiness to  $l_{13}$  and  $l_{40}$  respectively. The  $F_1$  of these autogamous  $F_2$  segregations with regard to body deformation phenotype were scored.

Table 1 (cont.)

Population (A)	Line no. (B)	Behaviour in culture medium (C)	Reaction to the Na solution (D)	Reaction to the Ba solution (E)	Complementation with $l_{13}$ ( <i>pwA</i> ) (F)	Complementation with $l_{40}$ ( <i>pwA</i> ) (G)	Independent stocks (H)
XIX	3-1-33	No AR	FM	FM	+	-	S
XX	3-5-23	No AR	FM	FM	+	-	S
XXI	7-1-21	No AR	FM	Leaky 2	-	+	S
XXII	5-1-27	No AR	FM	Leaky 2	-	+	S
XXIII	1-4-7	No AR	FM	FM	+	-	S
XXIV	11-2-10	No AR	FM	FM	+	-	S
	5-1-36†	No AR	FM	Leaky 2	-	+	S
	5-2-30†	Leaky	FM	Leaky 3	-	+	S
	12-3-9†	No AR	FM	FM	+	-	S

\* At least five separate attempts were made to cross these lines. No tight mating pairs could be found.

† Lines with same characteristics derived from a given population were as follows. VI: 10-5-9 (S), 14-5-1, 14-5-6, 14-5-24. VI: 14-5-43 (S), 10-1-9, 8-3-16, 14-5-11. VI: 14-4-4 (S), 8-1-5, 14-4-7, 14-4-21, 14-4-36, 14-4-41, 14-5-4 (S), 15-1-5, 15-1-6. VII: 11-1-12 (S), 11-1-42, 11-5-7, 11-5-28, 14-2-20, 16-1-48. VIII: 13-1-34 (S), 16-2-34, 12-2-9. IX: 15-4-24 (S), 12-3-27, 12-4-39, 13-2-33, 13-2-39, 13-3-48, 13-4-34, 13-5-46, 15-4-13, 15-4-29, 15-5-7, 16-3-30, 16-3-33, 16-3-48, 16-4-45, 16-4-12 (S), 16-5-7. IX: 16-5-17 (S), 13-5-32, 14-3-10, 16-3-34. XII: 6-4-8 (S), 6-3-18, 6-3-36, 6-4-2, 6-4-26. XIII: 6-5-41 (S), 6-5-35, 6-5-37, 6-5-38, 6-5-39, 6-5-43, 8-1-7. XV: 5-2-18 (S), 5-2-31, 5-2-42.

‡ These lines were of dubious origin. Lines 5-1-36 and 5-2-30 were derived from one of the populations III-V. Line 12-3-9 was from VI to IX.

it is important to be sure that fertilization has occurred after conjugation. Such assurance is especially important in crosses in which the two Pawn mutations involved are both recessive and do not complement. Because of this, the crosses must be genetically marked. In our studies, the gene *bd* was used as a marker.

We crossed the Pawn d4-94 (*pwA pwA*) and Pawn d4-95 (*pwB pwB*) respectively to the body-deformation mutant d4-93 (*bd bd*). Among the autogamous  $F_2$  the double mutants showing both the Pawn and the body-deformation phenotypes were chosen as tester stocks for the analyses of the Pawn lines in question.

The results of these two preparatory crosses were as follows: for d4-94 x d4-93 the  $F_1$  was wild-type and the autogamous  $F_2$  segregated into wild-type: Pawn: body-deformed:double mutant = 24:29:25:18. A double mutant clone,  $l_{13}$ , was chosen as the marked tester. In d4-95 x d4-93, the  $F_1$  was wild-type and the autogamous  $F_2$  segregated into wild-type: Pawn: body-deformed:double mutant = 20:22:25:28. The double mutant clone,  $l_{40}$ , was selected as the marked tester used below.

(iii) Crosses of representative lines to the tester strains

Pawn lines were selected among the 103, representing different mutagenized populations or different phenotypic leakiness. These selected lines were crossed to  $l_{13}$  and  $l_{40}$  respectively. The  $F_1$  of these crosses were examined and the exautogamous  $F_2$  segregations with regard to both the Pawn phenotype and the body-deformation phenotype were scored.

The results fell into two patterns: (A) the  $F_1$  were Pawns and there was no wild-type segregant among the autogamous  $F_2$ ; (B) the  $F_1$  were wild-type in phenotype and the  $F_2$  yielded a segregation of 3:1 of Pawn:wild-type. The simplest explanations of these patterns are the following: pattern A arose when the mutant line to be tested and the Pawn tester was not complementary and the two mutations were allelic with no observable intragenic recombination; pattern B resulted when the mutant line tested and the Pawn tester was complementary and the two mutations were completely unlinked with the double mutant also having a Pawn phenotype. Table 2 summarizes the results of these crosses. These crosses are valid since the marker gene, *bd*, segregated into the expected 1:1 ratio in the  $F_2$ .

It is important to notice the following from Table 2. Some lines gave the A pattern when crossed to  $l_{13}$  and the B pattern when crossed to  $l_{40}$ . For the rest of

Table 2.  $F_2$  segregations of crosses between the Pawn lines and the tester strains

(Pop.)	Line	Line $\times l_{13}$ ( <i>pwA/pwA bd/bd</i> )		Line $\times l_{40}$ ( <i>pwB/pwB bd/bd</i> )	
		Pawn: +	Deformed: +	Pawn: +	Deformed: +
(V)	6-3-47	95:0	55:40	70:16	40:46
(V)	3-4-29	63:32	47:48	92:0	46:46
(VI)	10-1-9	89:0	40:49	68:26	49:45
(VI)	10-5-9	96:0	48:48		
(VI)	14-5-1	89:0	44:45	62:32*	39:55
(VII)	11-1-12	86:0	45:41	43:48*	55:36
(VII)	14-2-20	48:0	26:22	43:42*	36:49
(VIII)	15-3-18	95:0	49:46	54:32*	52:34
(IX)	12-3-27	76:0	47:29*	71:21	46:46
(IX)	12-4-39	85:0	46:39	56:32	42:46
(IX)	13-2-39	94:0	46:48	43:23	33:33
(IX)	15-5-7	88:0	45:43		
(IX)	16-4-12	93:0	52:41		
(X)	3-1-3	93:0	46:47	70:20	41:49
(XI)	4-1-45	69:0	31:38	60:25	41:44
(XI)	4-1-46	96:0	44:52	71:21	38:54
(XII)	6-3-36	71:19	46:44		
(XII)	6-4-2	64:29	60:33	84:0	42:42
(XIII)	6-5-35	93:0	48:45	61:31	47:45
(XIII)	6-5-37	91:0	40:51		
(XIII)	6-5-41	91:0	47:44		
(XIV)	5-2-2	94:0	46:48		
(XV)	5-2-18	72:24	42:54		
(XV)	5-2-42			40:0	17:23
(XVII)	8-2-33	68:27	57:38	94:0	60:34*
(XVIII)	5-1-38	66:28	50:44	83:0	45:38
(XVIII)	5-1-40	58:36*	54:40	83:0	45:38
(XXII)	5-1-27	75:0	36:39	109:34	73:70
(XXIII)	1-4-7	66:21	47:40	88:0	46:42
(XXIV)	11-2-10	95:31	60:66	81:0	39:42
	12-3-9†	73:23	46:50	93:0	46:47

\* Ratios significantly different from the 3:1 or 1:1 expectations. See text for explanation.

† See footnote of Table 1.

the lines the reverse was the case. crosses. This means that the mutant is either allelic to *pwA* or to *pwB*.

A few of the crosses gave  $F_2$  segregation 3:1 expectation, with the wild-type. Such results may be due to selection when the cultural condition is such that a gene was established, it becomes unlinked in the segregating crosses.

#### (iv) Exhaustion

After we realized that most, if not all, of the two known loci, we restricted our crosses only to  $F_1$ . Each line (including  $l_{40}$ ) Wild-type from one of the lines tested was recessive. If the second mutation was judged to be in the same complementation group as the first, it was tested in the second tester.

The results of these complementation tests are given in Table 1. Plus (+) means that the mutant complemented the wild-type  $F_1$ . Minus (-) means that the mutant did not complement the wild-type  $F_1$  and the  $F_1$  was Pawn in phenotype. Complementation tests were scored only when the mutant was not a Pawn in the crosses.

Aside from six cases where exhaustion resulted, 59 lines did not complement the wild-type  $F_1$ . No line was found to complement  $l_{40}$ . No line was found to complement  $l_{40}$ .

Matching the phenotypes with the genotypes showed that all mutants belong to the same complementation group, *pwA*, or leaky, although the degree of leakiness varies.

#### 4. CONCLUSIONS

The breeding analyses indicate that in all breeding attempts, all the Pawn lines belong to the same complementation group of *pwA* or *pwB*. This was discovered among the 103 lines tested.

This study is the most extensive complementation analysis of independent mutations leading to the same phenotype. Two unlinked genic loci were identified. The mutants exhausted the system and have recessive viable Pawn phenotype. On the other hand, the mutants are basically by only one screening method, 'avoidance-minus mutants'. For example, the mutants on the cortical structures, resulting

the lines the reverse was the case. In no case did we find an *A* or *B* pattern in both crosses. This means that the mutations carried by the representative lines were either allelic to *pwA* or to *pwB*.

A few of the crosses gave  $F_2$  segregations that deviated significantly from the 3:1 expectation, with the wild-type group preponderant. Aside from pure chance, such results may be due to selective death of Pawns (or double mutant Pawns) when the cultural condition is suboptimal. Since in each case allelism to a known gene was established, it becomes unimportant to clarify the source of the skewness in the segregating crosses.

(iv) *Exhaustive complementation tests*

After we realized that most, if not all, of the lines carried mutations allelic to one of the two known loci, we employed much less laborious tests bringing the crosses only to  $F_1$ . Each line (including those shown in Table 2) was crossed to  $l_{13}$  and  $l_{40}$ . Wild-type from one of these crosses indicated that the mutation to be tested was recessive. If the second cross yielded  $F_1$  of Pawn phenotype, the mutation was judged to be in the same complementation group with the Pawn mutation in the second tester.

The results of these complementation tests are summarized in columns F and G of Table 1. Plus (+) means that the tested line complemented the tester to yield wild-type  $F_1$ . Minus (-) means that the tested line did not complement the tester and the  $F_1$  was Pawn in phenotype. All tests involved the *bd* marker. Complementation tests were scored only when all  $F_1$  were not body-deformed, indicating true crosses.

Aside from six cases where extensive attempts were made and no true conjugation resulted, 59 lines did not complement with  $l_{13}$  and the remaining 38 lines did not complement  $l_{40}$ . No line was found to complement both testers.

Matching the phenotypes with the genotypes identified, as listed in Table 1, it is clear that all mutants belong to the *pwA* complementation group tends to be leaky, although the degree of leakiness varies.

#### 4. CONCLUSIONS AND DISCUSSION

The breeding analyses indicated that, with the six exceptions which defy breeding attempts, all the Pawn lines studied carried mutations belonging to the complementation group of *pwA* or *pwB*. No alleles belonging to a third locus was discovered among the 103 lines tested.

This study is the most extensive one to date in ciliate genetics dealing with independent mutations leading to one basic phenotype. It is significant that only two unlinked genic loci were identified. The data could mean that we have exhausted the system and have retrieved all the genes whose mutations give the viable Pawn phenotype. On the other hand, these mutant lines were selected basically by only one screening method. This may bias our selection against other 'avoidance-minus mutants'. For example, Pawns with a strong pleiotropic effect on the cortical structures, resulting in severe body deformation, would be selected

against in our screening procedure, since they could not swim normally enough to perform geotaxis. Extremely slow and poor growers are also selected against by simple dilution with the post-mutagenized populations and by the feeding regime designed for routine maintenance of stocks. In fact, as mentioned above, 14 out of 117 lines were lost because they grew extremely poorly and even extra care could not keep them alive long enough for thorough investigation. The fact that a third Pawn locus, giving a temperature-sensitive phenotype, exists (Chang & Kung, 1973b), suggests that perhaps the system has not yet been exhausted. Our finding here shows that the commonly encountered Pawn mutants given our screening procedure are most likely mutants of two known loci, *pwA* and *pwB*.

It is interesting that among the 103 lines the two alternative genotypes can almost always be distinguished by a careful examination of the phenotype. Chang & Kung (1973b) noticed that d4-94 (*pwA pwA*) was slightly leaky. The cells tended to show weak avoiding reaction when they hit the wall of the vessels. All the mutants that showed similar leakiness turned out also to be mutated at the *pwA* locus. However, the phenotype was far from uniform. As described in detail above, there were those that barely show any slowing reaction to those that can show fairly obvious avoiding reaction upon very strong ionic stimulation although such avoidance disappeared when the clones were grown at 35 °C. In connexion with this, we should recall that although one of the five temperature-sensitive Pawn lines studied had mutation on the *pwC* locus, the other four were mutated at the *pwA* locus (Chang & Kung, 1973b). Thus these heat-sensitive Pawn mutants at the *pwA* locus, which are close to normal when grown at room temperature, can be viewed as an extreme of the spectrum of leaky phenotypes which the *pwA* locus offers.

All Pawns carry pleiotropic effects (Kung, 1971b). The Pawns studied here are no exception. Many lines have slightly body-deformed or truncated animals among their normal looking peers. Many lines have lower fission rates.

Among the 103 lines some may have descended from the same mutational events. We censored these lines for mutational origin based on the following criteria: (1) their independent origin from the different mutagenized populations; (2) their genotypic difference as revealed by the genetic analyses; and (3) their phenotypic difference, including degree of leakiness, reactions to test solutions and other pleiotropic phenotypes. A total of 45 lines were found to be of different origins from this conservative estimate. This means that at least 45 mutations were identified here, unless some are the descendants of a rare spontaneous mutation before the mutagen treatments, these lines are now considered derived stocks and are marked with an 'S' in column H of Table 1.

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