Genet. Res., Camb. (2000), **76**, *pp.* 125–133. Printed in the United Kingdom © 2000 Cambridge University Press

An unusual complementation in non-excitable mutants in *Paramecium*

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(*Received 6 January 2000 and in revised form 17 May 2000*)

Summary

A non-excitable behavioural mutant, d4-662, was previously characterized as the fourth pawn locus mutant *pwD* in *Paramecium tetraurelia*. We now provide data demonstrating that d4-662 is in fact controlled by a *pwB* allele that has the unusual feature of complementing other *pwB* alleles in heterozygous F_1 progeny. Neither the cytoplasm nor the nucleoplasm of d4-662 cured the mutational defects of *pwB* and in the reverse combination of d4-662 and *pwB*, the result was the same. On the other hand, *pwA*, another non-excitable mutant, was cured upon cross-injection with d4-662 and mutants carrying trichocyst non-discharge marker genes were also cured. This evidence suggests that d4-662 is a new mutant belonging to *pwB*, and would be better designated as pwB^{662}. Extensive crossbreeding analyses, however, showed an unusual genetic relationship between d4-662 and *pwB* (pwB^{95} or pwB^{96}). When d4-662 was crossed with *pwB* mutants, many progeny expressing wild-type phenotype or mixed clones of wild-type and pawn cells were obtained in the F_1. Less than 12·5% expressed the pawn phenotype. The appearance of wild-type progeny in this F_1 strongly suggests that an inter-allelic interaction between pwB^{662} and other *pwB* alleles may occur during development of the macronucleus.

1. Introduction

When cells of *Paramecium* encounter various stimuli, such as mechanical, chemical or thermal stimuli, they show an 'avoiding reaction' (Jennings, 1906). This avoiding reaction, a basic behavioural response of paramecia facing a stimulus, consists of a short period of backward swimming that is caused by the reversal of effective strokes of the cilia. Ciliary reversal is triggered by an increase in intraciliary Ca^{2+} that is tightly correlated with the generation of action potentials based on the activation of voltage-gated Ca^{2+} channels (Ca^{2+} channels) (Eckert, 1972), that is, membrane excitation.

Non-excitable mutants are called pawn in *Paramecium tetraurelia* (Kung, 1971) and CNR (caudatum non-reversal) in *P. caudatum* (Takahashi & Naitoh, 1978). Electrophysiological studies have shown that the mutational defect in all pawns and CNRs is the malfunction of the Ca^{2+} channels (Kung & Eckert, 1972; Takahashi & Naitoh, 1978). Seven single recessive loci affecting the function of Ca^{2+} channels have been obtained: *pwA*, *pwB* and *pwC* in *P. tetraurelia* (Kung, 1971; Chang & Kung, 1974), and *cnrA*, *cnrB*, *cnrC* and *cnrD* in *P. caudatum* (Takahashi, 1979; Takahashi *et al.*, 1985). Although crossbreeding analysis cannot be performed between two species, cytoplasmic transfer is effective for the analysis of the genetic relationships between pawns and CNRs over the species barrier (Haga et al., 1983). Three pawns (*pwA*, *pwB* and *pwC*) and four CNRs (*cnrA*, *cnrB*, *cnrC* and *cnrD*) have been found to be different mutants controlled by independent genic loci, because all of them complemented one another by cytoplasmic transfer (Haga *et al.*, 1983; Takahashi *et al.*, 1985). These results suggested that at least seven genes control the function of Ca^{2+} channels in *Paramecium*.

Non-Mendelian inheritance, such as cytoplasmic and caryonidal inheritance, is well known in *Paramecium* genetics. Cytoplasmic inheritance in *Paramecium* has

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https://doi.org/10.1017/S0016672300004699 Published online by Cambridge University Press

been reported not only for the killer and mitochondrial traits but also for mating types, trichocyst discharge and serotypes (Sonneborn, 1947, 1948; Sonneborn & Schneller, 1979; Epstein & Forney, 1984). Caryonides are lineages of paramecia that derive their macronuclei (somatic nuclei) from single macronuclear primordia (anlagen). Caryonidal inheritance, by which genetically identical homozygous exconjugants inherit different genetic characters in different caryonides, has been reported for mating types of *P. primaurelia* (reviewed in Kimball, 1943; Sonneborn, 1977) and *Tetrahymena thermophila* (Orias, 1981). Recent investigations have revealed that some of these examples of non-Mendelian inheritance involve developmentally controlled DNA rearrangements during macronuclear development (Duharcout *et al*., 1998; Forney *et al*., 1996; Meyer & Duharcourt, 1996; Rudman *et al*., 1991).

The macronucleus, which develops from fertilized micronuclei, undergoes large-scale DNA rearrangements, involving elimination of certain germ-nucleus-specific sequences (IESs or internal eliminated sequences), chromosome fragmentation and telomere addition (reviewed in Coyne *et al*., 1996; Klobutcher & Herrick, 1997), and degenerates during the next fertilization event. Ordinarily, the macronucleus controls the phenotype and does not participate in the transmission of genetic information, which is controlled by the transcriptionally silent micronucleus (germ nucleus). However, it has been suggested that, in some cases, the contents of the 'old' macronucleus influence the DNA rearrangement of the 'new' macronucleus, thus resulting in apparent cytoplasmic determination of the phenotype (see reviews by Forney *et al*., 1996; Meyer & Duharcourt, 1996).

One of the pawn mutants in *P. tetraurelia*, d4-662, formerly designated *pwD* (Saimi & Kung, 1987), has not previously been fully characterized in relation to the three other pawns. During our analysis, we discovered unusual complementation between d4-662 and pwB^{95} or pwB^{96}. The results suggest that specific allelic interactions between two alleles during macronuclear development may be involved in this phenomenon.

2. Materials and methods

(i) *Stocks and culture*

The stocks used in this study are listed in Table 1. All mutants used here are recessive. The culture medium was fresh lettuce juice (2·5% w/v) (Hiwatashi, 1968) in modified Dryl's solution (substituting NaH_2PO_4 for $K_2H\ PO_4$ as in original Dryl's solution (Dryl, 1959)), inoculated with *Klebsiella pneumoniae* 1 or 2 days before use. Cells were grown at 25 °C, except *pwC*, which is grown at 35 °C since it is a temperature-sensitive mutant expressing a mutant phenotype when grown at that temperature (Chang & Kung, 1974). Because d4-662 produces phenotypic revertants after autogamy (see Section 3), this mutant was grown in 0·4 ml culture medium in depression slides, instead of tube cultures, so as to avoid unwanted autogamy. Frequent transfers to excess culture medium in depression slides prevented the induction of autogamy in d4-662, and were effective in maintaining the pawn phenotype.

(ii) *Microinjection*

Microinjection was performed by the method described by Hori & Takahashi (1994). Cells for the transplantation of cytoplasm or macronucleoplasm were deciliated with 5% ethanol (Ogura, 1981) and embedded in mineral oil (Squibb & Sons). Cells in the log phase of the culture were used as recipients, while those in the stationary phase were used as donors. Cells of *P. tetraurelia* used were in the immature period, in which autogamy does not occur. About 20 pl or 40 pl of the cytoplasm of a donor was injected into recipient cells of *P. tetraurelia* or *P. caudatum*, respectively. Macronucleoplasm was injected at the approximate volume of over two-thirds of the macronucleus of the recipient. After injection, recipient cells were incubated in modified Dryl's solution containing 0·02% methylcellulose. Cilia regenerated within 1–3 h.

(iii) *Observation of the phenotypes*

The behavioural phenotype was examined by transferring the cells by micropipette into the stimulation solution (20 mM KCl in Dryl's solution) (Naitoh, 1968). When paramecia are transferred to the stimulation solution, cells of typical wild-type swim backwards for approximately 50 s. Cells showing clear backward swimming were thus classified as wild-type. Pawn or CNR mutants do not show backward swimming in the stimulation solution because they have a malfunction of the Ca^{2+} channels. Cells which showed only whirling or backward swimming for less than 3 s, were judged to be exhibiting the pawn phenotype.

The phenotype of exocytosis was tested by addition of a drop of saturated picric acid. Wild-type cells discharge massive trichocysts following this treatment, while non-discharge (*nd*) mutants do not behave in this way.

(iv) *Genetic analysis*

Each conjugating pair was isolated in fresh culture medium. After completion of the conjugation process, cells were allowed to pass through one post-con-

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Table 1. *Strains used in this study*

Strain	Mutant genes		Source
P. tetraurelia			
51s			University of Tsukuba
d4N-527	*nd169*		Takagi (Nara Women's University), originally isolated by Nyberg (1978)
d4N-526	*nd169*		Takagi
nd7	*ts111*	*nd7*	Cohen (CNRS, Gif-Sur-Yvette, France)
d4-502	*pwA*		Kung (University of Wisconsin, USA)
d4-95	pwB^{95}	*nd6*	Kung
a2001	pwB^{95}		F_2 segregant from d4-95 × nd7
a2071	pwB^{95}	*nd7*	F_2 segregant from d4-95 × nd7
d4-96	pwB^{96}		Kung
95ndE1	pwB^{96}	*nd169*	F_2 segregant from d4-96 × d4N-527
96ndE2	pwB^{96}	*nd169*	F_2 segregant from d4-96 × d4N-527
d4-649	*pwC*		Kung
d4-662	[a]pwB^{662}	*nd6*	Kung
YndE215	[a]pwB^{662}	*nd169*	F_2 segregant from d4-662Y × d4N-526
d4-662Y	[a]pwB^{662}	*nd6*	Kung
P. caudatum			
G3	*tnd2*		University of Tsukuba
16A1107	*cnrA*	*tnd2*	University of Tsukuba
16Bk102	*cnrB*	*tnd2*	University of Tsukuba
R16D305s-27	*cnrC*		University of Tsukuba
18D610	*cnrD*	*tnd2*	University of Tsukuba
18D621	*cnrD*	*tnd2*	University of Tsukuba

[a] Once called *pwD*. See Section 3.

jugational cell division, and the four cells thus produced from every conjugating pair were reisolated to establish caryonidal clones (clones that derive their macronuclei from a single macronuclear primordium). For the isolation of progeny, culture medium containing 5% rather than 2·5% lettuce juice was used because d4-662 does not grow well in medium with the lower concentration of lettuce juice. The phenotypes of the progeny were observed at about 9 cell divisions after conjugation. The parental cytoplasm of the progeny was determined by the mating type expressed, since mating types are known to be inherited cytoplasmically in this species (Sonneborn, 1947). Trichocyst non-discharge gene markers were used to confirm that conjugation had taken place normally.

F_2 were obtained by autogamy, during which gametic nuclei carrying an identical genotype are self-fertilized. Autogamy thus makes the progeny completely homozygous. Autogamy was induced by starvation, after cells had undergone more than 25 divisions following conjugation, so as to enter the maturity period. To confirm that 100% cells entering autogamy were present in the cultures, 20 or more cells were examined for macronuclear fragmentation by staining with Carbol fuchsin solution (Carr & Walker, 1961). Ex-autogamous cells were isolated in 0·4 ml fresh culture medium and allowed to grow for about 9 or 10 cell divisions to observe their phenotypes.

3. Results

(i) *Strain d4-662 is a mutant belonging to the* pwB *group*

One of the pawn mutants of *P. tetraurelia*, d4-662, was isolated by chemical mutagenesis with MNNG (*N*-methyl-*N′*-nitro-*N*-nitrosoguanidine) in the mid 1980s and at that time designated as *pwD* (Saimi & Kung, 1987). The mutational defect of d4-662 is found in the voltage-dependent Ca^{2+} channel, and the behavioural responses are very similar to those of the three other pawn mutants, but crosses with them showed complementation. Therefore, d4-662 was then considered to be a fourth pawn mutant *pwD*, though a full genetic analysis has not been published. However, we have now obtained results indicating that d4-662 belongs to *pwB,* rather than to *pwD*. Evidence for this is given in the following account.

First, we confirmed that d4-662 is controlled by a single recessive gene. When d4-662 was crossed with the wild-type, all the F_1 progeny expressed wild-type and F_2 obtained by autogamy (self-fertilization) from these F_1 organisms always showed a 1:1 ratio of wild-type to pawn (data not shown), indicating that d4-662 is controlled by a single Mendelian gene. However, in test tube cultures of d4-662, cells that were indistinguishable from wild-type cells in behavioural phenotype were often observed. Each phenotype, either pawn or wild-type, is very stable in vegetative growth, and never changes under various physiological

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Table 2. *Curing of the mutant phenotype of d4-662 by cytoplasmic transplantation from pawns and CNRs*

Donor	Duration of backward swimming (s)[a]
P. tetraurelia	
Wild-type	23·7 ± 10·4 (10)*
pwA	19·7 ± 10·6 (4)
pwB^{95}	0 (15)
pwC	21·6 ± 11·1 (5)
d4-662	0 (16)
P. caudatum	
Wild-type	10·0 ± 3·7 (7)*
cnrA	13·0 ± 5·3 (11)
cnrB	8·7 ± 1·6 (6)
cnrC	6·8 ± 1·7 (6)
cnrD	11·2 ± 2·3 (12)

* Numbers in parentheses indicate the number of injected cells.
[a] Duration of backward swimming (s ± SD) in 20 mM stimulation solution 3–5 h after cytoplasm had been injected into the recipients (d4-662).

conditions, such as temperature, starvation or clonal ageing. The appearance of these wild-type cells, which we denote R662, is not caused by a reverse mutation of the mutant to the wild-type allele but is phenotypic, because progeny from the cross between two cells of type R662 showed mostly the pawn phenotype (see Table 6). When d4-662 or R662 are subjected to sexual reproduction such as autogamy, some R662 types are produced in both parental phenotypes, but the frequency never exceeds 8 % (Table 6, fourth and fifth columns and data not shown).

(ii) *The* pwB *and d4-662 mutants do not show complementation when cytoplasm or macronucleoplasm is transplanted between them*

If d4-662 belonged to a different genic locus from other pawns, the mutational lesion of d4-662 might be cured by microinjection of cytoplasm from the other mutants. This approach has already been demonstrated successfully (Haga *et al.*, 1983). By transfer into a high K^+ stimulation solution (20 mM KCl in Dryl's solution) 3–8 h after microinjection of cytoplasm from the other mutants, transient restoration of the excitability of d4-662 cells was observed. Table 2 shows that not only the wild-type of *P. tetraurelia*, *pwA* or *pwC* but also all four CNR mutants rescued the defect of d4-662, with the exception of pwB^{95} and d4-662 itself. Reciprocal injections of the cytoplasm from d4-662 also showed curing effects to *pwA*, *pwC* and all four CNRs, but not to pwB^{95} (Table 3). In these experiments, the duration of backward swimming induced by K^+ solution was shorter than in the uninjected wild-type (35 s in *P. tetraurelia* and 90 s in *P. caudatum*) in most recipient cells, but clear responses of the recipients were observed. The entire backward swimming behavior in the recipients reverted to that of the level of the uninjected mutants in 48 h, indicating that the effects of the cytoplasmic injection were transient (Haga *et al.*, 1983). The most important evidence in these experiments is that d4-662 and pwB^{95} did not complement each other (Tables 2, 3). Rescue of d4-662 and pwB^{95} with wild-type cytoplasm showed that the amount of cytoplasm transfused was sufficient to complement the mutant phenotype. The absence of complementation between d4-662 and pwB^{95} may suggest that they belong to the same complementation group. These results contradict a previous report that d4-662 is a different mutant from *pwB* (Saimi & Kung, 1987).

The macronucleus of *Paramecium* is not only large enough for microinjection but also highly polygenomic (> 1000 copies) and transcriptionally active (see Wichterman, 1986). When the nucleoplasm of a macronucleus of d4-662 was transplanted into the macronucleus of pwB^{95}, pwB^{95} was never rescued, and vice versa. Lack of complementation between d4-662 and pwB^{95} was not due to the amount of nucleoplasm transplanted, because a similar amount of macro-

Table 3. *Restoration of excitability in mutants by cytoplasmic transplantation from d4-662 to pawns and CNRs*

Donor	Recipient						
	pwA	pwB^{95}	*pwC*	*cnrA*	*cnrB*	*cnrC*	*cnrD*
P. caudatum							
Wild-type	28·8 ± 13·3 (4)	11·1 ± 4·4 (6)	–	26·9 ± 12·9 (2)	17·0 ± 9·8 (13)	75·4 ± 25·9 (6)	9·3 ± 4·8 (6)
P. tetraurelia							
Wild-type	22·5 ± 4·4 (7)	19·4 ± 6·9 (11)	–	23·0 ± 9·7 (6)	11·5 ± 4·4 (6)	65·3 ± 32·1 (11)	16·7 ± 4·6 (3)
d4-662	29·2 ± 11·5 (6)	0 (14)	19·4 ± 5·8 (9)	18·2 ± 2·5 (3)	16·9 ± 8·1 (5)	46·7 ± 12·2 (3)	27·1 ± 12·8 (6)
No injection	0 (20)	0 (20)	1·0 ± 1·9 (20)	1·5 ± 2·4 (20)	2·4 ± 2·6 (20)	1·1 ± 1·9 (20)	0 (20)

Numbers are the duration of backward swimming (s) ± SD. –, not determined. Numbers in parentheses indicate the number of cells tested. Duration of backward swimming of the uninjected wild-type is about 35 s and 90 s in *P. tetraurelia* and *P. caudatum*, respectively.

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Table 4. *Complementation tests by nucleoplasmic transplantation*

Donor	Recipient pwB^{95}	d4-662	*cnrA*	*cnrD*
P. tetraurelia				
Wild-type	20·8 ± 13·6 (8)	23·2 ± 17·4 (23)	51·0 ± 35·1 (9)	93·5 ± 82·9 (14)
pwB^{95}	0 (14)	0 (32)	30·6 ± 15·0 (8)	63·4 ± 41·3 (14)
d4-662	0 (25)	0 (33)	37·1 ± 29·0 (15)	65·6 ± 40·5 (21)
P. caudatum				
Wild-type	13·6 ± 5·8 (9)	24·8 ± 16·6 (19)	84·2 ± 29·7 (7)	60·5 ± 34·6 (9)
cnrA	8·9 ± 3·1 (11)	16·0 ± 6·6 (23)	0 (11)	41·3 ± 21·5 (9)
cnrD	15·7 ± 11·1 (9)	23·7 ± 23·8 (17)	61·2 ± 30·6 (8)	0 (14)

One or two days after transplanting the macronucleoplasm, the behaviour of the recipient was examined. The volume of injection was more than two-thirds of the macronucleoplasm of the recipients. See footnotes to Table 2.

Table 5. *Nucleoplasmic transplantation with marker genes*

Donor	Recipient	No. of cells: Injected	Rescued: Trichocyst	Behaviour
Wild-type	d4-662	26	–	23
Wild-type	pwB^{95}	8	–	6
Wild-type	pwB^{96}	11	–	10
pwB^{95}; *nd7*	d4-662; *nd169*	15	6	0
d4-662; *nd169*	pwB^{95}; *nd7*	7	6	0
pwB^{96}; *nd*169	d4-662; *nd6*	13	7	0
d4-662; *nd6*	pwB^{96}; *nd*169	5	5	0

–, not determined. See footnotes to Table 4.

nucleoplasmic transfer from *cnrA* or *cnrD* of *P. caudatum* worked well (Table 4). Moreover, trichocyst non-discharge mutations (*nd6*, *nd7* and *nd169*) used as marker genes for d4-662 and other *pwB* (pwB^{95} and pwB^{96}) effectively rescued each other (Table 5).

The above observations strongly suggest that d4-662 is a different allele belonging to the *pwB* locus, and thus a better designation for it would be pwB^{662}.

(iii) *Progeny from crosses of d4-662 with* pwB *express predominantly the wild-type phenotype*

To know why d4-662 had previously been misjudged as *pwD*, the genetic relationship between d4-662 and *pwB* was re-examined by crossbreeding analysis. If d4-662 and *pwB* were mutants at the same locus, only pawn progeny would be produced after crosses between them. The results obtained were the opposite. When d4-662 was crossed with *pwB* mutants, pwB^{95} or two strains of pwB^{96} (crosses 1 and 2), many wild-type F_1 progeny were obtained and the percentages of pawn in the F_1 were less than 12·8% (Table 6). Wild-type phenotype observed in progeny from these crosses, however, showed a variable level of responses to stimulation solution (for example, see Table 9, third to sixth columns). We judged cells showing backward swimming for over 3 s as wild-type phenotype. In addition to the wild-type, many mixed clones of wild-type and pawn cells appeared in progeny obtained from both crosses. In the controls, a cross between pwB^{95} and pwB^{96} produced only pawn progeny, and a cross within d4-662 produced 3% of mixed clones from which the phenotypic wild-type (R662) of d4-662 was isolated. As shown in Table 6, the ratio of wild-type or mixed-clone F_1 progenies varied depending on the *pwB* strains used but was much greater than the frequency of R662 upon crosses between d4-662 (d4-662 × d4-662 in Table 6). Thus, the crossbreeding analyses show that d4-662 (pwB^{662}) heterozygotes with pwB^{95} or pwB^{96} express mostly the wild-type phenotype, irrespective of the fact that a single recessive gene controls each mutant phenotype. This may explain why d4-662 was once misjudged as *pwD*. The incidence of R662 in a cross within R662 was less than 8%. However, many wild-type progeny were obtained from the cross of R662 with pwB^{96} (Table 6),

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Table 6. *F_1 phenotype from crosses among* pwB *mutants*

Cross	Phenotypes of synclone[a]		
	Wild-type	Mixture	Pawn
d4-662 × pwB^{95}	43	36	0
d4-662 × pwB^{96}			
Cross 1	118	107	8
Cross 2	18	64	12
d4-662 × d4-662	0	2[b]	44
R662 × R662	0	8[b]	105
pwB^{95} × pwB^{96}	0	0	53
R662 × pwB^{96}	15	1	0

R662; d4-662 expressing wild-type phenotype.

[a] Two clones derived from a conjugating pair are called a synclone and the four cells from the first cell division of two exconjugants are called caryonides. These four F_1 caryonides were separated then the phenotype of the synclone was determined. For example, synclones of 'wild-type' contain four wild-type caryonides and those of 'mixture' contain wild-type and pawn caryonides. Survival of synclones was 100% in all crosses where at least one caryonide derived from both exconjugants survived. True crosses were confirmed by trichocyst marker genes.

[b] Probably a mixture of R662 and pawn.

suggesting that some maternal effects are involved in the inheritance.

The macronucleus is developed from a fertilized nucleus through extensive genomic rearrangement (Coyne *et al.*, 1996; Klobutcher & Herrick, 1997). To establish whether the above inheritances involve a problem in the macronuclear developmental process, the products of the first cell division after conjugation (caryonides) were grown separately (Table 7). Since a caryonide is a clone deriving from a single macronuclear primodium, four caryonides from a conjugated pair are produced and have an identical genotype but contain independently developed macronuclei. If the macronuclear developmental process associates with the inheritance of d4-662 and/or another *pwB*, the expressed phenotypes of the progeny should show a pattern of caryonidal or cytoplasmic inheritance. In crosses using *pwB*, the pattern of the expressed phenotypes was often caryonidal, as shown in Table 7. The mixed clones in Table 6 resulted from these clones, expressing different phenotypes in four caryonides. Thus, the macronuclear developmental process seems to be involved in the inheritance of d4-662 in the cross with either pwB^{95} or pwB^{96} examined in this study.

(iv) *Some F_1 progeny from the crosses of d4-662 with other* pwB *produce many wild-type progeny in the F_2*

Autogamy is a self-fertilization of *P. tetraurelia* in which meiotic products divide once and subsequently fuse to form a fertilized nucleus so that the progeny become completely homozygous for all genes and genes that in the F_1 are heterozygous, segregate. Therefore, when F_1 organisms are subjected to autogamy, the progeny might be all pawns if the F_1 progeny are diploid heterozygotes of d4-662 and either pwB^{95} or pwB^{96}. This segregation was observed in cross 1 of d4-662 with pwB^{96} (128 among 137 or 93·4% F_2 progeny expressed the pawn phenotype; Table 8), indicating that d4-662 is controlled by a gene that is allelic to *pwB*. However, only 45·2% (70 among 155 F_2) or 74·6% (194 among 260 F_2) of the progeny expressed the pawn phenotype in the crosses of d4-662 and pwB^{95} or pwB^{96} (cross 2), respectively (Table 8). To examine the nature of the F_2 expressing wild-type in the cross of d4-662 with pwB^{96} (cross 2), F_3 progeny were obtained

Table 7. *Distribution of phenotypes in four F_1 caryonides from the crosses between d4-662 and* pwB[95] *or* pwB[96]

Phenotypes	Distribution of phenotypes derived from cytoplasmic parents				No. of synclones		
	d4-662		pwB^{95} or pwB^{96}		d4-662 × pwB^{95}	d4-662 × pwB^{96}	
						Cross 1	Cross 2
All wild type	W	W	W	W	20	31	12
Mixture of wild-type and pawn	W	W	W	P	12	30	7
	W	P	W	W	2	10	4
	W	P	W	P	7	9	4
All pawn	P	P	P	P	0	0	0

Cytoplasmic parents were traced by mating types of the progeny because in this species, mating types are known to show cytoplasmic inheritance (Sonneborn, 1947). Only synclones where four caryonides survived are presented.
W, wild-type phenotype; P, pawn phenotype.

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Table 8. *Segregation of the F_2 phenotype from autogamy of the F_1*

Cross	Survival (%)	Behaviour			Trichocyst[b]			
		Wild-type	Mixture[a]	Pawn	Discharge	nd	χ^2	*P*
d4-662; *nd6* × *pwB*[95]	57	75	10	70	74	81	0·3	> 0·5
d4-662; *nd6* × *pwB*[96]; *nd169*								
Cross 1	69	5	4	128	29	108	1·1	> 0·2
Cross 2	90	49	17	194	68	192	0·2	> 0·5

Progeny were obtained from both cytoplasmic parents and from various phenotypes in the F_1.
[a] F_2 clones containing wild-type and pawn cells.
[b] Expected ratio is 1:1 for cross d4-662; *nd6* × *pwB*[95], and 1:3 for crosses d4-662; *nd6* × *pwB*[96]; *nd*169.

Table 9. *Transplantation of cytoplasm into d4-662 or* pwB[96] *from R662 or wild-type descendants of the cross between d4-662 and* pwB[96]

Donor		Recipient	
Strains	Duration of backward swimming (s)	d4-662	*pwB*[96]
R662	12·1 ± 6·9	0 (7)	–
	11·8 ± 2·8	–	0 (11)
d4-662 × *pwB*[96]			
F_5 clone 1	5·1 ± 1·8	1·4 ± 2·1 (5)	–
	6·0 ± 3·7	–	7·4 ± 3·8 (3)
F_5 clone 2	12·3 ± 2·2	20·2 ± 6·6 (4)	–
	17·6 ± 4·0	–	22·2 ± 11·8 (6)

Numbers are duration of backward swimming (s) ± SD in the stimulation solution. Numbers in parentheses indicate the number of cells examined. –, not determined.

by autogamy from 3 F_2 organisms. Again, 34 wild-type and 16 pawn progenies were obtained, suggesting that some wild-type F_2 are still heterozygous in spite of the fact that the F_2 was induced by autogamy. The phenotypic segregation in the F_3 is close to the ratio of wild-type to pawn, i.e. 2:1 ($P > 0·8$). This segregation continued to F_4 and F_5 and subsequent generations. Similarly, mass ex-autogamous clones from wild-type F_2 in the cross of d4-662 with *pwB*[95] became mixed clones of wild-type and pawn cells (data not shown). The genetic nature of this cross will be discussed in Section 4.

(v) *Wild-type progeny from crosses between d4-662 and* pwB *are not R662*

The segregant expressing wild-type in F_2 and the phenotypic wild-type of d4-662 (R662) are indistinguishable phenotypically, that is, in response to K^+ stimulation solution. However, the cytoplasm of R662 did not rescue the defects of d4-662 and *pwB* (Table 9). On the other hand, descendants from the F_2 progeny (F_5 by three successive rounds of autogamy of F_2) rescued the defects of these mutants, indicating that the wild-type segregants are not R662.

4. Discussion

The main focus of our work was to analyse the genetics of strain d4-662, belonging to the pawn class of mutants. To analyse the genetical relationship of two mutants we performed microinjection of cytoplasm and macronucleoplasm between mutants. Macronucleoplasmic transplantation was found to work well when it was difficult to rescue mutants with cytoplasmic transplantation, as shown in *cnrA* and *cnrD* of *P. caudatum* (Table 4). Evidence showing no complementation between d4-662 and *pwB* by microinjection of cytoplasm or macronucleoplasm (Tables 2–4) strongly suggests that the mutant d4-662 belongs to the same locus as *pwB* and, thus, the gene controlling d4-662 should be designated as *pwB*[662]. This conclusion is further supported by the recent observation that a molecular defect of d4-662 was found to be a single-base substitution inside an IES of the *pwB* gene, and, apparently, the mutation prevents excision of the IES from the *pwB* gene in the developing macronucleus (Haynes *et al.*, 2000). However, whether the presence of this IES in the coding region of the *pwB* gene abolishes the function of the gene product is not clear because the amount of the *pwB* gene transcripts is

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below the detectable level in this mutant (Haynes *et al*., 2000). We showed that this mutant produces phenotypic wild-type (R662) after sexual reproduction at low frequency. The cytoplasm of R662, however, did not rescue the defect of *pwB*^{662} and *pwB*^{96}. This may suggest that the *pwB* gene product of R662 qualitatively differs from that of the 'true' wild type.

d4-662 was misjudged as a new pawn mutant, *pwD* (Saimi & Kung, 1987), because the F_1 of the cross between d4-662 and *pwB* mutants expresses predominantly the wild-type. When the F_1 of a cross between two recessive mutants expresses the wild-type phenotype they are usually judged to be independent mutants controlled by two different genic loci. When the F_2 were obtained by autogamy of F_1, however, three different segregation ratios were observed depending on the *pwB* strains used. The simplest results of segregation observed among them was that almost all the progeny expressed pawn. This is consistent with d4-662 being a *pwB* mutant. In the second cross, many wild-type progeny (25·3%) were obtained. If *pwB* and d4-662 belonged to different loci, then the wild-type progeny in the F_2 should be homozygotes of wild-type alleles, because autogamy makes all progeny completely homozygous in the whole genome. However, when F_3 progeny were obtained by autogamy from the F_2 expressing the wild-type, they again produced wild-type and pawn progenies. Therefore, wild-type progeny in the F_2 were not genetically homozygous but heterozygous. In subsequent generations, the wild-type progeny continued to produce wild-type and pawn segregants in a ratio close to 2:1. Although the appearance of this heterozygous progeny is not yet fully understood, the segregation ratio suggests that the heterozygous wild-type might be not disomy but tetrasomy of a chromosome bearing *pwB*. If the genotype of wild-type progeny in the F_2 is $pwB^{662}/pwB^{662}/pwB^{96}/pwB^{96}$, heterozygous wild-type will continue to be produced in the next generation. These heterozygous progeny may express the wild-type phenotype as observed in F_1 heterozygotes. Thus, *pwB*^{96} strains may be in two states: disomy (cross 1 in Table 8) and tetrasomy (cross 2 in Table 8). Whether this prediction is correct is now under analysis. Finally, the F_2 from the cross between d4-662 and *pwB*^{95} showed a segregation ratio of almost 1:1. The nature of this wild-type F_2 progeny is not known, but again they became mixed clones in subsequent autogamous generations, indicating that these were not true wild-type homozygotes. In conclusion, it seems likely that the mutant gene of d4-662 is allelic to other *pwB* but has an unusual feature of complementing them in heterozygotes.

The question why the F_1 heterozygotes of *pwB*^{662} and *pwB*^{95} or *pwB*^{96} express the wild-type phenotype (Table 6) is still unsolved. Changes in the methylation pattern sometimes bring about unusual complementation (Schläppi et al., 1994), but *Paramecium* lacks cytosine methylation (Cummings *et al*., 1974), which is known to cause transcriptional inactivity (Laird & Jaenisch, 1996; Kass *et al*., 1997). Similarly, heterochromatin formation associated with deacetylation of histone is known to cause relatively stable repression of transcription (Kennison, 1995; Weiler & Wakimoto, 1995; Pirrotta, 1997; Klar, 1998). Although the involvement of these modifications in the expression of the *pwB* and *pwB*^{662} gene cannot be discounted, it is more reasonable to assume that the problems of nuclear dimorphism and development in ciliates is involved in the unusual inheritance of these mutants.

When the R662 was used for the cross, some maternal effects were observed (Table 6). Whether the cause of this phenomenon is connected with some property of the old macronucleus is unknown. However, in the crosses of *pwB*^{662} and *pwB*, the expression pattern of phenotypes in the F_1 was not cytoplasmic but partly caryonidal. These results suggest that inheritance involves some macronuclear developmental process.

The most important problem seems to be how the heterozygote of mutant alleles belonging to the same locus produced wild-type progeny. The results obtained strongly suggest that the inter-allelic interactions between *pwB*^{662} and *pwB*^{95} reveal the wild-type phenotype in the developing macronucleus. This phenomenon may be specific to the developmental process because no allelic interactions in the vegetative stage occurred when the macronucleoplasm from both mutants was directly mixed by microinjection (Table 4). An example of allelic interactions has been reported in a case of d12 and d48 mutants of surface antigen by Rudman *et al*. (1991). The heterozygotes of d12 and d48 expressed wild-type surface antigen. A similar example of *SerH1* gene in *Tetrahymena thermophila* suggests intragenic recombination during macronuclear development (Deak & Doerder, 1998). Molecular analysis may explain which case occurred in the inheritance of *pwB*^{662} and *pwB*^{95} or *pwB*^{96}. Our results suggest that the wild-type gene in the macronucleus did not result from the re-arrangement of different genes but from inside the same locus, and the wild-type phenotype is expressed neither by protein–protein interaction nor by recombination in the vegetative stage.

We wish to express our gratitude to Drs Ching Kung and Koichi Hiwatashi for helpful discussions and to Dr James D. Forney for critical reading of the manuscript. Thanks are also due to Drs Jean Cohen and Yoshiomi Takagi for supplying mutant strains.

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