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Inheritances of Behavioral Mutant *pawn-B* in *Paramecium tetraurelia*

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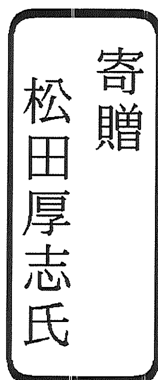

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ABBREVIATIONS

CNR	Caudatum non-reversal
DM	Double minute
ETDA	Ethylenediamine-N,N,N′,N′-tetraacetic acid
HRP	Horseradish peroxidase
IES	Internal eliminated sequence
MNNG	N-methyl-N′-nitro-N-nitrosoguanidine
PCR	Polymerase chain reaction
R662	Revertant of d4-662
RT-PCR	Reverse transcription-PCR
TE	Tris-EDTA

ABSTRACT

Pawn mutants in ciliate *Paramecium tetraurelia* have defect in the membrane excitation and do not show backward swimming upon any stimuli. One of pawn mutant, *pwB*, had long been considered to be a Mendelian mutant, but present study shows that all three strains belonging to *pwB* were genetically unusual in that they did not show conventional Mendelian inheritance. The way of inheritances of these three strains were different from each other and some of them was tightly involved in the nuclear differentiation in ciliate. In ciliates, gene expression is thought to take place only in the somatic macronucleus. The macronucleus, however, degenerates and newly develops in every sexual reproduction through massive genomic rearrangement of the germinal micronuclear genome including elimination of germ-line limited sequences (Internal Eliminated Sequences or IESs) and gene amplification.

One of *pwB* strains, d4-96, was found to be a "tetrasomy" of the micronuclear chromosome carrying pwB^{96} allele. Genetic analysis on the tetrasomy suggests that the tetrasomy of the chromosome carrying mutant *pwB* allele (pwB^{96}) was much more stable than the tetrasomy of the chromosome carrying wild-type *pwB* allele (pwB^{+}).

Another *pwB* strain, d4-95, showed non-Mendelian inheritance. In the cross of d4-95 with wild type, F_1 heterozygotes often expressed pawn phenotype. Furthermore, F_2 progeny that should be wild-type homozygotes produced pawn progeny after subsequent self-fertilizations. Molecular analysis performed on this

strain revealed that the strain had an amplified mutant *pwB* gene in its micronucleus. Repeated backcrossing of d4-95 to wild-type strain successfully reduced the frequency of occurrence of non-Mendelian inheritance in the resulted segregants and the segregants that show completely normal Mendelian inheritance were eventually obtained after seventh backcrossing. Therefore, the amplified mutant *pwB* gene in the micronucleus of strain d4-95 was heritable independently from the original *pwB* locus, and progeny receiving this bulk *pwB* gene copy seems to express pawn phenotype even if they had the wild-type *pwB* gene.

The third *pwB* strain, d4-662, was formerly thought to be a different pawn mutant from *pwB*, but I found that this strain is actually *pwB* mutant. Strain d4-662 and two other *pwB* strains (d4-95 and d4-96) did not show complementation when cytoplasm or macronucleoplasm was transplanted each other. When d4-662 was crossed with other *pwB* strains, however, they complemented and produced wild-type progeny. Results obtained from detailed genetic analyses strongly support the hypothesis that d4-662 have a recessive allele of pwB^{662} while this allele produces wild-type pwB^{+} gene in heterozygous compositions with other *pwB* alleles through developmental gene rearrangement of the macronucleus. Strain d4-662 also has a peculiar genetic feature, that is, cells phenotypically similar to wild type often appear in the culture. Genetic and molecular investigations on this phenotypic reversion of d4-662 showed that the reverted cells were produced only after sexual reproduction and a removal of the mutant IES in the coding region of pwB^{662} allele was associated with the appearance of reverted cells. Thus alternative excision of the mutant IES

seem to be a basis for the production of wild-type *pwB*$^{+}$ gene in this strain. All *pwB* strain thus independently showed unusual genetic features, and analyses on these genetic systems will provide an example for the amazing cellular regulation of unusual genetic systems in ciliate.

GENERAL INTRODUCTION

Ciliates, such as *Paramecium tetraurelia*, provide an opportunity to study unique genetical phenomena because of its nuclear dualism in a common cytoplasm. After the sexual process, two kinds of nuclei differentiate from the fertilized nucleus. The micronucleus (also called as the germ nucleus) transmits the genetic information while any transcriptional activities in the vegetative growth have not been demonstrated (Raikov, 1996). The macronucleus (also called as the somatic nucleus) originates from the micronuclear genome but it serves for a place where transcriptions of genes take place (Raikov, 1996). In addition to their apparent size difference, the micronucleus and the macronucleus have some different features, including their modes of division, i.e. the micronucleus divides mitotically while the macronucleus divides amitotically (Raikov, 1996). Furthermore, in most ciliate, the genome of the macronucleus is greatly different from that of the micronucleus because of a gene rearrangement and gene amplification in the developmental process of the macronucleus (Prescott, 1994; Raikov, 1996). The macronuclear genome is functional for gene expression but unable to transmit genetic information. Thus, the macronucleus degenerates and newly develops in every sexual reproduction.

In *P. tetraurelia*, gene rearrangement in the macronuclear anlagen includes *de novo* telomere addition to the fragmented chromosomes, elimination of IESs (Internal Eliminated Sequences) and gene amplification to about 800 - 1700 copies (Coyne *et al.*, 1996; Klobutcher and Herrick, 1997). Among these processes, the best understood

process is the IES elimination. *Paramecium* IES is a relatively short (26 bp to about 1 kb) and non-coding sequence found in and out of the protein coding regions. Over 50000 IESs are precisely eliminated from the micronuclear genome in every macronuclear development (Steele *et al.*, 1994; Duharcourt *et al.*, 1998). The mechanisms of how *Paramecium* IESs are recognized over the genome and how they are precisely excised remain largely unknown, but several elements required in *cis* and *trans* have been identified (Klobutcher and Herrick, 1995; Meyer and Keller, 1996; Duharcourt *et al.*, 1998; Ku *et al.*, 2000). Among the elements, statistically similar terminal inverted repeats consisting of eight base pairs are shared by all known *Paramecium* IESs and are similar to the terminal sequences found in *marinar*/Tc1 and *Euplotes* Tec transposable elements (Klobutcher and Herrick, 1995). The significance of this sequence has been experimentally confirmed by mutant analyses. IESs with mutations in the terminal sequence remained inserted in the mutant, indicating that this sequence is essential for the excision of the *Paramecium* IESs (Mayer *et al.*, 1998; Mayer and Forney, 1999).

P. tetraurelia has two types of sexual reproductions that make genetic manipulations in this organism very useful (Sonneborn, 1947; Sonneborn, 1970). In both sexual reproductions, one of the haploid meiotic products is selected to undergo an additional round of mitosis while the residual nuclei disappear. Two gametic pronuclei thus generated should be genetically identical. Conjugation is a process where one of the gametic pronuclei is exchanged from each conjugating partner. Alternative sexual reproduction in this species is a self-fertilization process called autogamy, where two gametic pronuclei fertilize in a single cell. Thus autogamous

progeny should be homozygous for all genome.

Several non-Mendelian inheritances have been known in *Paramecium* and in its close relative *Tetrahymena*, and studies of these genetic phenomena construct a large body of the history of the genetics in these ciliates (Preer, 2000). Two major classes of non-Mendelian inheritance are introduced below.

The first class is a peculiar inheritance called caryonidal inheritance. Caryonides are lineages of ciliates that derive their macronuclei (somatic nuclei) from single macronuclear primordia (anlagen), number of which is usually more than two in an exconjugant. Caryonidal inheritance, by which genetically identical exconjugants inherit different phenotypic characters in different caryonides, has been reported for mating types of *P. primaurelia* (Sonneborn, 1977) and *Tetrahymena thermophila* (Orias, 1981), and circadian rhythm of mating type reversals in some strains of *P. multimicronucleatum* (Barnett, 1966). The mechanisms that bring about caryonidal inheritance should involve macronuclear developmental process, though a detailed molecular mechanism involving alternative gene rearrangement waits to be confirmed (Orias, 1981).

The second, and the most divergent yet important class of non-Mendelian inheritances in ciliate is cytoplasmic inheritance. Cytoplasmic inheritance in *Paramecium* has been reported not only for the killer and mitochondrial traits but also for mating types, trichocyst discharge, and serotypes (Sonneborn, 1947, 1948; Sonneborn and Schneller, 1979; Epstein and Forney, 1984). In these inheritances, different mechanisms seem to underlie in different instances. Cytoplasmic inheritance may also be possible to include the inheritance of cortical structure

(Nanney, 1968; Sonneborn, 1975; Aufderheide *et al.*, 1980). Inverted ciliary rows in *Paramecium* and *Tetrahymena* propagated for hundreds or thousands of cell divisions (Beisson and Sonneborn, 1965; Ng and Frankel, 1977). The propagation of inverted ciliary row was demonstrated to be independent from genetic differences between the clones that bear ciliary inversion and that do not (Beisson and Sonneborn, 1965). This phenomenon is apparently brought about by the intrinsic polarity of the basal bodies that compose the ciliary rows and suggests the significant role of the preexisting structure in the inheritance of cortical structures (Sonneborn, 1975; Aufderheide *et al.*, 1980). Recent analysis on other cytoplasmic inheritance including mating type and serotype mutant in *P. tetraurelia* (Sonneborn, 1947; Epstein and Forney, 1984) provides molecular insight into another unique mechanism of cytoplasmic inheritance involving gene rearrangement (Forney *et al.*, 1996; Meyer and Duharcourt, 1996).

In mutant strain d48, that does not express A surface protein and shows cytoplasmic inheritance, a normal *A* antigen gene (*A* gene) is actually present in the micronucleus, but is missing in the macronucleus (Epstein and Forney, 1984; Forney and Blackburn, 1988). Injection of the normal *A* gene copies in the mutant macronucleus, however, permanently rescued this aberrant telomere addition upstream of the *A* gene (Harumoto, 1986; Koizumi and Kobayashi, 1989; Jessop-Murray *et al.*, 1991; Kim *et al.*, 1994; Scott *et al.*, 1994; You *et al.*, 1994). Alternatively, injection of a portion of *G* gene in the wild type macronucleus induced gene deletion in a sequence specific manner in the subsequent sexual generations (Meyer, 1992; Meyer *et al.*, 1997). This epigenetic effect of the old macronucleus on the process of

gene rearrangement was also observed for the excision of some IESs of *P. tetraurelia* and *T. thermophila* (Duharcourt *et al.*, 1995; Chalker and Yao, 1996; Duharcourt *et al.*, 1998). This mechanism seems to be involved in mating type determination in *P. tetraurelia* (Meyer and Keller, 1996). These evidences strongly suggest that DNA or RNA molecules in the old macronucleus enter the new macronucleus and guide the gene rearrangements in the next generation (Forney *et al.*, 1996; Meyer and Duharcourt, 1996). If this is really correct, this will be the first example of the inheritance of preexisting gene structures.

Although non-Mendelian inheritances are found in almost all organisms that participate in sexual reproductions, the mechanisms involving non-Mendelian inheritances of ciliates are quite unique in comparison to other organisms mainly because of their nuclear dimorphism. However, many unusual inheritances sometimes appear to be brought about by the unique features in other organisms, for example, homology-dependent gene silencing in plants and in *Neurospora*, transposon in maize, and transvection in *Drosophila*, but recent understandings of the mechanisms involved in these unusual genetic systems suggest that the basic mechanisms in these phenomena are rather common in eukaryotes. For example, Repeat-Induced Point mutation (RIP) in *Neurospora* was now known to be a byproduct of repeat-induced DNA methylation, and DNA methylation on repeated sequences is found in almost all eukaryotes (Selker, 1997). The mechanism of transvection in the bithorax complex of *Drosophila*, where correct gene expression requires allelic interaction such as pairing or proximity of the affected alleles, has not yet been fully understood (Henikoff, 1994; Kennison, 1995; Pirrotta, 1997), but the

phenomenon have been widely found in eukaryotes (Aramayo and Metzenberg, 1996, and references therein). Furthermore, alleles, which are subjected to imprinting, in the nucleus of mammalian diploid cells are closely located each other (LaSall and Lalande, 1996). Therefore non-Mendelian inheritances in ciliate does not necessarily emphasize that ciliates are radically different from other eukaryotes. But underlying mechanisms in non-Mendelian inheritances in ciliate seems to be also common. Basal body duplication is comparable to the centriol duplication, and its structural inheritance is widely observed in metazoa. The DNA rearrangements similar to caryonidal inheritance are found in cassette mechanisms in yeast (Nasmyth, 1982), and DNA-RNA interaction can be found in DNA replication and in gene rearrangement of bacteria, yeast and mammals (Griffiths, 1995; del Solar *et al.*, 1998; Dalgaard and Klar, 1999; Tracy *et al.*, 2000). Moreover, DNA rearrangements are similar to the process normally operated in DNA repair after double-strand break (Nasmyth, 1982; Mckim and Hayashi-Hagihara, 1998; Marsh *et al.*, 2000; Stavnezer, 2000). Thus, the similar mechanisms are differently used or expressed in different organisms. This implies that ciliates will help us to observe the different aspects of common objects occurring in eukaryotes.

Here analyses of unusual genetic phenomena in *P. tetraurelia* will be described. These genetic phenomena were found in behavioral mutant, "pawn," well known in *Paramecium* genetics.

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 behavioral 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 of intraciliary Ca^{2+} that is tightly correlated with the generation of action potentials based on the activation of voltage- dependent Ca^{2+} channels (Ca^{2+} channels) (Eckert, 1972), that is, membrane excitation.

Many behavioral mutants were isolated in *Paramecium*. Among them, the most important mutant class may be three pawn mutants in *P. tetraurelia* (*pwA*, *pwB* and *pwC*) and four CNR mutants in *P. caudatum* (*cnrA*, *cnrB*, *cnrC* and *cnrD*) (Kung, 1971; Chang and Kung, 1974; Schein, 1976; Takahashi, 1979; Takahashi *et al.*, 1985). Pawn and CNR mutants have a malfunction of Ca^{2+} channels and do not swim backward upon any stimuli (Kung *et al.*, 1975; Takahashi and Naitoh, 1978).

The genetic dissections of Ca^{2+} channel activity in *Paramecium* are particularly promising approach in the light of recent progress of studies in the regulatory system of voltage-gated Ca^{2+} channel activity in vertebrates. Calcium channels are found mainly in the synaptic terminus of vertebrates. Calcium currents generated by these channels strongly contribute the synaptic plasticity, thus they are main targets of synaptic modulators (Clapham, 1994; Hille, 1994; Tanaka and Nishizuka, 1994; Dolphin, 1996; Stanley, 1997). Although many modulators that alter Ca^{2+} channel activity were found, none of them seem to be absolutely required for Ca^{2+} channel gating.

Nucleotide sequences of *pwA* and *pwB* genes have already been determined

and found to code novel proteins (Haynes *et al.*, 1998; Haynes *et al.*, 2000). The *pwA* protein product is considered to be a GPI-anchored membrane protein and suggested to contribute correct anchoring of Ca^{2+} channels to the plasma membrane (Haynes *et al.*, 1998). The protein product of *pwB* is considered to be a membrane protein and pwB-GFP fusion protein localized throughout cytoplasm (Haynes *et al.*, 2000). The exact function of *pwB* gene product is, however, completely unclear.

The method to clone *Paramecium* genes was first established in these pawn genes indicating that the study of pawn mutants remains a leading field in both physiology and genetics of *Paramecium* (Preer, 1997; Meyer and Cohen, 1999). Pawn mutants have also been used for many genetic studies as Mendelian marker genes for more than 30 years.

I found, however, that strains of *pwB* are genetically unusual in that they did not show conventional Mendelian inheritance. The discovery of the unusual inheritances was accidental since the first aim of this study was whether a newly identified pawn strain, d4-662 (formerly designated as *pwD*; Saimi and Kung, 1987), is the same mutant as some CNR mutant in *P. caudatum* or not. However, this study eventually revealed that d4-662 is actually the same mutant as *pwB*, but shows complementation when they are crossed (Matsuda *et al.*, 2000). This starting study will be described in Part IV. Before going into this, it may be useful to understand the nature of the other two *pwB* strains because above study also suggested that two existing strains of *pwB*, namely d4-95 and d4-96, should have unusual genetic features. Further studies showed that the strain d4-96 was tetrasomy of the chromosome carrying *pwB* locus and stably maintained its tetrasomic genotypic composition

(Matsuda and Takahashi, 2001a). This will be described in Part I. In Part II, genetic and molecular investigation of non-Mendelian inheritance of d4-95 will be described. The inheritance of this strain was partly cytoplasmic as well as caryonidal, and involved gene amplification in the micronucleus. Finally, a phenotypic reversion of strain d4-662 will be described in Part IV (and also in Part III). A molecular investigation of phenotypic reversion in this strain revealed an imprecise excision of the mutated IES in macronuclear development (Matsuda and Takahashi, 2001b).

The present studies provide various mechanisms involved in unusual inheritances of *pwB*. The mechanisms ultimately shown to be involved in these inheritances are both common and uncommon. The phenomena were found in the context of *Paramecium* life, but may be able to be extended to other systems far from ciliates.

PART I

Stable maintenance of duplicated chromosomes carrying the mutant *pwB* gene in *Paramecium tetraurelia*

ABSTRACT

An allele of the behavioral mutant *pawn-B*^{96} has been reported as a typical recessive gene but was found to show a peculiar inheritance. When the F_2 progeny from crosses between the wild type and *pwB*^{96} were obtained by autogamy, the 1:1 phenotypic segregation ratio was observed as expected. However, two-thirds of the wild-type progeny in F_2 were thought to be heterozygotes because they became mixed progeny of wild-type and pawn clones in successive autogamies. Four marker genes showed the expected segregation ratio and stable phenotypes in these crossings. This result and the results of crossings using segregants from the above crosses indicated that parental *pwB*^{96} is a tetrasomy of the chromosome carrying the *pwB* gene. To determine the cause of chromosomal duplication in the mutant, the stability of the chromosome carrying the *pwB* locus was examined by genetic analyses. The disomy of both *pwB*^{96} and wild type and the tetrasomy of *pwB*^{96} showed genotypes that were relatively stable during several autogamous generations. However, in clones initially pure for the tetrasomy of wild type, disomic cells appeared within a few autogamous generations. The difference between the stabilities of the tetrasomy of *pwB*^{96} and that of the wild type might be partly due to differences between the growth rate of tetrasomy and disomy in *pwB*^{96} and the wild type but mostly the result of an unknown contribution of the chromosome carrying the *pwB*^{96} allele to the tetrasomic composition.

INTRODUCTION

Double sets of chromosomes are ordinarily maintained with accuracy. Duplication of mammalian chromosomes is thought to be one of the earliest events upon carcinogenesis or a cause of severe diseases (Lengauer *et al.*, 1998; Hernandez and Fisher, 1999). However, exceptions are found in insects, plants, and protozoa, where the ploidy or chromosome number can vary developmentally or by unknown reasons (de Rocher *et al.*, 1990; Lanzer *et al.*, 1995; Lu *et al.*, 2001). It is well known that it is easier to maintain stable polyploidy in a heterozygous or hybridized state, called allopolyploid, because of the tendency of chromosomes to pair with homologous chromosomes with their own species origin. On the other hand, an autopolyploid, in a homozygous state, shows reduced fertility due to unbalanced segregation of the chromosomes, which results in multivalents.

Many protozoa manifest indefinite chromosome number, karyotypes, and ploidy (Lanzer *et al.*, 1995). Ciliates, including *Paramecium*, are not an exception (Raikov, 1996). In the micronucleus of *Paramecium*, chromosome number is known to show inter- and intra-stock differences. Polyploidy was suggested in some races of *P. bursaria* and *P. caudatum* by cytological observations (Chen, 1940). In *P. tetraurelia*, cytological differences in chromosome number among stocks and among cells in a single stock have been reported (Dippell, 1954). Thus, polyploidy and aneuploidy might be common characteristics in *Paramecium.*

In spite of the results obtained from cytological observations, intra-stock aneuploidy had not been reported in genetic analyses in *Paramecium*. However, upon crossbreeding analysis, I found that a strain of the *pwB* mutant (d4-96) showed unusual inheritance, which implies that the strain was a tetrasomy of the

chromosome carrying the *pwB* locus.   I investigated the cause of duplication of the chromosome by genetic analyses and eventually found that the frequency of chromosome loss in the tetrasomy of the chromosomes carrying the mutant *pwB* allele and that carrying the wild-type allele was considerably different.

MATERIALS AND METHODS

Stocks and culture method: Table 1 shows the stocks used in this study.   All stocks are homozygotes.   Trichocyst non-discharge mutations (*nd6*, *nd7*, *nd9*, and *nd169*) and temperature-sensitive mutation (*ts111*) were used as recessive markers. Cells were cultured in lettuce juice medium in Dryl's solution (Dryl, 1959) inoculated with *Klebsiella pneumoniae* one or two days before use (Hiwatashi, 1968). Cells were grown at 25 – 27 °C unless otherwise noted.

Phenotypic observation: The behavioral phenotype of a clone was determined by transfer of more than 10 cells by micropipette into the stimulation solution (20 mM KCl in Dryl's solution).   When wild-type cells are transferred into the stimulation solution, they swim backward for 30 - 50 sec (Naitoh, 1968).   Cells of pawn mutants do not show backward swimming.   The discharge or non-discharge of the trichocyst was observed by adding a drop of saturated picric acid to the cells.   Temperature sensitivity was observed after growth for 2 days at 35 °C because the mutant dies in this condition.

Genetic analysis: Mating reactive cells of complementary mating types were mixed, and then conjugating pairs were isolated in a fresh culture medium.   In some experiments, both exconjugants of a pair were isolated and separately grown.

In every case, single cells were cloned after several postzygotic cell divisions. Phenotypes of F_1 clones were observed after they underwent more than 10 cell divisions from conjugation.

F_2 progeny were obtained from autogamy (self-fertilization) induced by starvation of mature F_1 cells (about 30 cell divisions after conjugation). One-hundred percent autogamy was determined when all >20 cells showed macronuclear fragmentation after being stained with Carbol fuchsin solution (Carr and Walker, 1961). Autogamous cells were isolated in a fresh culture medium, and phenotypes were observed after they had undergone 10 cell divisions.

After successive autogamies, some wild-type segregants in F_2 produced pawn as well as wild-type clones (see Results). These progeny were referred to as a "mixed" type. To examine the segregation of the non-mixed wild type vs. the mixed type in F_2, autogamy was induced in more than 50 cells of each F_2, and the cells were transferred to a fresh culture medium. After they had undergone about 10 cell divisions, the phenotype of F_3 was observed, and the mixed type and the non-mixed wild type were determined.

Counting the fission rate: A single cell was isolated in 0.4 ml of a fresh culture medium and allowed to grow. After 24 h, the cells were counted and again allowed to grow for 24 h. Cell divisions per day (r) were calculated by the following equation: $r = log_2 N$, where N is the number of cells produced by cell divisions in 24 h. The daily isolation procedure was continued for four days, and the numbers of cell division thus calculated were averaged.

RESULTS

Stock d4-96 is a tetrasomy of the chromosome carrying the *pwB* gene: Unlike wild-type cells, which show clear backward swimming for approximately 30 sec when transferred into the stimulation solution, pawn mutants do not show ciliary reversal leading to backward swimming. These behavioral responses in the stimulation solution were used to observe behavioral phenotypes.

As already reported by Kung (1971), the behavioral phenotype of strain d4-96, which has been known to carry the mutant allele of pwB^{96}, appeared to be controlled by a recessive gene. All F_1 progeny showed the wild-type phenotype in the crosses with the wild type (pwB^{+}/pwB^{+}) (see Figure 1A). Progeny from autogamy receive a diploid and completely homozygous nucleus resulted from a fertilization of two mitotic products of a single meiotic haploid product (Sonneborn, 1947). Therefore in autogamous progeny from single gene heterozygote, phenotypic segregation ratio should be 1:1. When the F_2 progeny were obtained by autogamy of F_1 of above cross, the segregation ratio of wild type vs. pawn was 1:1, as expected (Figure 1A and Table 2).

Nevertheless, I found an unusual inheritance of the original *pwB* strain, d4-96. Some wild-type F_2 progeny, which should be homozygotes, produced a mixed progeny of wild-type and pawn clones after successive autogamies (Figure 1 and Table 2). The segregation ratio of non-mixed vs. mixed progeny was close to 1:2 (Table 2).

To observe the appearance of the mixed clone, autogamous progeny were isolated from wild-type F_2 and from successive autogamous generations (Figure

1B). Table 3 shows the segregation of the phenotype in autogamous lineages from wild-type F_2's. Some wild-type parents produced only wild type, while others produced both wild type and pawn (close to the ratio of 5:1), and pawn parents produced only pawn progeny from autogamies. Parents which produced both wild-type and pawn clones from autogamy appeared in every autogamous generation.

The simplest interpretation of the inheritance of d4-96 is that the strain has four chromosomes carrying the *pwB* gene (Figure 2A). The model predicts that two kinds of wild-type genotypes are possible in F_2, +/+ or +/+/-/- if the trisomic F_1 chromosome carrying the *pwB* locus can perform normal meiosis (Figure 2A). The heterozygotes +/+/-/- should become mixed progeny in the next autogamy, and the ratio of non-mixed vs. mixed should be 1:2 (Table 2 and Figure 2A). Similarly, in a lineage analysis of the F_2 of heterozygous wild type, one homozygous wild type, four heterozygous wild type, and one homozygous pawn were segregated in F_3 (Figure 2B), consistently with the observed phenotypic segregation ratio of 5:1 ('wild type and pawn' column in Table 3). On the other hand, the segregation of marker genes (*nd6*, *nd7*, *nd169*, and *ts111*) showed the expected normal segregation ratio in F_2 (Table 4), and their phenotypes did not mix in the following autogamous generations of these crossings. Thus, genes other than *pwB* in strain d4-96 behaved as diploid, suggesting that chromosomes bearing other genes than the *pwB* gene are not duplicated in the strain; therefore, the strain is thought to be tetrasomic but not tetraploid.

The model shown in Figure 2 implies a number of predictions, the most

crucial of which were successfully tested.

(i) In the F_3 of autogamous lineages (Figure 2B), the ratio of segregation of the homozygous wild type (+/+/+/+), heterozygous wild type (+/+/-/-, to be mixed in the next generation), and homozygous pawn (-/-/-/-) should be 1:4:1. The observed segregation was 29 vs. 89 vs. 23 (χ^2=1.6, p=0.5).

(ii) Homozygotes of the wild type in F_2 should be ordinary disomic ('+/+' in Figure 2A), but homozygotes of the wild type in F_3 or F_4 derived from F_2 heterozygotes should be tetrasomic ('+/+/+/+' in Figure 2B). This was examined by crossing the wild-type segregants to the strain d4-96. Results are given in Table 5. Crosses using wild-type homozygous original F_2 segregants W7, W14, W24, and W27 showed a 1:1 segregation ratio of wild type vs. pawn in the F_2 in this cross and a 1:2 segregation ratio of non-mixed vs. mixed in the F_3 in this cross (Table 5). This was similar to the inheritance of original wild-type strains (+/+, see Table 2) and corroborated that they were disomic. On the other hand, when homozygous tetrasomy (+/+/+/+) was crossed with d4-96 (now assumed to be -/-/-/-), the genotype of F_1 should be +/+/-/-, and autogamy of this produce one homozygous wild type (+/+/+/+), four heterozygous wild type (+/+/-/-) and one tetrasomic pawn (-/-/-/-). Therefore expected segregation ratio of wild type vs. pawn should be 5:1 in F_2, and that of non-mixed vs. mixed should be 1:4 in F_3 (see Figure 2B). The crosses using wild-type original homozygous F_4 segregants WC-4a and WC-4b showed a 5:1 segregation ratio of wild type vs. pawn in the F_2 in this cross, while the ratio of non-mixed vs. mixed

was 1:4 in the F_3 in this cross, consistently with the predicted genotype of the F_1 (+/+/-/-) in these crosses (Table 5).

(iii) The pawn segregants in autogamous lineages should be tetrasomic (-/-/-/- in Figure 2B) and should thus behave similarly to the parental strain d4-96 (now assumed to be -/-/-/-). When segregants from F_3 and F_7 were crossed to the wild type, they showed similar inheritance to that of d4-96 (data not shown, see Figure 1).

(iv) Two-thirds of the pawn progeny in the original F_2 should be disomic (-/- in Figure 2A). When the predicted disomic pawns were crossed with the wild type, they could not produce heterozygous wild type in F_2. Table 6 shows the results obtained from the crosses between wild-type and pawn segregants (O4, O8, O9, O10, O18, O25, O29, O31, O35, E5, E6, E10, E14, E16, E17, E25, E28, E34, and E12N). Thirteen out of 19 pawn segregants are interpreted to be disomic, as expected (Table 6).

The instability of the chromosome carrying the *pwB* locus: In the course of the experiments, segregation of F_2 from more than 100 crosses was examined between wild type and pwB^{96}, and all cells from the wild-type strain were found to be disomic (data not shown). Therefore, whether duplication of the chromosome bearing the *pwB* gene in the d4-96 strain was an accidental event or an inevitable one was examined. First, disomic *pwB* segregants obtained from the above crosses were cultured, and whether duplication would occur in successive culturing was examined (Figure 3). Approximately every 30 cell divisions, cells were subjected to autogamy, and the total number of autogamous generations was

counted from the time when segregants were obtained from original crosses (generation 0, gen. 0 in Figure 3). After several autogamous generations, the progeny were crossed with the wild type to examine their genotype. If duplication of the chromosome had occurred, two-thirds of wild-type F_2 from the crosses should be heterozygotes. Thus, the phenotype in F_3 from the autogamy of wild-type F_2 was examined (see inheritance of disomy and tetrasomy in Table 6). Among 40 crossings tested using disomic *pwB* (O4, O9, O18, O35, and E12N) from the 1st to the 9th autogamous generations (gen. 1 to gen. 9), no heterozygous wild-type F_2's were found, indicating that all cells tested were still disomic (-/-). Thus, I concluded that the disomy of the chromosome carrying the *pwB* gene in these crosses was quite stable.

To test the stability of the duplicated chromosome, tetrasomic segregants carrying the wild-type allele of the *pwB* gene (+/+/+/+) were examined using the same method mentioned above (see Figure 3). The genotypes of four wild-type F_3 segregants (W1-3, W5-30, W12-10, and W12-36), which were isolated from the autogamous lineage of F_3 (see Table 3 and Figure 2B) and should thus be tetrasomic (+/+/+/+), were examined by crossing with the pawn d4-96. If the segregants maintain tetrasomic genotypes, the phenotype of F_2 of the cross should segregate at a ratio of 5:1, while if the segregants lose half of their chromosomes, the phenotype should segregate at a ratio of 1:1 (see inheritance of tetrasomic and disomic wild type in Table 5). Among 41 crosses tested from the 1st to the 8th autogamous generations, 13 crosses were identified as tetrasomic, while 23 were disomic, and the remaining 5 showed an ambiguous segregation ratio (among 41

crosses, 11 crosses are shown in Table 7). Cells thought to be disomic were observed as early as the 1st generation (Table 7). Clones of four segregants were thought to be a mixture of tetrasomy and disomy (or trisomy) at the 4th and the 8th generations (Table 7). In Table 5, I show the inheritance of the tetrasomy of the wild-type allele, pwB^{+} (crosses using WC-4a and WC-4b). Several crossings using WC-4a and WC-4b for one year repeatedly showed results indicating the presence of disomy in these clones (Table 7). These results suggest a considerable instability in the tetrasomy of the wild type.

In contrast to the tetrasomy of the wild type, the tetrasomy of pwB^{96} (-/-/-/-) showed a stable genotype. Among 56 crosses tested using tetrasomic pwB^{96} segregants (O25, O29, and 5 clones derived from the F_2 of d4-96 × *nd7*; *ts111*, as well as two subclones of the original d4-96) from gen. 3 to gen. 9, 53 crosses showed the inheritance of tetrasomy, and only three showed that of disomy. These results demonstrated that the tetrasomy of the mutated allele of the *pwB* locus was not unstable, differing considerably from that of the wild-type allele.

Tetrasomy and disomy differ in the fission rate: Some possible explanations can be drawn for the difference of stability between the tetrasomies of the wild type and the mutant. The first is that a particular genotype, like disomic pwB^{96} (-/-), can be negatively selected due to lower fitness, like lower fertility or slower growth rate in the culture. However, as compared in Table 8, survival from autogamy is almost the same among genotypes. Table 8 also shows the cell division per day of segregants from crosses between d4-96 and wild-type strains with different genotypes. The tetrasomy of pwB^{96} has some additive effect on the

fission rate of the *pwB* mutant ($t = 2.09$, d.f. = 50, $p = 0.04$, calculated from total of progeny from two crosses), while the tetrasomy of the wild-type allele of pwB^+ has little additive effect on the wild-type fission rate ($t = 0.68$, d.f. = 16, $p = 0.50$, +/+ vs. +/+/+/+ from one cross). Then, can this difference in additive effect on the fission rate be a basis for the difference in the stability of the tetrasomic genotypes of wild type and pwB^{96}? As discussed below (see Discussion), the difference in chromosome loss is not fully explained by such a slight difference in the fission rate. It is possible that other differences in property between chromosomes carrying wild-type and mutant *pwB* alleles might exist. Some of these could be pairing preferences owing to similarity and dissimilarity of four chromosomal sets of the tetrasomy. If such a difference exists, the crosses should show a more or less distorted segregation ratio from the expected one. For example, if chromosomes carrying the wild-type allele in the *pwB* locus preferentially pair with those carrying the wild-type allele, the phenotypic segregation ratio of the autogamy of '+/+/-/-' will deviate from 5:1 and become closer to 1:1. On the same context, the 1:2 segregation of non-mixed vs. mixed wild type should deviate closer to the ratio of 1:0. Even if the postulated bias may be too small to observe the hypothetical segregation distortion in small-scale data (say, 12 vs. 18 is still statistically 1:2), it will become obvious when the number of progeny is large enough (say, 120 vs. 180 is no more than statistically 1:2). To infer the preferable pairing among chromosomes in tetrasomy, pooled data of the phenotypic segregation of F_2 and subsequent generations of crosses are summarized in Table 9. The real data almost completely match the expected ratio and almost no preferable pairing between chromosomes carrying wild-type and mutant *pwB*

gene are found.

DISCUSSION

The inheritance of strain d4-96 *pwB* showed a theoretical segregation ratio when the number of chromosomes bearing the *pwB* gene was four. This theoretical segregation ratio is based on the assumption that the heterozygous tetrasomic chromosomes make bivalents and are not randomly assorted. If the four chromosomes make monovalents or trivalents as well as bivalents, the theoretical segregation of the progeny phenotype from the autogamy of '+/+/-/-' will be 11:3 for the wild type and *pwB* (possible genotypes are 2 +/+, 2 +/+/-/-/-/-, 2 +/+/+/+/-/-, 1 +/+/+/+, 4 +/+/-/-, 2 -/- and 1 -/-/-/-). However, in the autogamous lineages from heterozygous F_2, we observed a repeated segregation ratio of 5:1, which is the theoretical ratio if the chromosomes make only bivalents (Figure 2).

Although aneuploidy has been reported in inter-syngenic crosses of *P. caudatum* (Tsukii and Hiwatashi, 1985), the tetrasomy of *pwB* is the first aneuploidy reported in *P. tetraurelia*, though the proof of aneuploidy is indirect. Cytological observations showed that the chromosome number in *Paramecium* is not stable and the same species often show diverse chromosomal contents (Chen, 1940; Dippell, 1954). I showed that the original wild-type strains, when genetically examined, did not contain cells harboring four chromosomes carrying the *pwB* locus. Furthermore, my genetic analysis suggested that disomic cells appeared frequently in clones of the tetrasomy of wild type pwB^+. These results lead to the conclusion that tetrasomy, but not disomy, is unstable in this species.

Thus, although chromosome number is not cytologically constant in this species, it is reasonable to assume that micronuclear chromosomes carrying important genes might be stably diploid in the wild type of this species.

In contrast to the homozygous tetrasomy of *pwB*$^{+}$, the tetrasomy of the *pwB*^{96} mutant seems to maintain the tetrasomic genotype stably. Although I do not know whether chromosomal stability itself is different between *pwB*^{96} and its wild-type allele, one possible interpretation is that the difference in stability is brought about by selection of cells with a particular genotype. It is postulated that, in some cancers, trisomy with two copies of the mutated allele grows faster than heterozygous disomy, resulting in non-random duplication of the chromosome (Wirschubsky *et al.*, 1984; Bianchi *et al.*, 1990; Zhuang *et al.*, 1998). Indeed, the tetrasomy of *pwB*^{96} grew a little faster than its disomy, whereas the tetrasomy of *pwB*$^{+}$ grew nearly as fast as the disomy. Thus, the selection by higher fitness of the tetrasomy might be one of causes of the maintenance of the tetrasomy of the *pwB* mutant. However, absence of the selection by the fission rate in the disomy and tetrasomy of wild type does not necessarily increase the frequency of disomic cells in the culture. If it is assumed that chromosomal loss in tetrasomy occurs in meiosis, the rates of fission and survival from autogamy presented in Table 8 give a theoretical inference of the ratio of disomic cells in the culture that was initially pure for tetrasomy (Figure 4, for calculation of % of disomy, see Appendix). As shown in Figure 4, the difference in % of disomy of wild type and that of *pwB*^{96} in the culture of homozygous tetrasomy can be best observed in chromosome loss in the autogamy of tetrasomy above 5 %, where the

ratio of the disomy of wild type increases steadily while that of *pwB*^{96} reaches a plateau (Figure 4). This difference is what can be explained by the difference in the fission rate of wild type and *pwB*^{96} (Table 8). A comparison between models and real data, however, reveals a considerable difference in the frequency of chromosome loss between the homozygous tetrasomies of the wild type and *pwB*^{96} (Figure 4), though the real data are a rough estimate (see Appendix). According to the model, the frequency of chromosome loss in meiosis should be more than 30 % in the homozygous tetrasomy of the wild type, while it should be near 5 % in that of *pwB*^{96}. Therefore, there exists more than a six-fold difference between the stabilities of the homozygous wild type and mutant tetrasomies. The structural difference between chromosomes carrying the wild-type and mutant allele of the *pwB* gene is as yet unknown. As mentioned before, heterozygous tetrasomy makes mainly two bivalents, and the pair formation should be random among chromosomes carrying wild-type and mutant *pwB* alleles (see Table 9). Thus, chromosome loss was only observed in the homozygous tetrasomy of the wild type, in other words, tetrasomy without chromosomes carrying the mutant *pwB* allele. A reduction of chromosome loss was reported in autotetraploid maize cultivated for ten years (Gilles and Randolph, 1951). Although the exact time and cause of the chromosome duplication event that occurred in the micronucleus of strain d4-96 is not known, there is no reason to deny the possibility that the state of tetrasomy of the mutant can be long enough to acquire a stable chromosomal structure in tetrasomy as in disomy. A locus that ensures a correct meiotic synapsis is reported in polyploid wheat (Martinez-Perez *et al.*, 2001). The function of the *pwB* gene is still not known

(Haynes *et al.*, 2000). Studies on the chromosomal instability of the *pwB* mutant might shed light on a possible connection between the stability of chromosomes and the genes located on them.

APPENDIX

To examine the net effect of the difference in fission rate to the stability of tetrasomy, a simple model was established to simulate the appearance of disomic cells in a culture that was initially pure for tetrasomic cells. The model requires only a few parameters, including frequency of chromosome loss in tetrasomy, if the following assumptions are made:

(i) Number of cell divisions per day (r) and survival after autogamy (f; $0 \leq f \leq 1$) are counted as in Table 8.

(ii) For simplicity of the model, the effects of genetic drift are not assumed here.

(iii) Cell lines are cultured as in Figure 3.

(iv) Tetrasomy loses half of its chromosomes in meiosis at a constant frequency, l ($0 \leq l \leq 1$), while disomy is stable.

The model is as follows: At the end of generation 0 (gen. 0), the number of tetrasomic cells is N_{t0}, and that of disomic cells is 0. Disomic cells, whose number is N_d, should emerge after the autogamy of tetrasomy, depending on the frequency of chromosome loss in tetrasomy (l). Because survival from the autogamy of tetrasomy is f_t, the number of disomic cells at the beginning of gen. 1 is therefore lf_tN_{t0}. On the other hand, the number of tetrasomic cells at the beginning of gen. 1 is $(1\text{-}l)f_tN_{t0}$.

The cells are allowed to grow for ten days at a constant fission rate (r_t and

r_d for tetrasomy and disomy, respectively). Thus, the numbers of tetrasomic and disomic cells at the end of gen. 1 are $(1-l)f_t N_{t0}(2)^{10r_t}$ and $lf_t N_{t0}(2)^{10r_d}$, respectively. We called them N_{t1} and N_{d1}, which correspond to the number of tetrasomic and disomic cells, respectively, at the end of gen. 1.

After the second autogamy, the number of tetrasomic cells should again be $(1-l)f_t N_{t1}$, and that of disomic cells should be $lf_t N_{t1} + f_d N_{d1}$, where f_d is the survival from the autogamy of disomy. The cells are again allowed to grow for ten days, and at the end of gen. 2, the numbers of tetrasomic and disomic cells are $(1-l)f_t N_{t1}(2)^{10r_t}$ and $(lf_t N_{t1} + f_d N_{d1})(2)^{10r_d}$, respectively. The numbers of tetrasomic and disomic cells at the end of gen. 2 are again called N_{t2} and N_{d2}, respectively.

The genotype of the cells in the culture was determined by crossing the cells after they grew for two days after autogamy. Thus, the numbers of tetrasomic and disomic cells at the period of testing in, for instance, gen. 3, are $(1-l)f_t N_{t2}(2)^{2r_t}$ and $(lf_t N_{t2} + f_d N_{d2})(2)^{2r_d}$, respectively. The % of disomy in gen. 3 is calculated as follows:

$$100 \times (lf_t N_{t2} + f_d N_{d2})(2)^{2r_d} / \{(1-l)f_t N_{t2}(2)^{r_t} + (lf_t N_{t2} + f_d N_{d2})(2)^{2r_d}\}$$

The % of disomy in the culture predicted from this model with various generations and l values (presented in %, i.e., $l \times 100$) is given in Figure 4.

However, the % of disomy in the real data is inevitably influenced by genetic drift. For instance, the predominant presence of disomy in the culture of

W12-10 throughout 9 generations (data not shown) could be the result of genetic drift, i.e., a bottleneck effect by transfer of a drop containing predominantly disomic cells, which might be the minority in the parental culture. Indeed, in order to subject cells to constant vegetative growth, the number of cells transferred from parental culture to mass culture medium was often small, about 10-100 cells. Therefore, the % of disomy in real data should be considered as a rough estimate.

As mentioned above, I have assumed that chromosome loss occurs in meiosis of tetrasomy (assumption iv, see above), probably through non-disjunction. Non-disjunction of +/+/+/+ (tetrasomy) should produce gametes with genotype (instead of usual +/+) + and +/+/+, which results in progeny genotype +/+ (disomy) or +/+/+/+/+/+ (hexasomy) after autogamy (note that trisomy does not arise in the process). In the case of a cross of homozygous wild type of hexasomy (+/+/+/+/+/+) with tetrasomic pwB^{96} (-/-/-/-), wild type vs. pawn in F_2 should be 19:1 (with possible genotypes 6 +/+/-/-, 6 +/+/+/+/-/-, 3 +/+/-/-/-/-, 3 +/+/+/+, 1 +/+/+/+/+/+ and 1 -/-/-/-) and non-mixed vs. mixed in F_3 should be 15:4, while in the case of cross of pwB^{96} of hexasomy (-/-/-/-/-/-) with ordinary wild type (+/+), wild type vs. pawn in F_2 should be 1:1 (with possible genotypes 1 +/+/-/- and 1 -/-/-/-) and these wild type should be all mixed in F_3. Although I have some possible cases of the presence of hexasomy in the culture (data not shown), it was statistically difficult to distinguish the segregation ratio resulted from crosses involving tetrasomy and hexasomy without some additional test. Therefore, the crosses with possible involvement of hexasomy were classified as tetrasomy in this analysis. This, however, does not affect my model. Hexasomy was treated as tetrasomy in both

the real data and the model; therefore what this model shows is the % of disomy (among other possible genotypes including tetrasomy and hexasomy). This enable us a clear observation at only one definitive event of chromosome loss from tetrasomy to disomy. This is sufficient to compare chromosomal instability between wild type and mutant. Besides it, hexasomy, if they were so, was rare compared to disomy in my experimental cultures, suggesting that hexasomy is more unstable than tetrasomy and disomy therefore may be a transient and negligible state as a byproduct of non-disjunction.

PART II

Non-Mendelian inheritance induced by gene amplification in the germ nucleus of *Paramecium tetraurelia*

ABSTRACT

Genetic investigation of a strain d4-95 that carries a recessive mutant allele (*pwB*^{95}) of *pawn-B*, one of controlling elements of voltage-dependent calcium channels in *Paramecium tetraurelia*, revealed a non-Mendelian feature of this strain. Progeny of the cross between d4-95 and wild type often expressed mutant phenotype even if they had wild-type gene. The expression of mutant phenotype in these progeny was clonal but seems to be mostly random rather than cytoplasmic inheritance. No structural difference was found in the *pwB* gene of d4-95 compared with that of wild type, though single base-pair substitution was found in the coding region. Using molecular analysis, I found that copy number of *pwB* gene in the macro- and the micronucleus of d4-95 was much greater than that of wild type. The amplified, extra *pwB* gene in d4-95 was heritable independently from the original *pwB* locus in conjugation and autogamy, suggesting that it is not linked tightly. Repeated backcrossing of d4-95 with wild type to dilute extra *pwB* gene in the strain produced segregants with completely normal Mendelian trait in testcrosses. These results strongly suggest that non-Mendelian inheritance of d4-95 was induced by gene amplification in the micronucleus.

INTRODUCTION

Studies on non-Mendelian inheritances in *Paramecium tetraurelia* have ever brought the important concepts in genetics (Preer, 2000). Analysis on cytoplasmic inheritance of a killer trait led to the discovery of the first endosymbiont in *Paramecium* (Sonneborn, 1947; Preer *et al.*, 1974). Inheritance of inverted ciliary lows was also the first demonstration of a gene-independent, polarized propagation of the basal bodies and its accessory fibers (Beisson and Sonneborn, 1965; Sonneborn, 1975). Recent molecular investigations on other cytoplasmic inheritances including mating types (Sonneborn, 1947) and surface antigen mutant (Epstein and Forney, 1984) provide another mechanism involving gene rearrangement in their developmental process (Forney *et al.*, 1996; Meyer and Duharcourt, 1996). Caryonidal inheritance, which has been found in mating types of closely related species, such as *P. primaurelia* (Sonneborn, 1977), has, however, never been found in *P. tetraurelia*, though caryonides with different phenotypes were sometimes observed (Nanney, 1957; Sonneborn and Schneller, 1979; Rudman and Preer, 1996). Molecular mechanism of caryonidal inheritance in any species remains to be elucidated.

In this part, I report a genetic and molecular investigation of the second *pwB* strain, d4-95, that shows an inheritance different from other *pwB* strains as well as other non-Mendelian inheritances known in ciliate. The inheritance has partially cytoplasmic as well as caryonidal features and involves gene amplification in the micronucleus.

MATERIALS AND METHODS

Stocks and culture method: Stocks used in this study are summarized in Table 10. All stocks are homozygous. Trichocyst non-discharge mutations (*nd6*, *nd7* and *nd169*) and temperature-sensitive mutation (*ts111*) were used as recessive markers. Cells were cultured in lettuce juice medium in Dryl's solution (Dryl, 1959) inoculated with *Klebsiella pneumoniae* one or two days before use (Hiwatashi, 1968). Cells were grown at 25-27 °C unless otherwise noted.

Phenotypic observation: The behavioral phenotype of a clone was determined by transfer of more than 10 cells by micropipette into the stimulation solution (20 mM KCl in Dryl's solution). When wild-type cells are transferred into the stimulation solution, they swim backward for 30 - 50 sec (Naitoh, 1968). Cells which showed only whirling or backward swimming for less than 3 sec, were judged to be exhibiting the pawn phenotype. The discharge or non-discharge of the trichocyst was observed by adding a drop of saturated picric acid to the cells. Temperature sensitivity was observed after growth for 2 days at 35 °C because the mutant dies in this condition.

Genetic analysis: Mating reactive cells of complementary mating types were mixed, and then conjugating pairs were isolated in a fresh culture medium. After separation of the conjugating pair, both exconjugants were isolated and separately grown. In some experiments, two cells produced from the first cell division from conjugation (i.e. caryonides) were again isolated and separately grown. Phenotypes of F_1 clones were observed after they had undergone more than 10 cell divisions from conjugation.

F_2 progeny were obtained from autogamy induced by starvation of mature F_1 cells (about 30 cell divisions after conjugation). One-hundred percent autogamy was determined when all of at least 20 cells showed macronuclear fragmentation after being stained with Carbol fuchsin solution (Carr and Walker, 1961). Autogamous cells were isolated in a fresh culture medium, and phenotypes were observed after they had undergone 10 cell divisions.

After successive autogamies, some wild-type segregants in F_2 produced pawn as well as wild-type clones (see Results). These progeny were referred to as the "mixed" type. To examine the segregation of the non-mixed wild type vs. the mixed type in F_2, autogamy was induced in more than 50 cells of each F_2, and the cells were transferred to a fresh culture medium. After they had undergone about 10 cell divisions, the phenotype of F_3 was observed, and the mixed type and the non-mixed wild type were determined. For further one month, the clones were allowed to grow and undergo starvation every one or two days which induces natural autogamy in the culture.

Extraction of DNA and RNA: The cell pack from 10-100 ml of culture in the early stationary phase of the immature period ($5 \times 10^3 - 1 \times 10^5$ cells) was washed by sterilized Dryl's solution and lysed in NDS lysing solution (0.7 % sodium dodecyl sulfate, 0.3 M EDTA, 7 mM Tris-HCl, 0.7 mg/ml proteinase K, pH 8.0). After 2 h incubation of the mixture at 50 °C, DNA was extracted with phenol-chloroform twice and pelleted by addition of an equal volume of isopropanol followed by washing of

the pellet with 70 % ethanol.

PCR: Primers used for PCR were designed according to the nucleotide sequence of the *pwB* gene described by Haynes *et al.* (2000). For PCR amplification of most of the coding region of *pwB* gene, a sense primer *pwB*F-84 (5′-GGGCAATCCATTTAAGGCAAGTGG) and an antisense primer *pwB*R763 (5′-CGTCGTTTTCCTTATACTTCTCTTC) were used.

Southern blotting: The total genomic DNA was digested with restriction enzymes and then run on 1 % agarose gel electrophoresis. The gel was processed with a depurination solution (0.25 N HCl) followed by denaturation with a denaturation solution (1.5 N NaOH, 0.5 M NaCl) and blotted onto a Hybond N+ membrane (Amersham) in 0.4 N NaOH. PCR products used as probes were purified by electrophoresis on polyacrylamide gel, elution against TE, phenol extraction, and ethanol precipitation. The probe labeled with HRP (Horseradish Peroxidase) was prepared using ECL direct nucleic acid labeling and detection systems (Amersham), and hybridization and signal generation procedures were also performed according to the manufacturer's instructions. The signal was measured using Scion image (Scion).

RESULTS

Non-Mendelian inheritance that is partly cytoplasmic as well as caryonidal: Strain d4-95, homozygous for pwB^{95}, was crossed with wild-type strains (pwB^{+}/pwB^{+}). Because pwB^{95} is reported as a recessive allele, heterozygote of

pwB^{+}/*pwB*^{95} should show wild type phenotype, that is, backward swimming for 30 to 50 sec in the stimulation solution. Behavioral test performed on these F_1 progeny showed the appearance of clones showing reduced or complete absence of the behavioral response. No such aberrant result was observed for the marker genes (*nd6*, *nd7*, *nd169*) used in these crosses. Thus, any F_1 that contained pawn cells in at least one caryonide was regarded as F_1 showing non-Mendelian inheritance. Typical example of the pattern of the appearance of pawn cells and the % of F_1 showing non-Mendelian inheritance in the crosses between d4-95 and wild type are summarized in Table 11. The inheritance was often cytoplasmic since cells with pawn phenotype were more often found in the cytoplasmic derivatives from d4-95, but as often as caryonidal, i.e. different behavioral phenotype expressed in different caryonides (Table 11).

The ratio of F_1 that contained cells with pawn phenotype fluctuated in the period of these tests as shown in Figure 5. In a given short period such as one month, the % of non-Mendelian inheritance was relatively constant with minor fluctuations, yet it varied from zero to 100 % within four years. Examinations of the inheritance of d4-95 described below mainly include the crosses performed on the early period in 1998 and 2000 (C1 and C5 in Figure 5).

To test whether the F_1 progeny shown in Table 11 were really heterozygous at the *pwB* locus, F_2 progeny were taken by autogamy of two types of F_1. The first is those showing normal Mendelian inheritance in F_1 which will be described

immediately below. The second is those showing non-Mendelian inheritance in F_1, which will be described later.

F_2 progeny were obtained by autogamy of F_1, where a fusion of two haploid nuclei derived from a single meiotic product makes F_2 progeny completely homozygous for all genes. The 1:1 segregation is expected for the phenotype that is controlled by a single gene. As shown in Table 12 (column "F_2"), the ratio close to 1:1 was obtained in F_2 from all crosses that showed normal Mendelian inheritance in F_1, although some progeny were a mixture of wild-type and pawn cells. The segregation ratios do not seem to have been influenced by parental cytoplasm (Table 12). The marker genes (*nd6, nd169*) in these crosses segregated also in the expected ratios ($0.5 < \chi^2 < 3.5$, $0.05 < p < 0.5$). Therefore, F_1 should have been really heterozygotes and all F_2 thus obtained should be completely homozygous.

However, after mass autogamy of F_2, most wild-type clones in the F_2 became the mixed progeny of wild-type and pawn cells in F_3 or in the subsequent autogamous generations as shown in "subsequent autogamous generations" column in Table 12. The mixed progeny appeared at various frequencies in the F_3 depending on the crosses and parental cytoplasm, but they seem to be the ultimate destination of most wild-type F_2 after several rounds of autogamy, because autogamy should naturally occur in the culture condition used in this experiment within a month (see Materials and Methods). On the other hand, mass autogamy of pawn

clones in F_2 did not change their phenotype in F_3 and subsequent autogamous generations. A possible interpretation of these results may be that at least wild-type *pwB* genes had been segregated expectedly in F_2, while some exautogamous cells from almost all wild-type F_2 were suppressed to express the wild-type phenotype in the subsequent autogamous generations.

To examine the detailed origin of the mixed clones produced from the crosses of d4-95 with wild type, autogamous progeny from wild-type F_2, that should be exautogamous in F_2 verified with marker genes, were isolated, and the isolation of autogamous progeny was continued to several generations to make the autogamous lineages (Figure 6). Wild-type F_2 clones produced wild-type and pawn progeny after autogamy (F_3 in Figure 6). In the subsequent autogamous generations, however, both wild-type and pawn clones again produced wild-type and pawn progeny (F_4 – F_7 in Figure 6). The sum of progeny phenotype from F_3 to F_7 shows that each phenotypes were often expressed caryonidally and nearly random (Table 13). The caryonidal expression of different phenotype strongly suggests that this change of phenotype by autogamy is not associated with a change of the genotype in the micronucleus; rather the phenomenon is likely the matter of developmental process of the macronucleus. Similarly, the change of phenotype from pawn to wild type in these autogamous lineages is never explained by duplication of the micronuclear chromosome carrying the *pwB* locus as found in another strain of *pwB* (see Part I).

Similar inheritance was also observed in F_2 progeny obtained from autogamy of F_1 that showed non-Mendelian inheritance in F_1 mentioned above (see Table 11), though the suppression of the wild-type phenotype occurred earlier than the above instances. As shown in "F_2" column in Table 14, progeny phenotype was predominantly pawn in autogamous F_2. The tendency for this distorted phenotypic segregation was similar among F_1 parent for autogamy showing different phenotype in F_1 ("F_1/F_2 classified by its own phenotype" in Table 14) but was more enhanced if F_2 were obtained from d4-95 cytoplasmic descendants ("F_1 classified by parental cytoplasm" in Table 14), suggesting some somatic effect on the phenotypic segregation in F_2. The marker gene (*nd6*) in this cross, however, segregated expectedly in F_2 ($\chi^2 = 0.06$, $p = 0.80$). To examine whether the predominant appearance of pawn progeny in F_2 of this cross was some genetic change of *pwB* gene or some somatic alteration of pwB^+ gene expression in the progeny, all F_2 progeny were subjected to the next mass autogamy. After autogamy, most wild-type as well as some pawn F_2 became the mixed clones of wild-type and pawn cells in F_3. In total, if mixed progeny are counted as wild type, the distortion of segregation ratio of behavioral phenotype in F_2 was canceled out in F_3. As shown in "F_3" column in Table 14, the segregation ratio of "wild type and mixed type" vs. "pawn" was very close to the expected ratio of 1:1 in F_3. As described in above paragraphs, mixed

clones in F_3 are thought to have the wild-type *pwB*$^+$ gene in their micronucleus. On the other hand, the pawn progeny in F_3 did not change their phenotype by successive autogamies. Obviously, the single locus controls the pawn phenotype in these crosses, but the crosses of wild type with d4-95 often suppressed wild-type phenotype in some progeny cells not only in F_3 and in subsequent autogamous generations (Tables 12 and 13, and Figure 6), but also in F_1 (Table 11) and in F_2 (Table 14). In conclusion, the cross with d4-95 induces the cytoplasmic-like and caryonidal-like inheritances sometimes immediately in F_1, and sometimes later in the autogamous generations.

Extra *pwB* genes in the micronucleus of strain d4-95: Why did pawn progeny cells appear after autogamy of clones that must be wild-type homozygotes (see Figure 6 and Table 13)? They must be homozygotes because autogamy is a process where genetically identical, haploid nuclei are put together. However, as shown in a tetrasomic chromosomal composition of another *pwB* strain d4-96 (see Part I), it is possible that autogamous progeny are actually heterozygous. Although the inheritance of d4-95 cannot be explained by the inheritance of tetrasomy, I speculated that mixed clones were also actually heterozygous despite having been obtained by autogamy. The heterozygosity of autogamous progeny inevitably postulates a presence of the extra *pwB* gene copies in the micronucleus of d4-95 (see Figure 7A). If this extra *pwB* gene is heritable independently from the original *pwB* locus, it will produce homozygous and heterozygous F_2 progeny after autogamy (see Figure 7A).

To test this prediction, molecular identification of *pwB* alleles is necessary. Although single base pair substitution of pwB^{95} allele reported by Haynes *et al.* (2000) should be recognized by restriction enzyme *Alu*I, I could not produce this result. Sequencing of pwB^{95} gene revealed that the single base pair substitution of pwB^{95} was a C to G transversion in the 172nd rather than previously reported position of the 173rd nucleotide counted from the start codon. This was also confirmed in the Kung's laboratory (pers. commun.). This substitution is not recognized by any restriction enzymes, thus I took another approach for allele identification.

Molecular defect of another *pwB* strain d4-96, which has a recessive allele of pwB^{96}, should be specifically recognized by restriction enzyme *Ssp*I. A disomic derivative from strain d4-96 (strain a3093, see Table 10) was crossed with d4-95, and F_2 progeny were obtained by autogamy. Because strain d4-96 is also a *pwB* mutant, test of behavioral phenotype was not applicable in this cross, though marker genes (*nd6* and *nd9*) were segregated in the expected ratio ($\chi^2 = 0.3$, $p = 0.6$) showing that conjugation and autogamy in this cross had been normally performed. From F_2 progeny, *pwB* gene were amplified by PCR, and digested with the restriction enzyme. If F_2 progeny homozygous for pwB^{96} received the extra *pwB* gene derived from the micronucleus of d4-95, PCR fragments should not be completely cut by *Ssp*I (Figure 7A). An example of the result is shown in Figure 7B, where half of F_2 progeny had *pwB* gene species that were partly susceptible to *Ssp*I while another half did not. In total, five among 12 progeny were identified as clones having pwB^{96} allele as well as

pwB^{95} allele uncut by the enzyme, and residual seven clones were identified as clones having only *pwB*^{95} allele, showing close to 1:1 segregation of the *pwB*^{96} allele. No F_2 progeny having only *pwB*^{96} allele were found. Thus this experiment demonstrates that the extra *pwB* gene in d4-95 is present in the micronucleus of the strain and inherits independently from the original *pwB* locus.

Gene amplification in the macronucleus of d4-95: The presence of the extra *pwB* gene in the micronucleus of d4-95 suggests that *pwB* gene was amplified in the micronucleus of the strain. If this is the case, the amplified *pwB* gene may be detected from macronuclear DNA isolated from the strain. Total genomic DNA, which is predominantly macronuclear DNA, was digested with restriction enzymes, subjected to southern blotting and probed with *pwB* gene fragment labeled with HRP. The most extreme example is shown in Figure 8A to C where total DNA isolated from d4-95 contained more than 50 times more *pwB* gene copies than that from wild type (Figures 8A and 8B). Figure 8C shows the same blot as Figure 8A but with short exposure time. Several sizes of restriction fragments are visible in Figure 8C, indicating a heterogeneous feature of the amplified *pwB* gene in the macronucleus. Because coding region of *pwB* gene is approximately 1 kb, the amplified unit should be much larger than the *pwB* gene (at least 7.5 kb).

However, *pwB* gene dosage in the macronucleus of strain d4-95 is not always so high as in Figure 8A, and the sizes of heterogeneous restriction fragments (indicated by black arrowheads in Figure 8C) also varied among separately isolated DNA samples (data not shown). The absence of reproducibility of the macronuclear

pwB gene dosage and heterogeneity was partly explained by clonal difference in the original culture of d4-95. Difference of *pwB* gene dosage and heterogeneity of restriction fragments in the total DNA were indeed observed in three caryonides isolated from autogamy of d4-95 (lanes 2 to 4 in Figure 8D). After subsequent mass autogamies of these clones, however, the original restriction patterns were not well reproduced (lanes 5 to 10 in Figure 8D). Because two of the clones (clone 1 and 2) showed the similar restriction patterns flanking *pwB* gene in at least one autogamous generation (compare lanes 5 and 9), they might contain similar set of amplified fragments in the micronucleus, while they could show sometimes nearly normal restriction pattern as wild type (e.g. lanes 3 and 8) suggesting a macronuclear modification of gene amplification. Clone 3 showed slightly different restriction pattern compared to other two clones (the upper-most fragment in lane 4 in Figure 8D) suggesting that this clone had different amplified fragments from other two clones. In conclusion, each clone in the culture of d4-95 may have different amplified fragments in the macronucleus and likely in the micronucleus, as well.

Amplified gene may involve in the non-Mendelian inheritance of d4-95: If massive extra *pwB* gene in the micronucleus of strain d4-95 was responsible for the non-Mendelian inheritance of the strain, repeated backcrossings of d4-95 to wild type should dilute the *pwB* gene copies in the micronucleus and thus should reduce the non-Mendelian inheritance. Strain d4-95 was repeatedly backcrossed with wild type. Because the progeny may not faithfully express their phenotype, one round of backcross consisted of three generations (conjugation with wild type followed by two rounds of autogamy) to obtain homozygous pawn segregants. This procedure of

backcrossing was repeated for eight times from the original strain d4-95 and then % of F_1 showing non-Mendelian inheritance of the segregants from each round was plotted (Figure 9A). In the first round of backcrossing, % of non-Mendelian inheritance increased steeply (Figure 9A). The reason for this is not understood. However, in the subsequent rounds of backcrossings, the % of non-Mendelian inheritance decreased gradually, and had disappeared by the sixth round (Figure 9A). The suppression of wild-type phenotype in the F_2 and the subsequent autogamous generations in the test cross was, however, still observed for the crosses of segregants from sixth round of backcross with wild type. The complete disappearance of the suppression of wild-type phenotype in the subsequent autogamous generations (i.e. completely normal Mendelian inheritance) was achieved after seventh round of backcross (data not shown). The original strain d4-95 was also characterized by a low survival after crossing (see Tables 12 and 15), but segregants with higher survival after crossings (almost 100 %) had been obtained in the second round of backcrossing (data not shown).

The *pwB* gene dosages in the total DNA samples isolated from the segregants were shown in Figure 9B. All segregants have generally similar macronuclear *pwB* gene dosage to wild type, but some of them seem to have heterogeneous restriction sites around extra *pwB* gene. The *pwB* gene in the segregants from the seventh round of backcross was indistinguishable from that in wild type, consistently with the result of cross breeding analysis (Figure 9A and data not shown). These results strongly suggest that amplified or extra *pwB* gene in the

micronucleus is responsible for the non-Mendelian trait of strain d4-95.

DISCUSSION

The inheritance of strain d4-95 manifested both cytoplasmic and caryonidal pattern when crossed with wild type. The most surprising feature of this inheritance may be a nearly random determination of caryonidal phenotype (Figure 6 and Table 13) that is particularly unique in this strain. Although, similarity of inheritance of d4-95 to the caryonidal determination of mating types in other *Paramecium* and *Tetrahymena* species is apparent, important differences exist between them. In mating type determinations of *P. primaurelia* and *T. thermophila*, the ratios of progeny mating-types are well predicted depending on the environmental factor such as temperature and timing of feeding after sexual reproduction (Sonneborn, 1977; Orias, 1981; Orias and Baum, 1984). On the contrary, the ratio of progeny phenotype was not constant in the autogamous lineages derived from the cross between wild type and d4-95 without obvious differences in the environmental conditions. They produced sometimes exclusively pawn progeny (Figure 6), while sometimes mostly wild-type progeny, though the total ratio of caryonidal phenotype appears random (Table 13). Furthermore, the caryonidal inheritance did not always appear in F_1 and F_2 in crosses of d4-95 with wild type (Tables 11 and 12), and % of F_1 showing non-Mendelian inheritance fluctuated during four years (Figure 5). Therefore, I hesitate to insist the apparent parallelism between caryonidal determination of mating types in other species and the inheritance of d4-95. Instead, I understand the inheritance of d4-95 as a novel one in ciliate with a novel mechanism involving amplified mutant gene.

I demonstrated the amplified, extra *pwB* gene in the micro- and the macronucleus of strain d4-95. By repeated backcrossings, I could obtain a pwB^{95} segregants with wild-type genetic background, and these segregants showed completely normal Mendelian inheritance (Figure 9), suggesting that amplified mutant *pwB* gene was the responsive element for the non-Mendelian inheritance of d4-95.

However, how amplified mutant gene suppressed the wild-type phenotype is not yet clear. Because phenotypic suppression was often caryonidal, the mechanism likely involves macronuclear developmental process. If, in *Paramecium*, there is a mechanism compensating macronuclear gene dosage in developmental process, this mechanism may be a strong candidate for the suppression of wild-type phenotype, since absolute pwB^{+} gene copies in the macronucleus will be significantly lowered if macronuclear *pwB* gene is amplified less than the actual *pwB* gene dosage in the micronucleus. Alternatively, there are many examples of dosage-dependent gene-silencing mechanisms in almost all eukaryotes (Ruiz *et al.*, 1998; Bastin *et al.*, 2001) and these transcriptional controls may also be involved in this case. Whatever the cause is, if the bulk copies of mutant gene in the micronucleus of d4-95 are really the responsible element for the non-Mendelian inheritance, this would be the first report that shows a non-Mendelian inheritance resulted from gene amplification in ciliate.

This is the first gene amplification in the micronucleus reported in ciliate. An interesting question arises as to whether gene amplification of *pwB* gene in the

micronucleus is related to the gene amplification in the developmental process of the macronucleus. Although the exact size of the macronuclear chromosome carrying *pwB* locus is not known, it may not greatly differ from the mean size of the macronuclear chromosomes in *P. tetraurelia*, i.e. 450 kb (Phan *et al.*, 1989). On the other hands, the size of the major amplicon carrying *pwB* gene in the micronucleus of d4-95 should be more than the largest restriction fragment tested here revealed by southern blotting of digested DNA (Figure 8). Therefore, a possibility that the actual size of amplicon carrying *pwB* gene in the micronucleus is more than tens or hundreds kb and thus comparable to the macronuclear chromosome cannot be denied. On the other hand, gene amplification is commonly observed both in prokaryotes (Romero and Palacios, 1997) and eukaryotes (Beverley, 1991; Hamlin *et al.*, 1991). Thus alternative interpretation of the gene amplification in d4-95 may be similar to gene amplifications in other organisms rather than the gene amplification in the macronucleus of ciliates. In the gene amplifications of mammalian cultured cells, the sizes of amplicons are usually over 200 kb and sometimes beyond 10 000 kb (Hamlin *et al.*, 1991), and thus may also be large enough to cover an average macronuclear chromosomes in *P. tetraurelia*. The characters shared by present case and gene amplification in mammalian cells are also found in the heterogeneity of amplified units (Figures 8C and D) and its probable instability (Figure 8D) that are widely observed in gene amplifications (Stark *et al.*, 1989; Hamlin *et al.*, 1991). Because amplified DNA makes aberrant cytological characteristics such as abnormally banding chromosome regions and double-minute chromatin bodies (DMs) (Stark *et al.*, 1989; Beverley, 1991; Hamlin *et al.*, 1991), it may be worth to

observe the cytological character of amplified *pwB* gene in the micronucleus of d4-95. Concerning to this point, dot-like chromosomes found in the micronucleus of some wild stocks of *P. tetraurelia* (Dippell, 1954) is also worth to test whether they are actually DMs and contain amplified DNA. If it is the case, gene amplification similar to other organisms would frequently occur in the natural stocks. However, the possibility that the mechanism for gene amplification in the present case is related to the macronuclear gene amplification also remains. Since similarity between macronuclear rDNA amplification in *Tetrahymena* and common DNA amplification mechanism in other organisms has been experimentally suggested (Butler *et al.*, 1996), it will be an interesting challenge to test whether the mechanism for macronuclear gene amplification is used for the DNA amplification in the micronucleus.

In this part, a description of non-Mendelian inheritance of *pwB* strain d4-95 was made. The inheritance is new in ciliate genetics and amplified *pwB* gene in the micronucleus of the strain seems to involve the generation of non-Mendelian trait of this strain.

PART III

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

ABSTRACT

A non-excitable behavioral mutant, d4-662, was previously characterized as the fourth pawn locus mutant *pwD* in *Paramecium tetraurelia*. I 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.

INTRODUCTION

Seven single recessive loci affecting the function of voltage-dependent Ca^{2+} channels have been so far obtained in *Paramecium*. They are *pwA*, *pwB*, and *pwC* in *P. tetraurelia* (Kung, 1971; Chang and 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 controlled the function of Ca^{2+} channels in *Paramecium*.

One of the pawn mutants in *P. tetraurelia*, d4-662, formerly designated *pwD* (Saimi and Kung, 1987), has not previously been fully characterized in relation to the three other pawns. During my analysis, I 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.

MATERIALS AND METHODS

Stocks and Culture: The stocks used in this study are listed in Table 15. 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* one or two 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 and Kung, 1974). Because d4-662 produces phenotypic revertants after autogamy (see Results), 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 of d4-662, and were effective in maintaining the pawn phenotype.

Microinjection: Microinjection was performed by the method described by Hori and Takahashi (1994). Cells for the transplantation of cytoplasm or macronucleoplasm were deciliated with 5 % ethanol (Ogura, 1981) and embedded in mineral oil (Squibb & Sons, Inc.). 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.

Observation of the phenotypes: The behavioral 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 backward for approximately 50 sec. 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 sec, 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 mutants do not behave in this way.

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-conjugational cell division, and the four cells thus produced from every conjugating pair were reisolated to establish caryonidal clones. 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's were obtained by autogamy. 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 macronuclear fragmentation by staining with Carbol fuchsin solution (Carr and Walker, 1961). Exautogamous cells were isolated in 0.4 ml fresh culture medium and allowed to grow for about 9-10 cell divisions to observe their phenotypes.

RESULTS

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 1980's and at that time designated as *pwD* (Saimi and Kung, 1987). The mutational defect of d4-662 is found in the voltage-dependent Ca^{2+} channel, and the behavioral responses are very similar to those of 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, I 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, I 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's 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 behavioral phenotype were often observed. Each phenotype, either pawn or wild type, is very stable in

vegetative growth, and never changes under various physiological conditions, such as temperature, starvation or clonal aging. The appearance of these wild-type cells which I 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 (Table 20). 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 20, the third and forth columns, and Part IV deal with this issue in more detail).

The *pwB* and d4-662 mutants do not show complementation when cytoplasm or macronucleoplasm are 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 transferring into a high K^+ stimulation solution (20 mM KCl in Dryl's solution) 3-8 h after microinjection of cytoplasm from the other mutants, the transient restoration of the excitability of d4-662 cells was observed. Table 16 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, except 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 17). In these experiments, the duration of backward swimming induced by K^+ solution was shorter than in the uninjected wild type (35 sec in *P. tetraurelia* and 90 sec 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 hrs, indicating that the effects of the cytoplasmic injection were transient (Haga *et al.*, 1983). The most important evidence in these experiments, d4-662 and *pwB*^{95} did not complement each other (Tables 16 and 17). 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 and 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 similar amount of macronucleoplasmic transfer from *cnrA* or *cnrD* of *P. caudatum* worked well (Table 18). 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 19).

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}.

Progeny from crosses d4-662 with *pwB* express predominantly the wild-type phenotype: To know why d4-662 had been previously 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 F_1 were less than 12. 8 % (Table 20). Wild-type phenotype observed in progeny from these crosses, however, showed variable level of responses to stimulation solution (for example, see Table 23, from third to sixth column). I judged cells showing backward swimming for over 3 sec 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 20, 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 20). 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*. Appearance of R662 on a cross within R662 was less than 8 %. However, many wild-type progeny were obtained from the cross of R662 with pwB^{96} (Table 20), suggesting that some maternal effects are involved in the inheritance.

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 21). 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 would associate 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 21. The mixed clones in Table 20 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.

Some F_1 progeny from the crosses of d4-662 with other *pwB* produce many wild-type progeny in 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 contained in F_1 as heterozygous composition segregate. Therefore, when F_1's 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 pawn phenotype, Table 22), 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 pawn phenotype in the crosses of d4-662 and pwB^{95} or pwB^{96} (cross 2), respectively (Table 22). To examine the nature of the F_2 expressing wild type in the cross of d4-662 with pwB^{96} (cross 2), F_3 were obtained by autogamy from 3 F_2's. Again, 34 wild-type and 16 pawn progenies were obtained, suggesting that some wild-type F_2 is still heterozygous in spite of the fact that the F_2 was induced by autogamy. The phenotypic segregation in F_3 was close to the ratio of wild type to pawn, 2:1 ($p > 0.8$). This segregation continued to F_4 and F_5 and subsequent generations. Similarly, mass exautogamous 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 the 'Discussion.'

Wild-type progeny from crosses between d4-662 and *pwB* is 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 23). 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.

DISCUSSION

The main focus of my work was to analyze the genetics of strain d4-662, belonging to the pawn class of mutants. To analyze the genetical relationship of two mutants, I performed microinjection of cytoplasm and macronucleoplasm between mutants. Macronucleoplasmic transplantation was found to work well when cytoplasmic transplantation was difficult to rescue mutants as shown in *cnrA* and *cnrD* of *P. caudatum* (Table 18). Evidence showing no complementation between d4-662 and *pwB* by microinjection of cytoplasm or macronucleoplasm (Tables 16, 17, 18 and 19) 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). I showed that this mutant produce phenotypic wild type (R662) after sexual reproduction at low frequency. The cytoplasm of R662, however, did not rescue the defect of the pwB^{662} and pwB^{96} (Table 23). Probably, this difference was resulted from smaller amount of the wild type *pwB* gene product in R662 (see Part IV).

The reason that d4-662 was misjudged as a new pawn mutant, *pwD* (Saimi and Kung, 1987), resulted from the evidence that the F_1 of the cross between d4-662 and *pwB* mutants expresses predominantly the wild type. When F_1 of a cross between two recessive mutants expresses wild-type phenotype, they are usually judged to be independent mutants controlled by two different genic loci. When F_2 were obtained by autogamy of F_1, however, three different segregation ratio were observed depending on *pwB* strains used. The simplest

result of segregation observed among them was that almost all progeny expressed pawn. This is consistent if d4-662 is in fact 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 F_2 should be homozygotes of wild-type alleles, because autogamy makes all progeny completely homozygous in whole genome. As shown in Parts I and II, the segregation ratio will be explained if pwB^{96} strain used in this cross was a tetrasomy of the chromosome carrying *pwB* gene. If the genotype of wild-type progeny in F_2 is $pwB^{662}/pwB^{662}/pwB^{96}/pwB^{96}$, heterozygous wild type will continue to be produced in the next generation at a ratio of 2:1 (see Results). These heterozygous progeny may express wild-type phenotype as observed in F_1 heterozygotes. Thus, pwB^{96} strains used here should be in two states, disomy (cross 1 in Table 22) and tetrasomy (cross 2 in Table 22). Finally, the F_2 from cross between d4-662 and pwB^{95} showed almost 1:1 segregation ratio and again these wild-type F_2's became mixed clones in subsequent autogamous generations, indicating that these were not true wild-type homozygotes. As demonstrated in Part II, original strain d4-95 was shown to have amplified *pwB* gene in the micronucleus and this extra *pwB* gene inherits independently from the original *pwB* locus. Thus, half of F_2 progeny harboring pwB^{662} should be heterozygous with the extra pwB^{95} gene and these F_2 would express wild-type phenotype. These considerations lead to a conclusion that 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 20) 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 (Kass *et al.*, 1997; Laird and Jaenisch, 1996). Similarly, heterochromatin formation associated with deacetylation of histone is known to cause relatively stable repression of transcription (Kennison, 1995; Weiler and Wakimoto, 1995; Pirrotta, 1997; Klar, 1998). Although the involvement of these modifications in the gene expression of the *pwB* and *pwB*^{662} genes 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 20). Whether the cause of this phenomenon is connected with some property of the old macronucleus is still unknown. However, in the crosses of *pwB*^{662} and *pwB*, the expression pattern of phenotypes in 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 in developmental process because no allelic

interactions in the vegetative stage occurred when the macronucleoplasm from both mutants was directly mixed by microinjection (Table 18). 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 *T. thermophila* suggests intragenic recombination during macronuclear development (Deak and Doeder, 1998). Molecular analysis may explain which case occurred in the inheritance of pwB^{662} and pwB^{95} or pwB^{96}. Present 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.

PART IV

A molecular basis for the alternative stable phenotype in a behavioral mutant of *Paramecium tetraurelia*

ABSTRACT

In the sexual reproduction of *Paramecium tetraurelia*, the somatic nucleus (macronucleus) undergoes massive genomic rearrangement, including gene amplification and excision of internal eliminated sequences (IESs), in its normal developmental process. Strain d4-662, one of the pawn mutants, is a behavioral mutant of *P. tetraurelia* that carries a recessive allele of pwB^{662}. The *pwB* gene in the macronucleus of the strain has an insertion of the IES because a base substitution within the IES prevents its excision during gene rearrangement. The culture of this strain frequently contains cells reverting to the wild type in the behavioral phenotype. The mutant and revertant maintained stable clonal phenotypes under the various environmental conditions examined unless they underwent sexual reproduction. After sexual reproduction, both mutant and revertant produced 2.7-7.1 % of reverted progeny. A molecular analysis performed on the macronuclear DNA of the mutant and revertant of d4-662 showed that much less than 1 % of the mutant IES was precisely excised at every sexual reproduction of the strain. Therefore, the alternative phenotype of strain d4-662 seems to be caused by an alternative excision of the mutant IES.

INTRODUCTION

Recently, mutation in the 5th nucleotide of the terminal sequence of IES was found in the 44-bp IES (IES427) interrupting the protein-coding region of the *pwB662* allele (Haynes *et al.*, 2000). Eventually, the IES is not removed in the macronuclear *pwB* gene, abolishing the normal function of the protein product (Haynes *et al.*, 2000). As described in Part IV, I found a stable phenotypic reversion of strain d4-662, carrying the recessive allele of pwB^{662}. A genetic and molecular investigation to explore the phenotypic reversion revealed a spontaneous excision of the mutant IES in its macronucleus during sexual reproduction.

MATERIALS AND METHODS

Stocks and culture method: Strain d4-662 homozygous for *nd6* (non-discharge of trichocyst) and pwB^{662} (formerly *pwD*; Saimi and Kung, 1987) is supplied by Dr. C. Kung (Univ. Wisconsin). For behavioral wild type (pwB^{+}/pwB^{+}), stocks 51s in our laboratory and *nd6*, homozygous mutant for *nd6*, which was supplied by Dr. T. Hamasaki (Albert Einstein College of Medicine of Yeshiva University, Bronx, New York) were used. Cells were grown at 25-27 °C in 3 % lettuce juice medium in Dryl's solution (Dryl, 1959) inoculated with *Klebsiella pneumoniae* one or two days before use (Hiwatashi, 1968).

Genetic analysis: Autogamy was induced by starvation of mature cells (about 30 cell divisions after the previous autogamy). Autogamous progeny were isolated in a fresh culture medium from a 100 % autogamous culture judged by the

observation where all >20 cells showed macronuclear fragmentation stained with Carbol fuchsin solution (Carr and Walker, 1961). Phenotypes were observed after exautogamous progeny underwent 10 cell divisions.

Observation of phenotype: The behavioral phenotype was observed after transfer of cells into the stimulation solution (20 mM KCl in Dryl's solution) using a micropipette (Naitoh, 1968). Cells showing clear backward swimming or whirling (more than 5 sec) were judged as revertant and designated as "R662". Cells with a tight pawn phenotype (no response of backward swimming or whirling of less than 5 sec) were judged as pawn and designated as "P662".

Calculation of fission rate: A single cell was isolated in 0.4 ml of a fresh culture medium and allowed to grow for 24 h. The cells were daily counted and re-isolated in a fresh culture medium (cell line). Cell division per day was calculated by the following formula; $log_2 N$, where N is the number of cells produced by cell division within 24 h. The daily isolation procedure was performed on several cell lines and continued for a few days.

Extraction of DNA and RNA: The cell pack from 10-100 ml of culture in the early stationary phase of the immature period ($5 \times 10^3 - 1 \times 10^5$ cells) was washed with sterilized Dryl's solution and lysed in NDS lysing solution (0.7 % sodium dodecyl sulfate, 0.3 M EDTA, 7 mM Tris-HCl, 0.7 mg / ml proteinase K, pH 8.0). After a 2h incubation of the mixture at 50 °C, DNA was extracted with phenol-chloroform twice and pelleted by addition of an equal volume of isopropanol followed by washing of the pellet with 70 % ethanol. The DNA was solubilized in distilled water and then used as a template for PCR. For the isolation of RNA,

approximately 100 µl of the cell pack (1 x 10^5 cells) obtained with gentle centrifugation was lysed in 1 ml of ISOGEN (Nippon Gene). The protocol for RNA isolation was provided by the same company. The procedure using ISOGEN described above was repeated once to remove residual proteins and DNA.

PCR and RT-PCR: Primers used for PCR and RT-PCR were designed according to the nucleotide sequence of the *pwB* gene described by Haynes *et al.* (2000). For PCR amplification of the region around IES427 of *pwB* gene, a sense primer *pwB*F395 (5′-CAATCATTAAGGATTTGGAATAATTGGAAG) and an antisense primer *pwB*R763 (5′-CGTCGTTTTCCTTATACTTCTCTTC) were used. SuperScript II (Gibco BRL) was used to generate first-strand cDNA primed by oligo-d(T) from 1.4 µg of total RNA. For the RT-PCR of most of the coding region of the *pwB* gene, two sense primers (with an antisense primer *pwB*R763 mentioned above) were used; *pwB*F-84 (5′-GGGCAATCCATTTAAGGCAAGTGG) and *pwB*F7 (5′-CTAGGAAAAGCAGGGGTTATGGC) were the sense primers from –84 to –60 bp upstream of and 7 to 30 bp of the coding region of the *pwB* gene sequence, respectively.

Southern blotting: The PCR product was digested with *Hin*fI and then run on 8 % polyacrylamide gel electrophoresis. The gel was processed with a denaturation solution (1.5 N NaOH, 0.5 M NaCl) and blotted onto a Hybond N+ membrane (Amersham) in 0.4 N NaOH. PCR products used as probes were purified by electrophoresis on polyacrylamide gel, elution against TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), phenol extraction, and ethanol precipitation. The labeled probe was prepared using ECL direct nucleic acid labeling and detection

systems (Amersham), and hybridization and signal generation procedures were also performed according to the manufacturer's instructions. The signal was measured using Scion image (Scion).

RESULTS

When the homozygotes of *pwB*^{662} were transferred to a stimulation solution, cells with different behavioral phenotypes were often observed. Cells showing no response (tight pawn), backward swimming (wild type), and the intermediate between wild type and pawn were present in a single culture. The mutant phenotype of d4-662 was so tight that it did not show any responses against the stimulation solution. I designated the cells with the pawn phenotype as "P662". However, the duration of backward swimming of some cells of d4-662 sometimes reaches the same length as the wild type. The appearance of cells showing any conspicuous responses to stimulation was considered to be caused by a reversion of *pwB*^{662}, and those thought to be reverted were called revertant or "R662".

Reverted cells originated from sexual reproduction: R662 predominated in the tube culture of d4-662. When cells pure for P662 were transferred to 20 tubes containing a fresh culture medium, R662 cells appeared in all tubes unless the cells died out (Table 24). Similarly, when cells pure for R662 were transferred to new tubes, P662 cells reappeared, though R662 cells were predominant (Table 24). Thus, the phenotypic conversion was bi-directional. To know the exact cause of the reversion of d4-662, how R662 emerges from P662 as well as how R662 converts to P662 was investigated.

Although I examined various environmental conditions that might

induce conversion of the phenotype of d4-662, including starvation, clonal aging, and temperature, it was only sexual reproduction that induced conversion of their cellular phenotype. Autogamy naturally occurs in ordinary tube cultures, thus providing a plausible explanation for the above experiment (Table 24). As shown in Table 25, the ratio of phenotypes in the autogamous progeny was similar between both P662 and R662 used as parents for induction of autogamy and was mostly P662 (94.9-97.3 %). The residual progeny were R662 (2.7-5.1 %). The ratio of progeny phenotype from autogamy was similar to that from conjugation (Table 20 in Part III).

It is apparent, however, that the ratio of R662 cells from autogamy was different from those observed in tube cultures. In the tube cultures, R662 cells were predominant, whereas R662 cells in the progeny from autogamy were the minority (compare Tables 24 and 25). The difference was most likely brought about by the selection of R662 cells. The average numbers of cell division, dead cells, and deformed cells per day were different between P662 and R662 (Table 26). Moreover, R662 cells were more resistant to starvation than P662 cells (Figure 10). Thus, the predominant appearance of R662 cells in tube cultures was likely the result of the selection of revertant cells produced from autogamy, which naturally occurs in the cultures.

Removal of the defective IES in the macronucleus of the reverted cells: The mutation of pwB^{662} is known to be a single base-pair substitution within an IES (IES427) of the *pwB* gene (Haynes *et al.*, 2000). Thus, the wild-type pwB^{+} gene would be generated if this IES were normally removed in the gene rearrangement. To test the possibility of the removal of IES427 in R662, *pwB* genes in total DNA

isolated from R662 and P662 were amplified by PCR. Because this IES is too short (44 bp) to resolve clearly in gel electrophoresis, the PCR products were digested with *Hin*fI, which recognizes IES427, and then loaded on polyacrylamide gel electrophoresis (PAGE), blotted to a nylon membrane, and hybridized with a *pwB* gene probe labeled with HRP (Figure 11). A small amount of a DNA fragment escaping from the cleavage by *Hin*fI (IES-removed, 96 bp in Figure 11) was detected in all three PCR products from R662 (clones 1 – 3) although the major bands were 67 bp and 73 bp (IES-containing). The band of 96 bp was never detected in the PCR products from P662. Comparisons of signal intensities of 96-bp bands (wild type) with IES-containing 67- and 73-bp bands of the Southern blot indicated that 2.9-7.7 % of the macronuclear *pwB* gene in R662 was the wild-type gene, i.e., IES-removed.

Expression of the IES-removed *pwB* gene in the reverted cells: If the IES-removed pwB^{+} gene in the macronucleus of R662 is responsible for the reversion of pwB^{662}, then the pwB^{+} gene in R662 cells should be transcribed. It is reported that transcripts of the *pwB* gene in d4-662, whether IES-removed or –containing, were below detectable levels on Northern blot (Haynes *et al.*, 2000). To examine transcription of wild-type gene in R662 cells, total RNAs obtained from R662 and P662 were subjected to oligo-d(T)-primed reverse transcription, and the *pwB* gene was amplified by PCR (RT-PCR). Unexpectedly against the report by Haynes *et al.* (2000), transcription of the *pwB* gene in P662 was detected; its amount was similar to that from R662 but much smaller than that from the wild type (Figure 12A, primer F7). RT-PCR using another primer (F-84), which effectively amplified the genomic *pwB* gene, did not give a detectable signal (Figure 12A),

demonstrating an absence of contamination from genomic DNA in the RNA samples.

To characterize the *pwB* gene fragments amplified by RT-PCR using primer F7 (756 bp – 863 bp in Figure 12A), they were digested with *Hin*fI restriction enzyme and loaded on PAGE (Figure 12B). Since many restriction sites were present in the coding region of the *pwB* gene as well as in IES427, the restriction enzyme is useful to detect the removal of both IES and intron. Two fragments in preparation from the wild type correspond to IES-removed (124 bp) and intron-removed (112 bp) fragments. The intron-removed fragments (112 bp) were visible in all RT-PCR products, showing that most of transcripts were mature. However, a faint 141-bp band was visible in the RT-PCR product of P662 (Figure 12B), showing that a small number of transcripts were not intron-removed. As expected, preparation from R662 contained an IES-removed fragment (124 bp in Figure 12B) that is not found in P662. These data suggest that expression of the wild-type phenotype in R662 is caused by a transcription of the wild-type gene (pwB^{+}) produced by a spontaneous excision of the defective IES in the macronuclear development.

DISCUSSION

The causes leading to reversion of mutant phenotypes are various. The most prevalent cases are suppression mutation of other genes, often tRNA genes, which suppress the mutational phenotype of the original gene. Many cases of reversion are genetic, though reverse mutations are less frequent. Others can be included as a conversion of the wild-type and mutant phenotype; mixture of wild type and mutant would appear under different environmental or physiological conditions

when the penetrance of the gene is not complete. I showed that the pawn mutant strain of d4-662 converts its behavioral phenotype only after sexual reproductions of autogamy and conjugation. The phenotypic conversion itself was not genic because progeny from autogamy of reverted cells (R662) showed mostly pawn phenotype. Thus, the phenomenon in d4-662 is due to incomplete penetrance of the *pwB* locus. The penetrance of the *pwB* phenotype of d4-662 is 92.9-97.3 %.

Present molecular analysis on the revertant of d4-662 provides a plausible explanation of this phenomenon. Reversion of d4-662 was tightly associated with a removal of the mutated IES427 in the macronuclear *pwB* gene and its transcript. The IES-removed form of the pwB^{662} allele, which was found in the macronucleus of revertants, should be completely the same as that of the wild-type gene and should thus code the functional protein product. Therefore, spontaneous excision of the IES should be the basis for the penetrance of the mutant phenotype. Because IES-removed and IES-containing DNA species were amplified by the same primer pair in PCR cycles, the ratio of restriction fragments observed in Figure 11 should reflect the amount of IES-removed *pwB* gene in the macronucleus of R662. Thus, the fraction of the IES-removed *pwB* gene (the 96-bp band in Figure 11) in the macronucleus of R662 is estimated to be roughly 5 %. If 1000 copies of the *pwB* gene are present in the macronucleus, R662 should have about 50 copies of the wild-type pwB^{+} gene. On the other hand, R662 cells are produced at a frequency of 2.7–7.1 % from sexual reproductions (Table 20 in Part III, and Table 25). Thus, the rate of spontaneous excision of the mutant IES in the process of gene rearrangement should be multiples of 'a fraction of the pwB^{+} gene in R662 cells (about 5 %)'

with 'a rate of appearance of R662 after sexual reproductions (2.7-7.1 %),' or much less than 1 % per gene.

It is suggested that IESs of the A^{51} and G^{51} gene of *Paramecium* are excised before they are amplified to 16 copies (Bétermier *et al.*, 2000). If the timing of excision of the IES427 of the *pwB* gene during macronuclear development is supposed to be 16 gene copies, one excision event will generate 1/16 or 6.25 % of the wild-type pwB^+ gene in the fully developed macronucleus. Alternatively, if it is supposed that the excision was more or less earlier than above (such as 8 copies), a greater number of wild-type gene copies would be amplified (such as 1/8 or 12.5 %). These percentages of wild-type copies are probably enough for the expression of the wild-type phenotype, since one of R662 clones, for example, clone 1 in Figure 11, possessed only about 3 % of the pwB^+ gene while showing backward swimming in the stimulation solution. However, macronuclear division is amitotic, and unequal assortment of pwB^+ allele may result in either concentration or dilution of it during cell divisions (Harumoto and Hiwatashi, 1992; Wong *et al.*, 2000). This unequal assortment likely brings about mixed clones of R662 and P662 often observed in progeny from sexual reproductions ("M" and "m" clones in Table 24).

The molecular lesion of d4-662 (pwB^{662}) is a single-base substitution at the 5th nucleotide inside the terminal consensus sequence of IES427 (Haynes *et al.*, 2000). Although present molecular analysis of the *pwB* gene in R662 cells revealed that a very small fraction of mutant IES performed their normal excision, the 5th position of the consensus sequence seems to be critical for the excision of *Paramecium* IESs, since more than 99 % of the *pwB* gene was IES-not-

removed in the strain. Exactly the same mutational site and base substitution (C to T) of the terminal consensus sequence of IES was reported in the *AIM-1* mutant of the surface antigen A^{51} gene in this species (Mayer *et al.*, 1998). Excision of the mutant IES has not yet been found in the *AIM-1* mutant. Since the terminal sequence of the IES427 of the *pwB* gene differs slightly from that of the IES2591 of the A^{51} gene, the importance of the 5th nucleotide for proper excision of the IES may depend on the surrounding sequences (Mayer *et al.*, 1998; Haynes *et al.*, 2000). Alternatively, it is also possible that they actually harbor the IES-removed, wild-type A^{51} gene in their macronuclei, even though the number may be very small. Because expression of the surface antigen genes is under as yet unknown control (Preer, 1986; Caron and Meyer, 1989), similarly to the mutually exclusive antigen expression in *Plasmodium* and *Trypanosome*, detection of the wild-type A^{51} gene by the phenotype or their transcripts in *AIM-1* mutant may be difficult.

Many alternative phenotypes in genomically identical clones similar to the present case were reported in trichocyst and the behavioral mutant of *P. tetraurelia* (Sonneborn and Schneller, 1979; Nyberg, 1980; Rudman and Preer, 1996). With respect to their ways of inheritance, the above-mentioned researchers suggested that the strains have acquired an unknown property in the macronuclear gene that tends to inherit along with the macronuclei. Recent studies on IESs of *Paramecium* A^{51} and the G^{51} surface antigen gene revealed that the presence of some IESs in the old macronucleus maternally inhibits the excision of the IES in a sequence-specific and copy number-dependent manner (Duharcourt *et al.*, 1995; Duharcourt *et al.*, 1998). Although d4-662 showed Mendelian

inheritance in crosses with the wild type (Saimi and Kung, 1987; Matsuda *et al.*, 2000), frequency of R662 produced from the autogamy of P662 was slightly smaller than that produced from the autogamy of R662 (Table 20 in Part III, and Table 24). Whether this was caused by a cytoplasmic effect of the insertion of the IES in the macronuclear *pwB* gene is yet unknown.

In this last part, I suggest that mutant IES was excised at much less than 1 % per pwB^{662} gene in macronuclear development. This is the molecular basis for the phenotypic conversion of the pawn mutant d4-662.

GENERAL DISCUSSION

This study showed that all existing strains of *pwB* showed unusual genetic phenomena. These inheritances as well as underlying mechanisms differ from each other and also from non-Mendelian inheritances known in ciliates. Strain d4-96 is a first aneuploidy in *P. tetraurelia* and has unusual genetic property in the chromosome carrying *pwB* locus (Matsuda and Takahashi, 2001a). Strain d4-95 had amplified extra *pwB* gene in the micronucleus and this amplified mutant gene seems to induce non-Mendelian inheritance. Strain d4-662 was found to have a mutant pwB^{662} allele, while heterozygotes of pwB^{662} with other *pwB* alleles expressed wild-type phenotype (Matsuda *et al.*, 2000). Study on a reversion frequently found in d4-662 suggested that a few copies of mutant IES are precisely excised in the gene rearrangement (Matsuda and Takahashi, 2001b).

The reason why all *pwB* strain has acquired unusual genetic peculiarity is unclear. Does *pwB* gene product have some feature inducing these unusual genetic phenomena? The key to solve this problem may be whether genetic phenomena found in *pwB* strains are mutually related or not. If they are related, the phenomena must owe to the *pwB* gene product. However, as summarized above, it does not seem to be related each other. One possible relationship, if any, may be that all genetic phenomena in *pwB* strains got to increase *pwB* gene dosage in the macronucleus or got closer to wild type phenotype. In other words, only *pwB* strains became closer to wild type phenotype now exist. This suggests that more *pwB* gene copies in the micronucleus, more fitness for the strain will have. In support to this, *pwB* strains are generally slow grower and, moreover, both

tetrasomy of *pwB*^{96} and reverted cells of *pwB*^{662} showed greater fission rates than the normal mutant strains (Matsuda and Takahashi, 2001a, 2001b). Therefore, higher fitness owing to increased or wild type *pwB* gene product may be the primary cause for these unusual genetic phenomena. Alternatively, involvement of genetic backgrounds of these *pwB* strains may also be possible. At present, no information to discriminate these possibilities is available.

Although genetic phenomena found in *pwB* strains appear to be uncommon in Eukaryotes, following discussions could bring these phenomena into more common biological ground. Genetic mechanisms involved in the unusual inheritances are usually operated in often unrecognized, normal processes. For instance, transgene-induced gene silencing in plants and fungi as well as RNA interference (RNAi) in *C. elegans* was found to be a normal genome defense system against foreign DNA invasion such as viruses (Bastin *et al.*, 2001). Similarly, cellular mechanisms such as DNA methylation and histon deacetylation involved in most of epigenetic phenomena (Flavell, 1994; Matzke and Matzke, 1995; Barlow, 1997; Hollick *et al.*, 1997; Jaenisch, 1997; Selker, 1997; Sherman and Pillus, 1997; Klar, 1998) are used in the cellular differentiation in development, chromosome condensation, centromere formation and also contribute to the repression of transposable elements (Karpen and Allshire, 1997; Birchler *et al.*, 1999). No one can deny the possibility that the unusual genetic mechanisms found in this study are also operated in the normal processes not only in *Paramecium* but also in other eukaryotic organisms. The study of non-Mendelian inheritances of *pwB* thus opened up many ways to study the genetic systems in *Paramecium*.

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TABLE 1

Stocks used in this study

Stock	Mutant genes		Source
d4N-527	*nd169*		From Takagi (Nara Woman's University), originally isolated by Nyberg (University of Illinois) (Nyberg, 1978)
nd6	*nd6*		From Hamasaki (Albert Einstein University)
$nd9^c$	$nd9^c$		From Cohen (CNRS, Gif-sur-Yvette)
nd7; ts111	*nd7*	*ts111*	From Cohen
d4-96		pwB^{96}	From Kung (University of Wisconsin)

TABLE 2

Segregation of behavioral phenotypes in F_2 and those of mixed type in F_3

Cross	No. of F_1 synclones examined	F_2 phenotype: Wild type	F_2 phenotype: Pawn	Expected ratio	p	F_3 phenotypes from wild-type F_2: Non-mixed	F_3 phenotypes from wild-type F_2: Mixed[a]	Expected ratio	p
d4-96 × *nd169*	8	135	133	1:1	0.9	41	94	1:2	0.5
d4-96 × *nd6*	3	42	47	1:1	0.6	10	32	1:2	0.2
d4-96 × *nd7; ts111*	3	93	92	1:1	0.9	34	56	1:2	0.4

Probability (p) was calculated by χ^2 test.

[a] Progeny containing wild-type and pawn clones. The ratio of segregation of non-mixed vs. mixed was close to 1:2.

TABLE 3

Segregation of behavioral phenotypes in mixed clones

Autogamous generation	Phenotypic segregation of autogamous progeny[a]					
	All wild type		Wild type and pawn[b]		All pawn	
	W	P	W	P	W	P
F_3			39	13		
F_4	63	0	103	24	0	54
F_5	26	0	89	24	0	48
F_6	71	0	67	10	0	41
F_7			9	2	0	18

W, wild type; P, pawn.

[a] Parents for induction of autogamy were classified into three categories depending on the segregation of the progeny phenotype. "All wild type" did not produce progeny of pawn. "All pawn" did not produce progeny of wild type. "Wild type and pawn" produced both wild-type and pawn progeny. Wild-type parents for autogamy in successive generations were obtained from clones in the "wild-type and pawn" category (see Figure 1B).

[b] The ratio of segregation of wild type vs. pawn was close to 5:1 ($0<\chi^2<2.6$, $0.1<p<0.99$).

TABLE 4

Segregations of marker genes used in this study

Cross	Survival in F_2 (%)	Segregation of F_2 phenotype by autogamy							
		Trichocyst				Temperature sensitivity			
		Discharge	Non-discharge	χ^2		Resistant	Sensitive	χ^2	
				1:1[a]	5:1[b]			1:1[a]	5:1[b]
d4-96 × *nd169*	91.4	191	204	0.4*	286				
d4-96 × *nd6*	87.3	49	40	0.9*	51				
d4-96 × *nd7; ts111*	86.1	99	87	0.8*	121	87	99	0.8*	179

The χ^2 value, which indicates a probability (p) higher than 0.05, is indicated by an asterisk. Segregation ratios of behavioral phenotype in F_2 were close to 1:1 in all crosses ($0.0<\chi^2<0.3$, $0.5<p<0.99$, see Table 2).

[a] Expected ratio of the disomy of chromosomes carrying marker genes in the strain d4-96.

[b] Expected ratio of the tetrasomy of chromosomes carrying marker genes in the strain d4-96.

TABLE 5

Tests of tetrasomy and disomy in wild-type segregants by crossing with d4-96

Wild-type segregants from original cross	F_2 in crosses with d4-96								
		Segregation of behavior in F_2				F_3 of wild type			
	Survival (%)	Wild type	Pawn	Expected ratio	χ^2	Non-mixed	Mixed	Expected ratio	χ^2
W7	97.2	11	24	1:1	4.8	3	8	1:2	0.2 *
W14	100.0	13	23	1:1	2.8 *	5	8	1:2	0.2 *
W24	100.0	17	19	1:1	0.1 *	1	16	1:2	5.8
W27	100.0	17	19	1:1	0.1 *	3	14	1:2	1.9 *
WC-4a	100.0	59	13	5:1	0.1 *	5	25	1:4	0.2 *
WC-4b	96.3	84	20	5:1	0.5 *	10	33	1:4	0.3 *

Progeny W7, W14, W24, and W27 are wild-type segregants in the original F_2, and WC-4a and WC-4b are wild-type segregants in the original F_4, both from crosses between d4-96 and wild type. The χ^2 value, which indicates a probability (p) higher than 0.05, is indicated by an asterisk.

TABLE 6

Tests of tetrasomic and disomic *pwB* segregants by crossing with wild type

Pawn segregants from original F_2	F_2 in crosses with wild type					Deduced genotype
	Survival (%)	Segregation of F_2		F_3 of wild type		
		Wild type	Pawn	Non-mixed	Mixed	
O4	94.4	19	15	19	0	Disomy
O8	95.8	34	35	33	0	Disomy
O9	97.2	40	30	37	0	Disomy
O10	97.2	15	20	4	11	Tetrasomy
O18	100.0	41	30	41	0	Disomy
O25	100.0	37	35	18	18	Tetrasomy
O29	47.1	19	14	6	12	Tetrasomy
O31	94.4	30	38	30	0	Disomy
O35	93.1	32	35	28	0	Disomy
E5	91.7	34	32	34	0	Disomy
E6	61.1	21	23	6	12	Tetrasomy
E10	88.9	25	39	25	0	Disomy
E14	97.2	41	29	41	0	Disomy
E16	69.4	9	16	4	5	Tetrasomy
E17	98.6	33	38	32	0	Disomy
E25	100.0	37	35	37	0	Disomy
E28	100.0	37	35	17	20	Tetrasomy
E34	95.8	34	35	33	0	Disomy
E12N	22.2	13	3	10	0	Disomy
				No. of segregants		
				6		Tetrasomy
				13		Disomy

TABLE 7

Tests of tetrasomy and disomy in wild-type segregants after several autogamous generations

Autogamous generation in which strains were crossed	Wild-type F_3 or F_4 segregants[a]	Survival in F_2 (%)	Phenotype in F_2 of this cross: Wild type	Pawn	χ^2 5:1 [b]	χ^2 1:1 [c]	Wild type in F_3 of this cross: Non-mixed	Mixed	χ^2 1:4 [b]	χ^2 1:2 [c]	Deduced genotype of wild type
gen. 1	W1-3	100.0	29	7	0.2 *	13.4	4	25	0.7 *	5.0	Tetrasomy
	W5-30	88.9	26	6	0.1 *	12.5	7	19	0.8 *	0.5 *	Tetrasomy
	W12-36	100.0	11	13	24.3	0.2 *	6	5	8.2	2.2 *	Disomy
gen. 4	W1-3	100.0	22	1	2.5 *	19.2	3	19	0.6 *	3.8	Tetrasomy
		100.0	14	8	6.1	1.6 *	2	12	0.3 *	2.3 *	Disomy
	W5-30	100.0	21	2	1.1 *	15.1	3	18	0.4 *	3.4	Tetrasomy
		100.0	10	13	26.3	0.4 *	4	6	2.5 *	0.2 *	Disomy
gen. 8	W1-3	100.0	20	4	0.0 *	10.7	5	15	0.3 *	0.6 *	Tetrasomy
		88.2	6	9	20.3	0.6 *	3	3	3.4	0.8 *	Disomy
	W5-30	95.8	19	4	0.0 *	9.8	6	13	1.6 *	0.0 *	Tetrasomy
		100.0	14	9	8.4	1.1 *	0	14	3.5	7.0	Disomy
Unknown[d]	WC-4a	100.0	19	17	24.2	0.1 *	3	9	0.2 *	0.4 *	Disomy
	WC-4b	100.0	19	17	24.2	0.1 *	9	10	8.9	1.7 *	Disomy

The wild-type segregants obtained from original crosses were crossed with d4-96 after they had undergone the indicated autogamous generations (gen., see Figure 3). The χ^2 value, which indicates a probability (p) higher than 0.1, is indicated by an asterisk.

[a] Segregants derived from F_3 or F_4 of original crosses (see Figure 1).

[b] Expected ratio of tetrasomy of the wild type.

[c] Expected ratio of disomy of the wild type.

[d] The crosses were carried out after the clones WC-4a and WC-4b had been cultured for several months.

TABLE 8

Comparison of cell division per day and % of survival from the autogamy of progenies from crosses between d4-96 and wild type with various genotypic compositions

	d4-96 × *nd169*		d4-96 × *nd7; ts111*				
	-/-	-/-/-/-	-/-	-/-/-/-	+/+	+/+/-/-	+/+/+/+
Cell division/day ± S.D.	2.8 ± 0.3	3.0 ± 0.3	2.8 ± 0.6	3.0 ± 0.3	3.5 ± 0.2	3.5 ± 0.3	3.6 ± 0.3
No. of F_2 progeny examined	12	6	11	5	9	5	5
No. of cell lines examined	24	12	11	5	9	63	6
			d4-96 × *nd169*				
			-/-	-/-/-/-	+/+	+/+/-/-	+/+/+/+
Survival from autogamy (%)			86.1	79.8	94.0	92.9	93.9
No. of F_2 progeny examined			4	3	12	13	3
No. of cell lines examined				16		40	20

TABLE 9

Segregation ratio of pooled data in the crosses between wild type and pwB^{96}

Cross		No. of crosses	Average of survival (%)	Segregation of behavioral phenotype in F_2				Segregation of wild type in subsequent generation			
Wild type	× pwB^{96}			Wild type	Pawn	Expected	*p*	Non-mixed	Mixed	Expected	*p*
Disomy	× Disomy	77	89.8	1258	1219	1:1	0.6				
	Expected			*1239*	*1239*						
Disomy	× Tetrasomy	109	93.4	1551	1629	1:1	0.2	487	1027	1:2	0.3
	Expected			*1590*	*1590*			*505*	*1009*		
Tetrasomy	× Tetrasomy	13	98.3	347	62	5:1	0.4	59	218	1:4	0.6
	Expected			*340*	*68*			*55*	*222*		
								Wild type	Pawn		
Autogamous Lineage		31	93.0					405	99	5:1	0.1
	Expected							*420*	*84*		

TABLE 10

Stocks used in this study

Stock	Genes		Source
d4-95	*pwB*^{95}	*nd6*	Kung, C. (Univ. of Wisconsin)
51s			Our laboratory
d4N-527	*nd169*		Takagi, Y. (Nara Woman's Univ.)
nd6	*nd6*		Hamasaki, T. (Albert Einstein Univ.)
nd7; ts111	*nd7*	*ts111*	Cohen, J. (CNRS, gif-ser-yvette)
nd9	*nd9*		Cohen, J. (CNRS, gif-ser-yvette)
d4-96	*pwB*^{96}		Kung, C. (Univ. of Wisconsin)
a3093	*pwB*^{96}	*nd9*	F_2 segregant from cross of d4-96 with nd9, disomic for the chromosome carrying *pwB* locus

TABLE 11

Pedigree analysis on the F_1 caryonides derived from crosses of wild type with d4-95

Parental cytoplasm[a]				Cross[b]		
Wild type		d4-95		C1	C2	C3
Mendelian inheritance						
W	W	W	W	10	21	25
			%	22.2	46.7	55.6
Pawn progeny appeared in d4-95 cytoplasmic descendants						
W	W	W	M	4	4	4
W	W	W	P	1		
W	W	M	M	7		1
W	W	M	P	11	3	
W	W	P	P	1	1	
			%	53.3	25.0	14.7
Pawn progeny appeared in both cytoplasmic descendants						
W	M	W	P		1	
W	M	M	M	1		
W	M	M	P	1		
W	M	W	M	1		
W	M	P	P			1
W	P	M	M	1		
M	M	W	M	1		
M	M	W	P			1
M	M	P	P	2		
M	P	M	M	1		
M	P	P	P			1
			%	17.8	3.1	8.8
Pawn progeny appeared in wild-type cytoplasmic descendants						
W	M	W	W	1	2	
M	M	W	W			1
M	P	W	W	1		
P	P	W	W	1		
			%	6.7	6.3	2.9
Total % of non-Mendelian inheritance				77.8	34.4	26.5

[a] Because one exconjugant produces two caryonides, four F_1 caryonides were isolated and separately grown. Then the phenotype of caryonides are aligned horizontally; "W", wild type, "P", pawn; "M", mixed clone of wild-type and pawn cells. Parental cytoplasms of caryonides were determined by mating type that shows cytoplasmic inheritance. Note that pawn progeny appeared predominantly in d4-95 cytoplasmic descendants.

[b] For three independent crosses between d4-95 with wild type (C1, C2 and C3), see Figure 5.

TABLE 12

Segregation of behavioral phenotype in F_2 and those of mixed type in the subsequent autogamous generations from F_1

Cross[a]	Cytoplasm	F_2: Survival (%)	F_2 Behavior: Wild type	F_2 Behavior: Mixed[b]	F_2 Behavior: (Wild type and mixed)	F_2 Behavior: Pawn	F_2 Behavior: p^c	Subsequent autogamous generations: No. of wild-type F_2 progeny examined	% of wild type clone to be mixed: In F_3	% of wild type clone to be mixed: In a month
C1	Wild type	74	21	4	(25)	27	0.8	12	58	83
	d4-95	33	10	2	(12)	12	1.0	5	60	100
C4	Wild type	65	23	2	(25)	22	0.7	7	100	100
	d4-95	82	28	1	(29)	31	0.8	8	88	100
C5	Wild type	31	28	0	(28)	17	0.1	28	7	100
	d4-95	31	16	5	(21)	25	0.6	17	82	100

[a] Marked in Figure 1.

[b] Clones containing wild-type and pawn cells. For χ^2 test, mixed type was counted as wild type.

[c] Probability (p) was calculated with χ^2 test with the expected phenotypic segregation ratio of 1:1.

TABLE 13

Summary of the phenotypic segregation ratios in the autogamous lineages from F_3 to F_7

Cross from which lineages were derived[a]	Phenotype of parents for induction of autogamy	No. of autogamous progeny examined	Phenotype of exautogamous caryonidal pair					
			Wild type Wild type	Wild type Mixed	Wild type Pawn	Mixed Mixed	Mixed Pawn	Pawn Pawn
C5	Wild type	185	51 (28%)	13 (7%)	33 (18%)	6 (3%)	23 (12%)	59 (32%)
	Pawn	116	37 (32%)	7 (6%)	16 (14%)	3 (3%)	9 (8%)	44 (38%)
C1	Wild type	264	130 (49%)	0 (0%)	27 (10%)	2 (1%)	1 (0.4%)	104 (39%)
	Pawn	64	16 (25%)	0 (0%)	3 (5%)	2 (3%)	0 (0%)	43 (67%)
Total		629	234 (37%)	20 (3%)	79 (13%)	13 (2%)	33 (5%)	250 (40%)

[a] For the name of crosses, see Figure 5.

TABLE 14

Phenotypic segregation of autogamous F_2 and F_3 from the non-Mendelian inheritance F_1 of the cross between d4-95 and wild type

	No. of F_1 caryonide examined	Clones: Isolated	Clones: Survived	F_2: Wild type (Wild type and mixed)	F_2: Mixed	F_2: Pawn	F_2: p^a	No. of F_2 clone[b]	F_3: Wild type (Wild type and mixed)	F_3: Mixed	F_3: Pawn	F_3: Died	F_3: p^a
F_1 classified by parental cytoplasm													
wild type	13	360	212	43	35	134	<0.01	212	32	78	95	7	0.3
				(78)					(110)				
d4-95	14	396	175	15	20	140	<0.01	175	6	72	85	12	0.6
				(35)					(78)				
F_1/F_2 classified by its own phenotype[c]													
Wild type	11	288	178	38	22	118	<0.01	58	17	38	2	1	
				(60)					(55)				
Mixture	1	36	17	4	3	10	0.47	55	7	43	4	1	
				(7)					(50)				
Defective wild type	5	180	71	5	14	52	<0.01						
				(19)									
Pawn	10	252	121	11	16	94	<0.01	274	14	69	174	17	
				(27)					(83)				
Total	27	756	387	58	55	274	<0.01	387	38	150	180	19	0.7
				(113)					(188)				

F_2 and F_3 autogamous progeny from the cross of C1 (see Figure 5). Marker gene was segregated expectedly in F_2.

[a] For χ^2 test, mixed phenotype was taken as wild type and the expected ratio was 1:1.

[b] Numbers of the F_2 clones of each entry correspond to them summarized in the "F_2" column.

[c] Progeny were classified according to the behavioral phenotype of parents (F_1 or F_2) for induction of autogamy.

TABLE 15

Strains used in this study

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

[a] Once called as *pwD*. See Results.

TABLE 16

Curing of the mutant phenotype of d4-662 by cytoplasmic transplantation from pawns and CNRs

Donor	Duration of backward swimming (sec) [a]		Donor	Duration of backward swimming (sec) [a]	
P. tetraurelia			*P. caudatum*		
Wild type	23.7 ± 10.4	(10) [b]	Wild type	10.0 ± 3.7	(7) [b]
pwA	19.7 ± 10.6	(4)	*cnrA*	13.0 ± 5.3	(11)
pwB^{95}	0	(15)	*cnrB*	8.7 ± 1.6	(6)
pwC	21.6 ± 11.1	(5)	*cnrC*	6.8 ± 1.7	(6)
d4-662	0	(16)	*cnrD*	11.2 ± 2.3	(12)

[a] Duration of backward swimming (sec ± SD) in 20-mM stimulation solution 3-5 hr after cytoplasms had been injected to the recipients (d4-662).

[b] Numbers in parentheses indicate number of injected cells.

TABLE 17

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

Donor	Recipient			
	pwA	*pwB*^{95}	*pwC*	
P. caudatum Wild type	28.8 ± 13.3 (4)	11.1 ± 4.4 (6)	—	
P. tetraurelia Wild type	22.5 ± 4.4 (7)	19.4 ± 6.9 (11)	—	
d4-662	29.2 ± 11.5 (6)	0 (14)	19.4 ± 5.8 (9)	
No injection	0 (20)	0 (20)	1.0 ± 1.9 (20)	
	cnrA	*cnrB*	*cnrC*	*cnrD*
P. caudatum Wild type	26.9 ± 12.9 (2)	17.0 ± 9.8 (13)	75.4 ± 25.9 (6)	9.3 ± 4.8 (6)
P. tetraurelia Wild type	23.0 ± 9.7 (6)	11.5 ± 4.4 (6)	65.3 ± 32.1 (11)	16.7 ± 4.6 (3)
d4-662	18.2 ± 2.5 (3)	16.9 ± 8.1 (5)	46.7 ± 12.2 (3)	27.1 ± 12.8 (6)
No injection	1.5 ± 2.4 (20)	2.4 ± 2.6 (20)	1.1 ± 1.9 (20)	0 (20)

Numbers are the duration of backward swimming (sec) ± SD. "—", not determined.

Numbers in parentheses indicate the number of cells tested. Duration of backward swimming of uninjected wild type is about 35 sec and 90 sec in *P. tetraurelia* and *P. caudatum*, respectively.

TABLE 18

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 of macronucleoplasm, the behavior of the recipient was examined. Volume of injection was more than two-thirds of the macronucleoplasm of the recipients. See footnotes in Table 16.

TABLE 19

Nucleoplasmic transplantation with marker genes

		No. of cells		
			Rescued	
Donor	Recipient	Injected	Trichocyst	Behavior
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}; *nd169*	d4-662; *nd6*	13	7	0
d4-662; *nd6*	*pwB*^{96}; *nd169*	5	5	0

—; not determined. See footnotes in Table 16

TABLE 20

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 synclone and the four cells from the first cell division of two exconjugants are called caryonides. These four F_1 caryonides were separated then phenotype of 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 synclone 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 mixture of R662 and pawn.

TABLE 21

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: d4-662		Distribution of phenotypes derived from cytoplasmic parents: *pwB*^{95} or *pwB*^{96}		No. of synclones: d4-662 × *pwB*^{95}	No. of synclones: d4-662 × *pwB*^{96} Cross 1	No. of synclones: d4-662 × *pwB*^{96} 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 "indicates wild-type phenotype, and "P" indicates pawn phenotype.

TABLE 22

Segregation of F_2 phenotype from autogamy of F_1

Cross	Survival (%)	Behavior: Wild type	Mixture [a]	Pawn	Trichocyst [b]: Discharge	non-discharge	χ^2	*p*
d4-662; *nd6* × *pwB*^{95}	57	75	10	70	74	81	0.3	0.6
d4-662; *nd6* × *pwB*^{96}; *nd169* Cross 1	69	5	4	128	29	108	1.1	0.3
d4-662; *nd6* × *pwB*^{96}; *nd169* Cross 2	90	49	17	194	68	192	0.2	0.7

Progeny were obtained from both cytoplasmic parents and from various phenotypes in 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}; *nd169*.

TABLE 23

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 (sec)	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 (sec) ± SD in the stimulation solution.

Numbers in parenthesis indicate the number of cells examined. —; not determined.

TABLE 24

Appearance of reverted cells from homogenous culture of either pawn or revertant

Original homogeneous culture	% of reverted cells in the tube culture							
	100	90	70	50	30	0	Died	Total
P662	2	5	4	3	3	0	3	20
R662	3	11	5	1	0	0	0	20

P662 means cells of the pawn phenotype, while R662 means revertants of d4-662. Approximately 50-100 cells were transferred to new tubes containing 2 ml of a fresh culture medium and fed the double volume of fresh culture medium when food bacteria in the culture medium became to be exhausted. This feeding procedure was further repeated twice. The phenotype was observed when cells entered in stationary phase after the final feeding. Data are presented with number of tubes.

TABLE 25

Alternative phenotypes in the autogamous progeny of d4-662

Parents for induction of autogamy	Progeny (%)			
	R662[a]			
	R	M	m	P662
P662	7 (0.8)	12 (1.3)	6 (0.6)	915 (97.3)
R662	14 (2.5)	11 (1.9)	4 (0.7)	539 (94.9)

[a] Progeny containing R662 cells are classified into three categories: clones pure for R662 cells (R), clones containing more than half of R662 cells (M), and clones containing less than half of R662 cells (m). Progeny means exautogamous clones.

TABLE 26

Number of cell division, frequency of cell death, and deformed cells per day in cell lines of mutant and revertant of d4-662

Clone	Number of cell lines examined	Cell division ± S. D.	% of cell line terminated by cell death	% of deformed cells
Wild type	42	4.2 ± 0.7	0	0
R662	126	3.3 ± 0.5	0	0.1
P662	109	2.4 ± 0.2	8	10

See Materials and Methods for experimental procedure of cell lines.

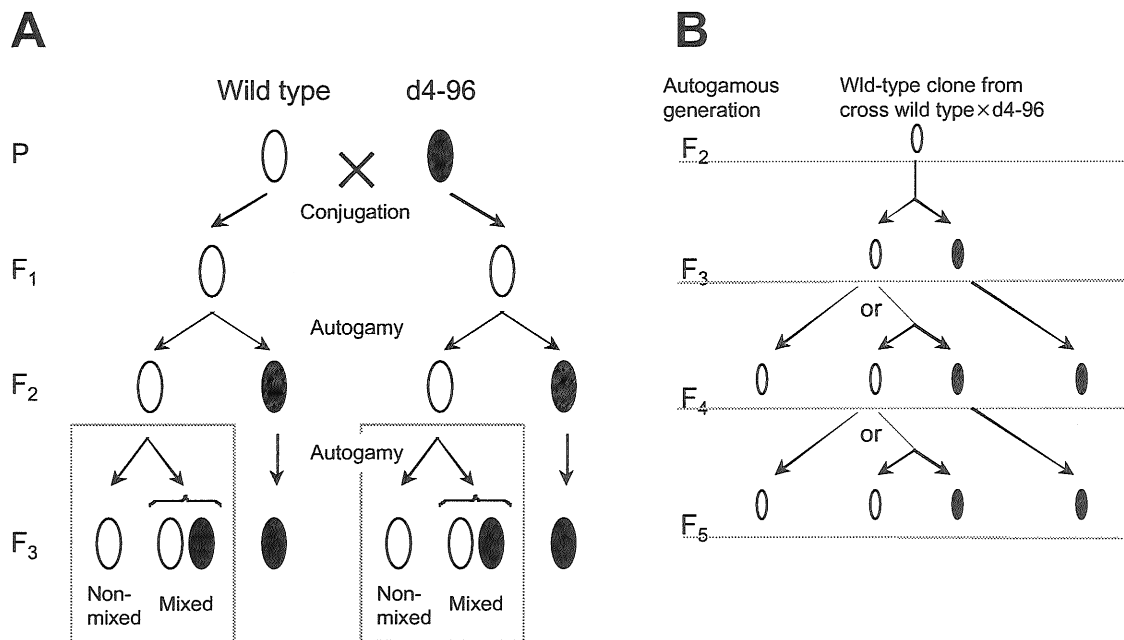
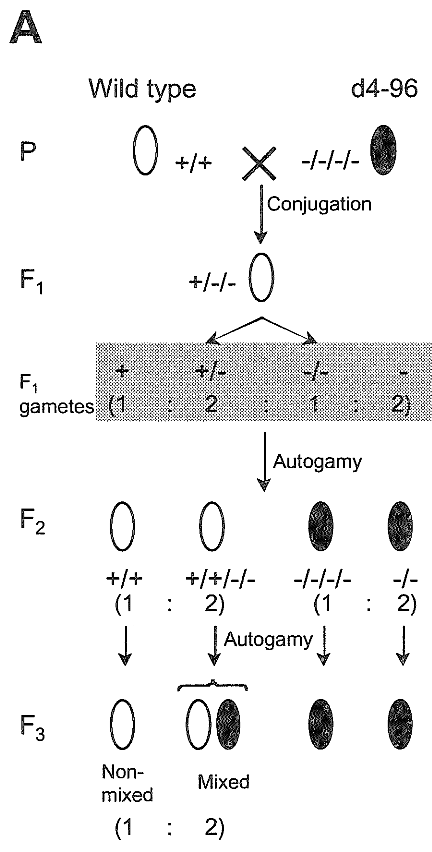
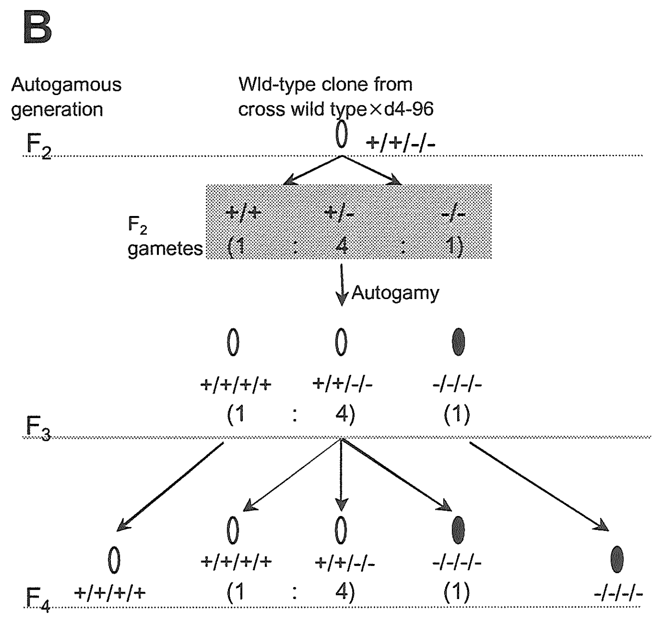


FIGURE 1. --------Inheritance of the strain d4-96. Clones with wild-type and pawn phenotypes are indicated by white and black ovals, respectively. (**A**) When looking only at F_1 and F_2, the inheritance observed in the cross of d4-96 with wild type resembles that of a typical recessive gene. Some wild-type progeny in F_2, however, become mixed clones of wild-type and pawn cells in subsequent autogamous generations. (**B**) Autogamous progeny was isolated from wild-type F_2 and subsequent generations to observe the segreagtion of behavioral phenotype. In the autogamous lineage thus obtained, some wild-type parents produced only wild type, while others produced both wild type and pawn from autogamy.

FIGURE 2. --------The inheritance of d4-96 might be explained if the strain was a tetrasomy of the chromosome carrying the *pwB* gene. White oval, wild type; black oval, pawn. Symbols "+" and "-" indicate chromosomes carrying the wild-type and mutant alleles of *pwB*, respectively. (A) and (B) correspond to those in Figure I-1. (**A**) A cross of ordinary wild type (+/+) with tetrasomy of pwB^{96} (-/-/-/-) will produce trisomic F_1 (+/-/-). Since two mutant chromosomes (-) are present, four kinds of gametes should be produced with indicated ratio (shaded area) from meiosis of this F_1. Autogamy will simply duplicate the genotypic composition of gametes and produce disomic and tetrasomic F_2 progeny, including unusual heterozygous wild-type F_2 (+/+/-/-). This heterozygous F_2 will produce wild-type and pawn cells after autogamy, resulting in mixed progeny in F_3. The detailed analysis of the mixed progeny was shown in (**B**). Three kinds of gametes should be produced (the ratio is indicated in the shaded area) two of which become homozygous tetrasomy for either wild type or mutant while one of which becomes heterozygous tetrasomy with identical genotype to the parent (F_2) after autogamy. After the next round of autogamy of heterozygous tetrasomy, again three kinds of progeny genotype are possible like F_3.


A
Wild type
d4-96
P
+/+
-/-/-/-
Conjugation
F1
+/-/-
F1 gametes
+ +/- -/- -
(1 : 2 : 1 : 2)
Autogamy
F2
+/+ +/+/-/- -/-/-/- -/-
(1 : 2) (1 : 2)
Autogamy
F3
Non-mixed
Mixed
(1 : 2)


B
Autogamous generation
Wld-type clone from cross wild type×d4-96
F2
+/+/-/-
F2 gametes
+/+ +/- -/-
(1 : 4 : 1)
Autogamy
+/+/+/+ +/+/-/- -/-/-/-
(1 : 4) (1)
F3
+/+/+/+ +/+/-/- -/-/-/-
(1 : 4) (1)
F4
+/+/+/+
-/-/-/-

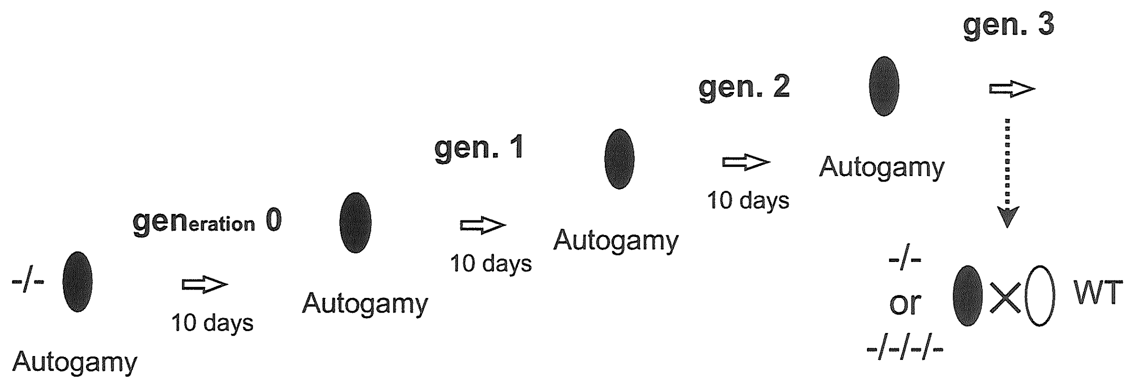


FIGURE 3. --------Pawn segregants were cultured to examine the change of their genotypes during culturing. The nutritional condition of the segregants was controlled to induce autogamy every 10 days, corresponding to approximately every 30 cell divisions. Autogamous generations (gen.) of the segregants were counted from F_2 in the original cross between wild type and d4-96. Cells were crossed with the wild type, and then the phenotypic segregation in F_2 and non-mixed vs. mixed in F_3 from wild-type F_2 were examined (see Figure 2A). Wild-type segregants from F_3 were also examined in the same way and tested with d4-96.

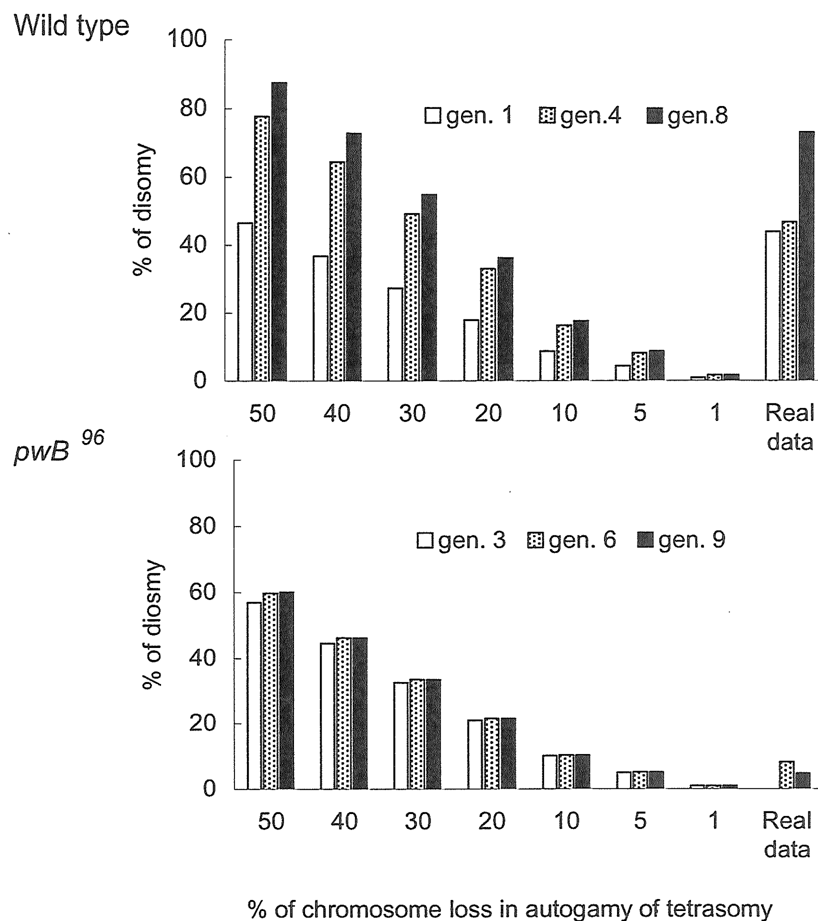


FIGURE 4. --------Simulated and real appearance of disomy in the culture that was initially pure for homozygous tetrasomy through several autogamous generations (gen., see Figure 3). The % of disomy (ordinate) is presented individually as a function of the hypothetical parameter of chromosome loss in the autogamy of tetrasomy (abscissa; *l*, see Appendix). If chromosome loss in the autogamy of tetrasomy (*l*) in wild type is supposed to be, for example, 40 % in every meiosis, the ratio of disomy in a culture calculated from fission rates and survivals from autogamy (Table 8) should be 37 %, 64 %, and 73 % in gen. 1, gen. 4, and gen. 8, respectively (see Appendix). The real data were obtained by crossing the cells from the culture. Some of them are presented in Table 7. The appearance of disomy in the real data from the culture initiated with tetrasomy of wild type is similar to those predicted by higher *l* values (more than 30 %), while that from the culture initiated with tetrasomy of pwB^{96} is similar to those predicted by lower *l* values (near 5 %).

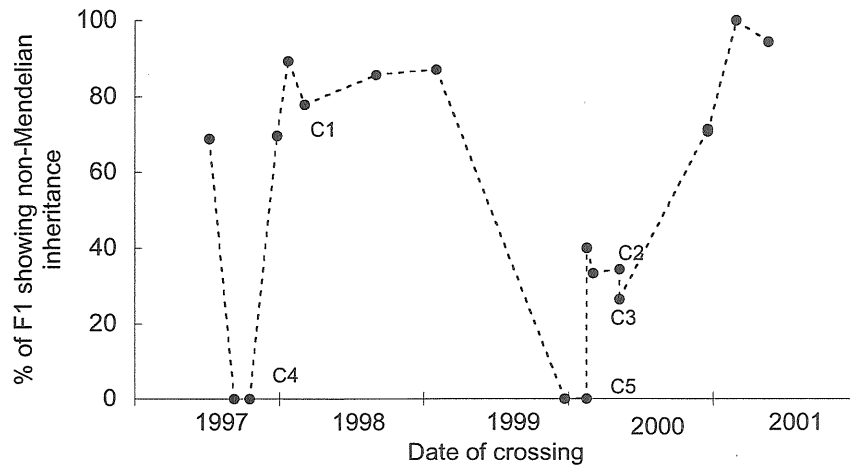


FIGURE 5. --------For about four years, strain d4-95 had been crossed with wild type at 17 time points and % of F_1 showing non-Mendelian inheritance was plotted. Although cultivation of the strain was not always constant during the experimental period (such as long time starvation followed by continuous growth and transfer of small number of cells), F_1 progeny from the crosses sometimes showed complete Mendelian inheritance and sometimes showed complete non-Mendelian inheritance. More than 11 F_1's were examined in each cross with at least one marker gene. Cross numbers (C1 to C5) were labeled along with the time point of the cross (black circles).

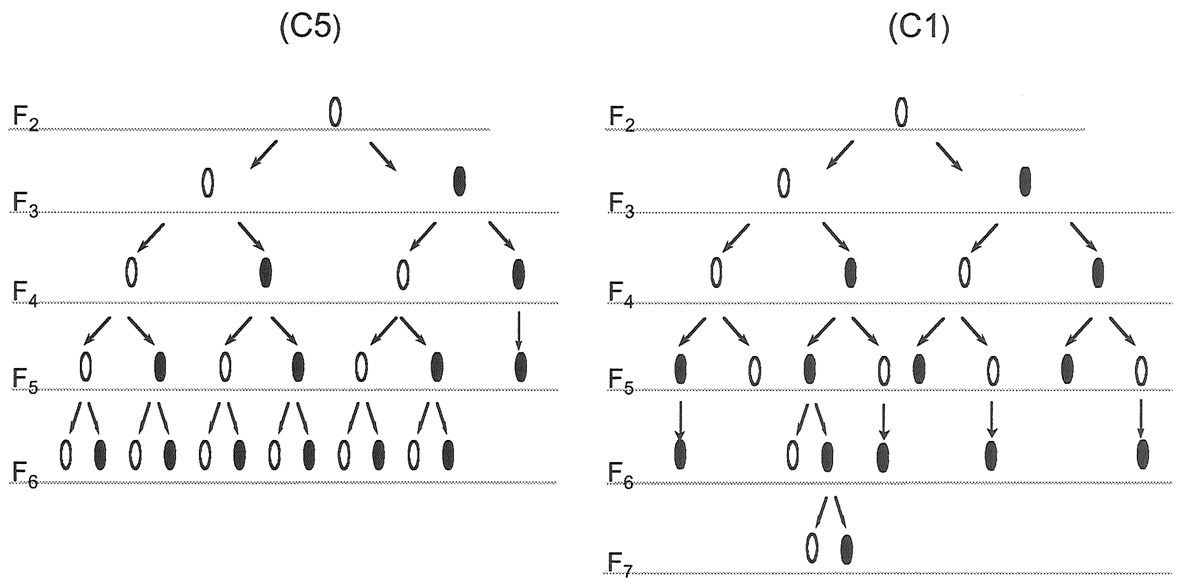
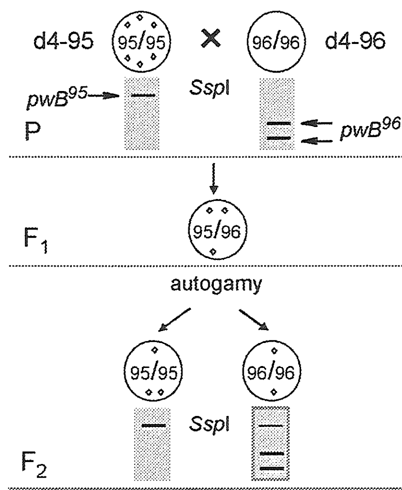


FIGURE 6. --------Examples of pattern of phenotypic segregation in autogamous lineages derived from wild-type F_2's from the crosses between d4-95 with wild type. White oval indicates wild-type progeny and black oval indicates pawn progeny. Cross names (C5 and C1) were indicated in Figure 5. For ratio of wild-type and pawn progeny, see Table 13.

FIGURE 7.--------Identification of *pwB* alleles in the autogamous F_2 progeny from cross using d4-95. (**A**) Strain d4-96 had a *pwB*^{96} allele specifically recognized by a restriction enzyme, *Ssp*I. d4-96 was crossed with d4-95 and F_2 were obtained by autogamy. The suspected extra *pwB* gene copies in the micronucleus of d4-95 are indicated as dots, and they are supposed to be inherited independently from the original *pwB* locus. In the absence of the extra *pwB* gene, all F_2 progeny will be homozygous, as shown by genotypes. But if this extra copy is present in the micronucleus of d4-95, it will produce homozygous and heterozygous F_2 progeny at the ratio of 1:1. From the F_2 progeny, the *pwB* gene was amplified by PCR and cleaved by the restriction enzyme. If the hypothetical extra *pwB* gene in d4-95 is heritable independently from the original locus, F_2 progeny harboring the *pwB* gene derived from d4-96 should be heterozygous with the extra *pwB* gene derived from d4-95. (**B**) An example of the restriction analysis for the F_2 progeny. PCR products amplified from d4-96, d4-95, and F_2 segregants from a cross of d4-95 with a disomic derivative of d4-96 were digested with *Ssp*I, loaded on 5 % polyacrylamide gel electrophoresis and stained with Ethidium Bromide. Clones 1, 2 and 4 contained two types of *pwB* alleles (*pwB*^{95} type and *pwB*^{96} type), i.e. heterozygous, while clones 3, 5 and 6 only contained *pwB*^{95} allele.

A



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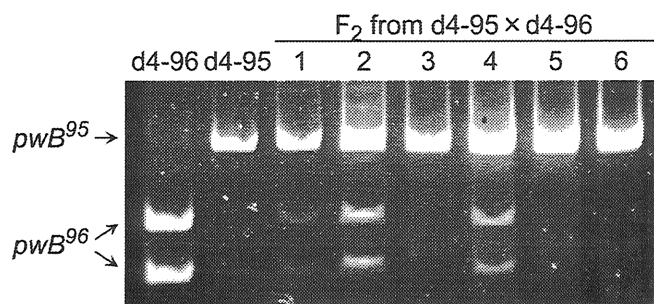

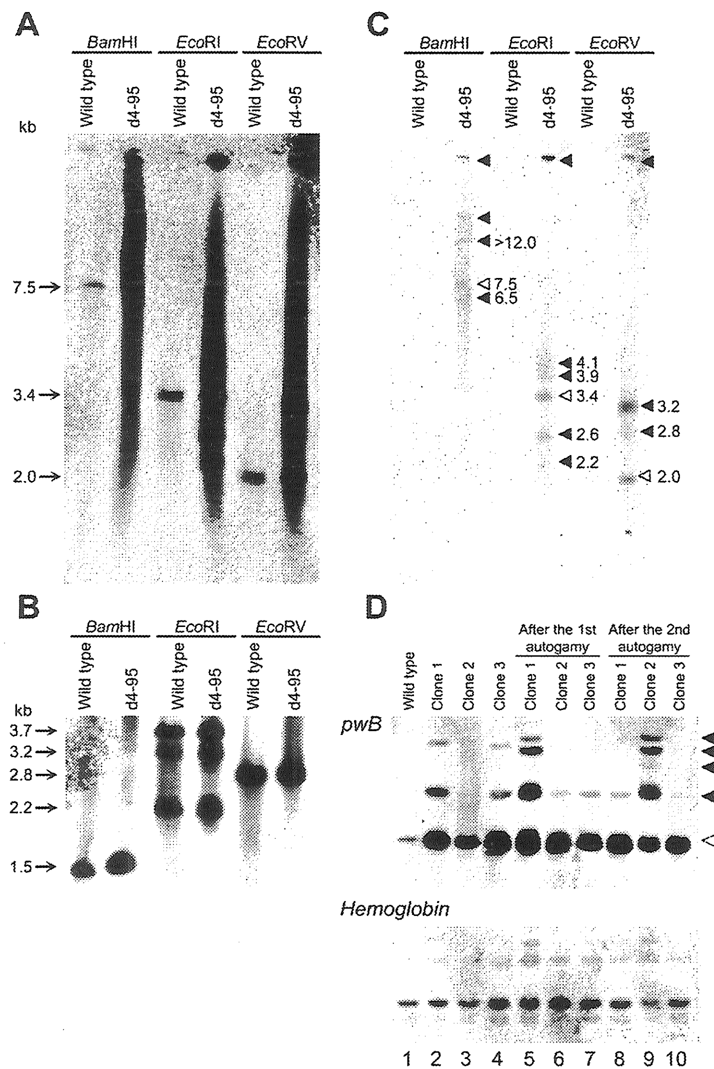

FIGURE 8. --------- (A to C) The same Southern blot of total genomic DNA, which is exclusively macronuclear DNA, digested with the indicated restriction enzymes were probed with either *pwB* (A and C) or the hemoglobin gene (B). (C) Shorter exposure time than A (60 times less) where heterogeneous amplified units together with normal restriction fragments indicated with black or white arrowheads, respectively, can bee seen. (D) Southern blot of *Eco*RI digested total genomic DNA extracted from wild type and series of clones derived from independent three caryonides from autogamy of d4-95. Arrowheads are as in C. Lanes 2 to 4, the original caryonides; lanes 5 to 7, clones after mass autogamy; lanes 8 to 10, clones after the second round of mass autogamy.

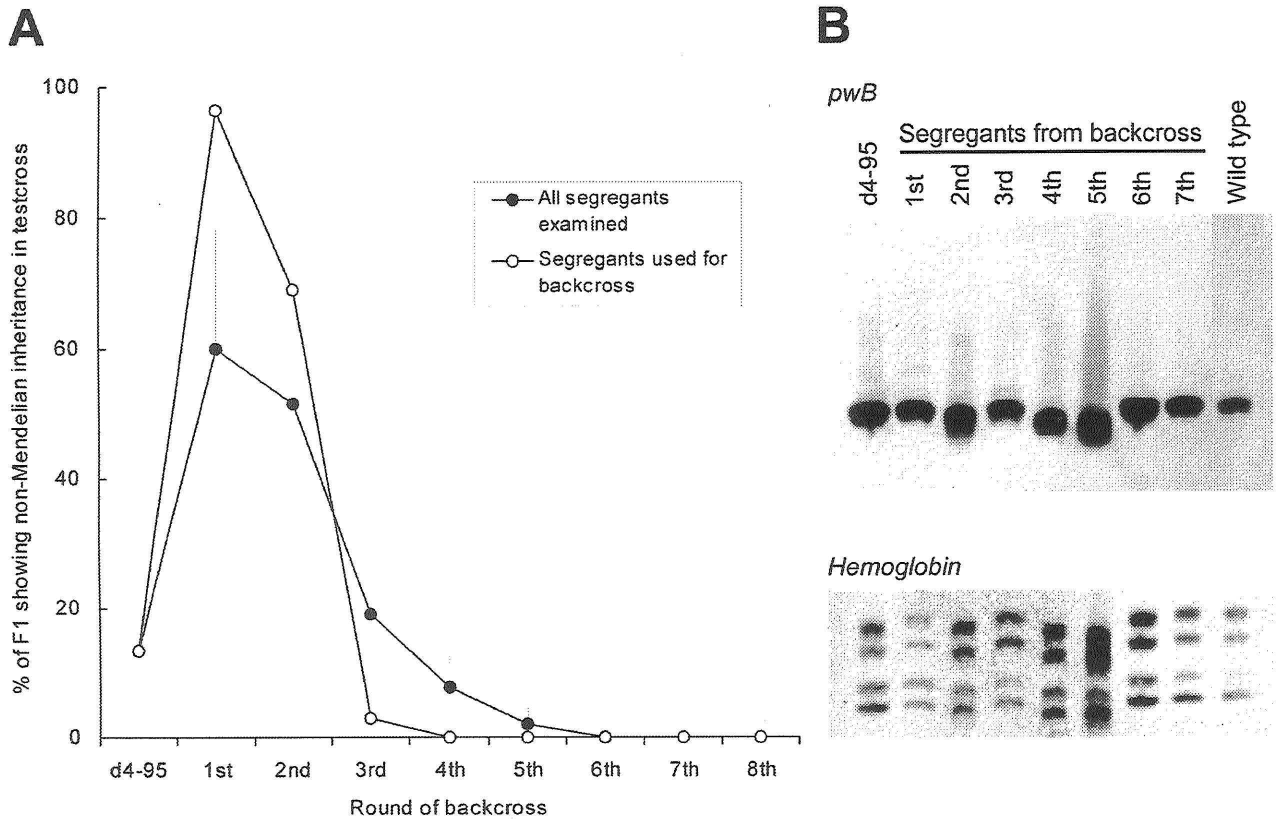

FIGURE 9. ---------d4-95 was repeatedly crossed with wild type, and the genetic background of this strain was replaced with that of wild type. Segregants thus obtained were again crossed with wild type and the % of F_1 showing non-Mendelian inheritance was plotted (**A**). Bar in (A) indicates confidence limit. % of non-Mendelian inheritance of all segregants examined was shown by black symbol, while that of segregants used for the next round of backcross was shown by white symbol. (**B**) Southern blot of *Eco*RI-digested DNA extracted from segregants (shown by white symbols in (A)) derived from backcrossings of d4-95 (left-most lane) to wild type (right-most lane). Heterogenity of *pwB* gene had disappeared by seventh round of backcrossing.

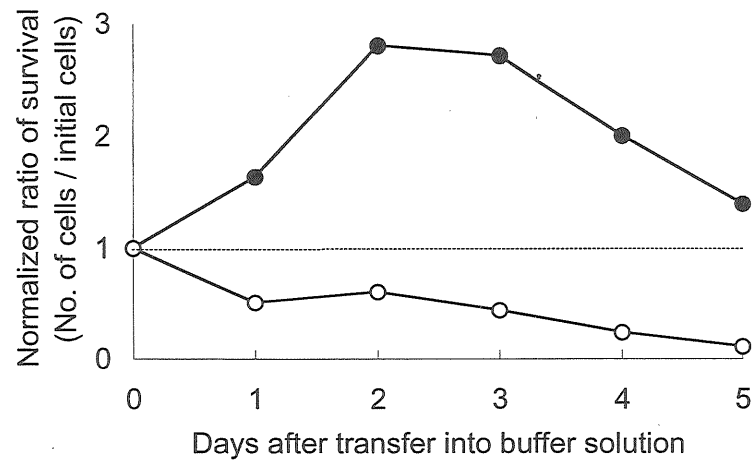

FIGURE 10. --------Difference of resistance to starvation depending on the phenotypic states of *pwB*^{662}. Cells of P662 (white circle) or R662 (black circle) at the stationary phase were transferred into a sterilized Dryl's solution. Initial cell number (day 0) was set as 1 (broken line).

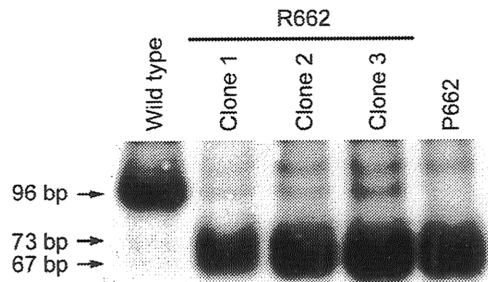

FIGURE 11. --------Southern blot of PCR products loaded on 8 % polyacrylamide gel electrophoresis after digestion with *Hin*fI. Preparations from three independent R662 clones showing backward swimming were used. An IES-removed fragment (96 bp) was found in all R662 clones examined with abundant 44-bp-IES-containing fragments (67 and 73 bp, i.e., cleaved by *Hin*fI).

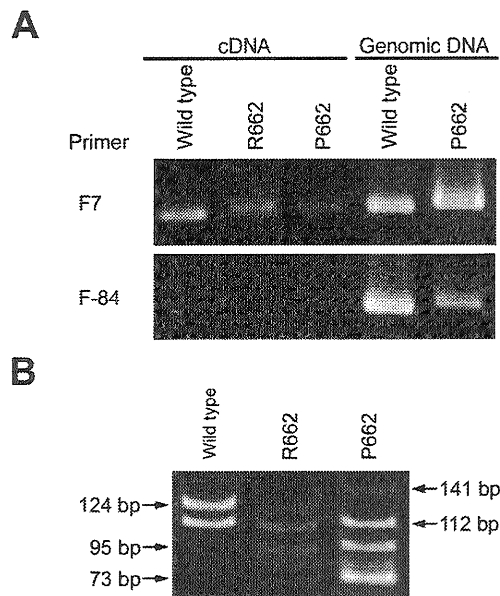

FIGURE 12. --------The *pwB* gene expression of the mutant and revertant of d4-662. (A) RT-PCR and PCR products were loaded on 1.5 % agarose gel electrophoresis. Sense primer F7 amplifies the coding region, while sense primer F-84 amplifies the region including the upstream of the *pwB* gene. RT-PCR using primer F-84 did not amplify any detectable signal, demonstrating that RNA samples were free from genomic DNA contamination. (B) Transcription of the IES-removed *pwB* gene in R662. RT-PCR products were digested with *Hin*fI and subjected to 5 % polyacrylamide gel electrophoresis. The five bands correspond to fragments containing intron (141 bp), IES-removed (pwB^{+}; 124 bp), intron-removed (112 bp), and IES-not-removed (pwB^{662}; 95 and 73 bp). Note that the preparation of R662 contains a 124-bp fragment.