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

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Summary

An allele of the behavioural mutant *pawn-B*^{96} has been reported as a typical recessive gene but was found to show a peculiar inheritance. When the F2 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 the F2 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* and wild-type and the tetrasomy of *pwB* 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 due partly 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.

1. Introduction

Double sets of chromosomes are ordinarily maintained with accuracy. Duplication of mammalian chromosomes is thought to be one of the earliest events in carcinogenesis or a cause of severe diseases (Lengauer *et al.*, 1998; Hernandez & Fisher, 1999). However, exceptions are found in insects, plants and protozoa, where the ploidy or chromosome number can vary developmentally or for unknown reasons (De Rocher *et al.*, 1990; Lanzer *et al.*, 1995). 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.

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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*. Many mutants are known in *P. tetraurelia* (see Sonneborn, 1974), including the well-studied behavioural mutants known as 'pawn'. Pawn mutants are unable to show ciliary reversal due to malfunction of the voltage-dependent calcium channels (Kung *et al.*, 1975). One of the pawn mutants, *pwB*, was isolated about 30

years ago and reported as a mutant controlled by a single recessive gene (Kung, 1971; Schein, 1976). Upon crossbreeding analysis, we found that a strain of the *pwB* mutant showed unusual inheritance, which implies that the strain was a tetrasomy of the chromosome carrying the *pwB* locus. We 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.

2. Materials and methods

(i) *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 a 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* 1 or 2 days before use (Hiwatashi, 1968). Cells were grown at 25–27 °C unless otherwise noted.

(ii) *Phenotypic observation*

The behavioural 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 s (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.

(iii) *Genetic analysis*

Mating reactive cells of complementary mating types were mixed, and then conjugating pairs were isolated in fresh culture medium. In some experiments, both exconjugants of a pair were isolated and grown separately. In each case, single cells were cloned after several postzygotic cell divisions. Phenotypes of F1 clones were observed after they had undergone more than 10 cell divisions from conjugation.

F2 progeny were obtained from autogamy (self-fertilization) induced by starvation of mature F1 cells (about 30 cell divisions after conjugation). One hundred per cent autogamy was determined when all 20+ cells showed macronuclear fragmentation after being stained with carbol fuchsin solution (Carr & Walker, 1961). Autogamous cells were isolated in fresh culture medium, and phenotypes were observed after they had undergone 10 cell divisions.

After successive autogamies, some wild-type segregants in the F2 produced pawn as well as wild-type clones (see Section 3). These progeny were referred to as a 'mixed' type. To examine the segregation of the non-mixed wild-type versus the mixed type in the F2, autogamy was induced in more than 50 cells of each F2, and the cells were transferred to fresh culture medium. After they had undergone about 10 cell divisions, the phenotype of the F3 was observed, and the mixed type and the non-mixed wild-type were determined.

(iv) *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 4 days, and the numbers of cell divisions thus calculated were averaged.

3. Results

(i) *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 s when transferred into the stimulation solution, pawn mutants do not

Table 1. *Stocks used in this study*

Stock	Mutant genes		Source
d4N-527	*nd169*		Y. Takagi (Nara Women's University), originally isolated by D. Nyberg (University of Illinois) (Nyberg, 1974)
nd6	*nd6*		T. Hamasaki (Albert Einstein University)
nd9^{c}	*nd9*^{c}		J. Cohen (CNRS, Gif-sur-Yvette)
nd7; ts111	*nd7*	*ts111*	J. Cohen (CNRS, Gif-sur-Yvette)
d4-96		*pwB*^{96}	C. Kung (University of Wisconsin)

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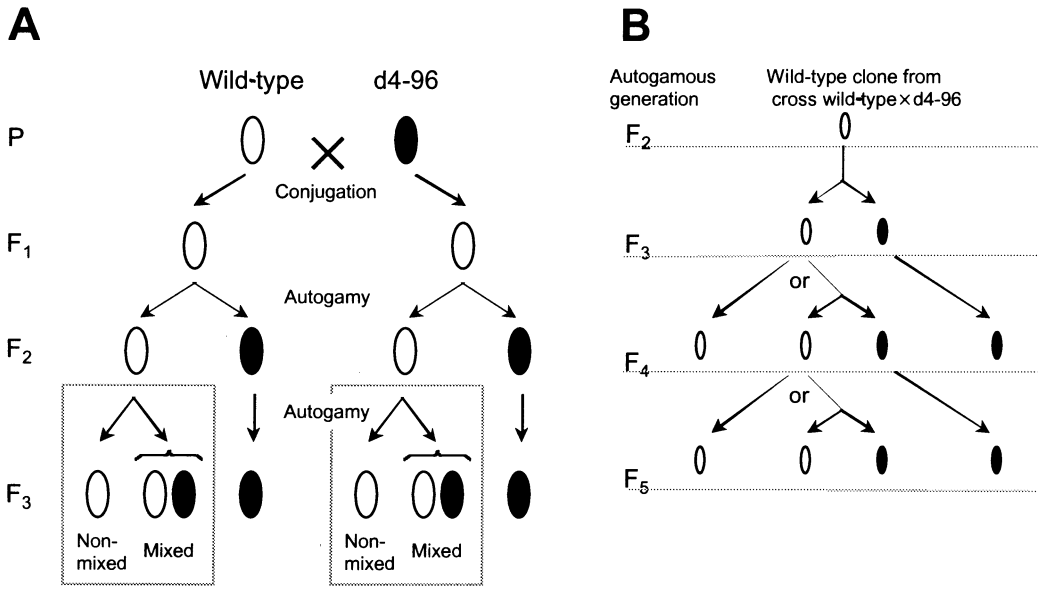

Fig. 1. Inheritance of the strain d4-96. Clones with wild-type and pawn phenotypes are indicated by white and black ovals, respectively. (*A*) When looking only at the F1 and F2, the inheritance observed in the cross of d4-96 with wild-type resembles that of a typical recessive gene. Some wild-type progeny in the F2, however, become mixed clones of wild-type and pawn cells in subsequent autogamous generations. (*B*) Autogamous progeny were isolated from wild-type F2 and subsequent generations to observe the segregation of behavioural 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.

Table 2. *Segregation of behavioural phenotypes in the F2 and those of mixed type in the F3*

Cross	No. of F1 synclones examined	F2 phenotype		Expected ratio	*P*	F3 phenotypes from wild-type F2		Expected ratio	*P*
		Wild-type	Pawn			Non-mixed	Mixed[a]		
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 segregation ratio of non-mixed versus mixed was close to 1:2.

show ciliary reversal leading to backward swimming. These behavioural responses in the stimulation solution were used to observe behavioural phenotypes.

As already reported by Kung (1971), the behavioural phenotype of strain d4-96, which is known to carry the mutant allele of pwB^{96}, appears to be controlled by a recessive gene. All F1 progeny showed the wild-type phenotype in crosses with the wild-type (pwB^{+}/pwB^{+}) (Fig. 1 *A*). A self-fertilization called autogamy makes *Paramecium* useful organism for genetics, because progeny from autogamy receive a diploid and completely homozygous nucleus resulting from fertilization of two mitotic products of a single meiotic haploid product (Sonneborn, 1947). Therefore in autogamous progeny from a single gene heterozygote, the phenotypic segregation ratio should be 1:1. When the F2 progeny were obtained by autogamy of the F1 of the above cross, the segregation ratio of wild-type versus pawn was 1:1, as expected (Fig. 1 *A*, Table 2).

Nevertheless, we found an unusual inheritance of the original *pwB* strain, d4-96. Some wild-type F2 progeny, which should be homozygotes, produced a mixed progeny of wild-type and pawn clones after successive autogamies (Fig. 1, Table 2). The segregation ratio of non-mixed versus mixed progeny was close to 1:2 (Table 2).

To observe the appearance of the mixed clone, autogamous progeny were isolated from wild-type F2 and from successive autogamous generations (Fig. 1 *B*). Table 3 shows the segregation of the phenotype in autogamous lineages from wild-type F2 generations. Some wild-type parents produced only wild-type, while others produced both wild-type and pawn (at a ratio of approximately 5:1), and pawn parents produced only pawn progeny from autogamies.

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Table 3. *Segregation of behavioural 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
F3			39	13		
F4	63	0	103	24	0	54
F5	26	0	89	24	0	48
F6	71	0	67	10	0	41
F7			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 pawn progeny; 'All pawn' did not produce wild-type progeny; '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 (Fig. 1 *B*).

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

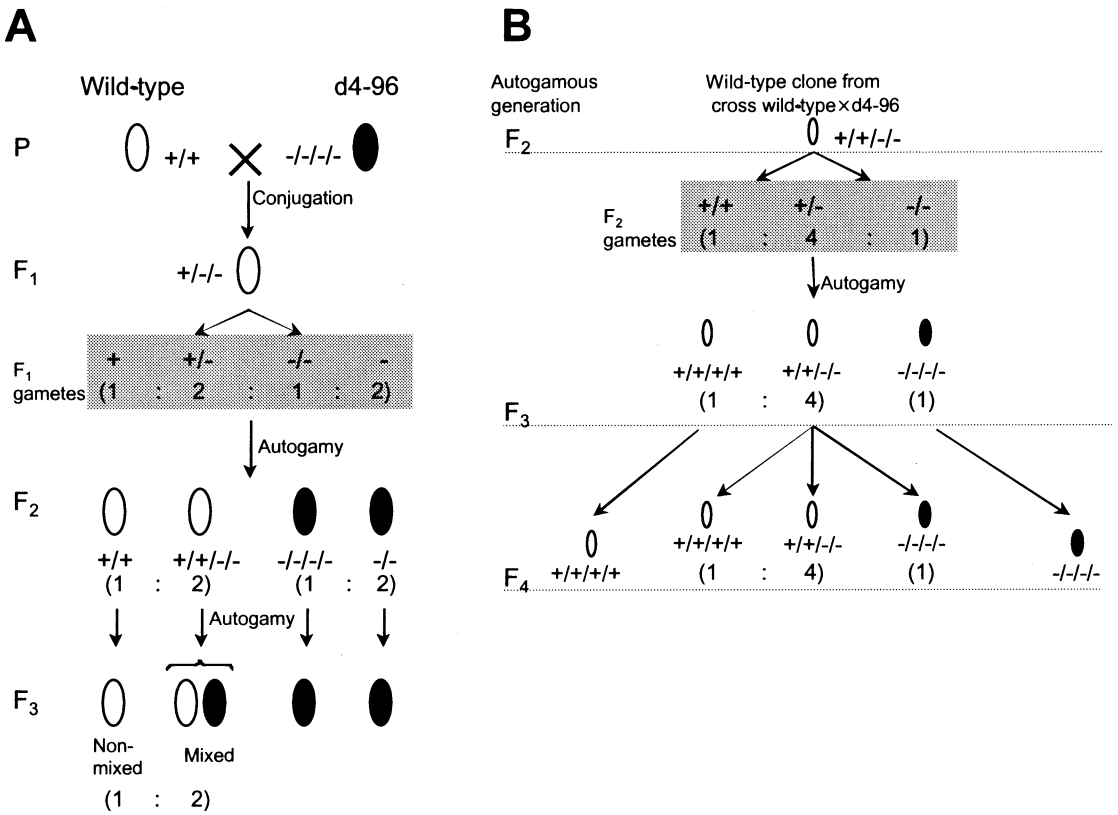

Fig. 2. The inheritance of d4-96 might be explained if the strain were a tetrasomy of the chromosome carrying the *pwB* gene. White oval, wild-type; black oval, pawn. Symbols '+' and '−' indicate chromosomes carrying the wild-type and mutant alleles of *pwB*, respectively. (*A*) and (*B*) correspond to those in Fig. 1. (*A*) A cross of ordinary wild-type (+/+) with tetrasomy of pwB^{96} (−/−/−/−) will produce trisomic F1 (+/−/−). Since two mutant chromosomes (−) are present, four kinds of gametes should be produced with the indicated ratio (shaded area) from meiosis of this F1. Autogamy will simply duplicate the genotypic composition of gametes and produce disomic and tetrasomic F2 progeny, including unusual heterozygous wild-type F2 (+/+/−/−). This heterozygous F2 will produce wild-type and pawn cells after autogamy, resulting in mixed progeny in the F3. The detailed analysis of the mixed progeny is 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 becomes heterozygous tetrasomy with identical genotype to the parent (F2) after autogamy. After the next round of autogamy of heterozygous tetrasomy, again three kinds of progeny genotype are possible as in the F3.

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Table 4. *Segregations of marker genes used in this study*

Cross	Survival in F2 (%)	Segregation of F2 phenotype by autogamy: Trichocyst: Discharge	Non-discharge	χ^2 1:1[a]	χ^2 5:1[b]	Temperature sensitivity: Resistant	Sensitive	χ^2 1:1[a]	χ^2 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

χ^2 values which indicate a probability (P) higher than 0.05 are indicated by an asterisk. Segregation ratios of behavioural phenotype in the F2 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	F2 in crosses wiht d4-96: Survival (%)	Segregation of behaviour in F2: Wild-type	Pawn	Expected ratio	χ^2	F3 of wild-type: 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 F2, and WC-4a and WC-4b are wild-type segregants in the original F4, both from crosses between d4-96 and wild-type. χ^2 values which indicate a probability (P) higher than 0.05 are indicated by an asterisk.

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 (Fig. 2*A*). The model predicts that two kinds of wild-type genotypes are possible in the F2: $+/+$ or $+/+/-/-$ if the trisomic F1 chromosome carrying the *pwB* locus can perform normal meiosis (Fig. 2*A*). The heterozygotes $+/+/-/-$ should become mixed progeny in the next autogamy, and the ratio of non-mixed versus mixed should be 1:2 (Table 2, Fig. 2*A*). Similarly, in a lineage analysis of the F2 of heterozygous wild-type, one homozygous wild-type, four heterozygous wild-type and one homozygous pawn were segregated in the F3 (Fig. 2*B*), consistent 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 the F2 (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 Fig. 2 implies a number of predictions, the most crucial of which were successfully tested.

(i) In the F3 of autogamous lineages (Fig. 2*B*), the segregation ratio 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 versus 89 versus 23 ($\chi^2 = 1{\cdot}6$, $P = 0{\cdot}5$).

(ii) Homozygotes of the wild-type in F2 should be ordinary disomic ('$+/+$' in Fig. 2*A*), but homozygotes of the wild-type in F3 or F4 derived from F2 heterozygotes should be tetrasomic ('$+/+/+/+$' in Fig. 2*B*). 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

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Table 6. *Tests of tetrasomic and disomic* pwB *segregants by crossing with wild-type*

Pawn segregants from original F2	F2 in crosses with wild-type					
		Segregation of F2		F3 of wild-type		
	Survival (%)	Wild-type	Pawn	Non-mixed	Mixed	Deduced genotype
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

F2 segregants W7, W14, W24 and W27 showed a 1:1 segregation ratio of wild-type versus pawn in the F2 in this cross and a 1:2 segregation ratio of non-mixed versus mixed in the F3 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 the F1 should be +/+/−/−, and autogamy of this produces one homozygous wild-type (+/+/+/+), four heterozygous wild-type (+/+/−/−) and one tetrasomic pawn (−/−/−/−). Therefore the expected segregation ratio of wild-type versus pawn would be 5:1 in the F2, and that of non-mixed versus mixed would be 1:4 in the F3 (Fig. 2*B*). The crosses using wild-type original homozygous F4 segregants WC-4a and WC-4b showed a 5:1 segregation ratio of wild-type versus pawn in the F2 in this cross, while the ratio of non-mixed versus mixed was 1:4 in the F3 in this cross, consistent with the predicted genotype of the F1 (+/+/−/−) in these crosses (Table 5).

(iii) The pawn segregants in autogamous lineages should be tetrasomic (−/−/−/− in Fig. 2*B*) and should thus behave similarly to the parental strain d4-96 (now assumed to be −/−/−/−). When segregants from the F3 and F7 were crossed to the wild-type, they showed similar inheritance to that of d4-96 (data not shown; see Fig. 1).

(iv) Two-thirds of the pawn progeny in the original F2 should be disomic (−/− in Fig. 2*A*). When the predicted disomic pawns were crossed with the wild-type, they could not produce heterozygous wild-type in the F2. 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 of 19 pawn segregants are interpreted to be disomic, as expected (Table 6).

(ii) *The instability of the chromosome carrying the* pwB *locus*

In the course of the experiments, segregation of F2 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

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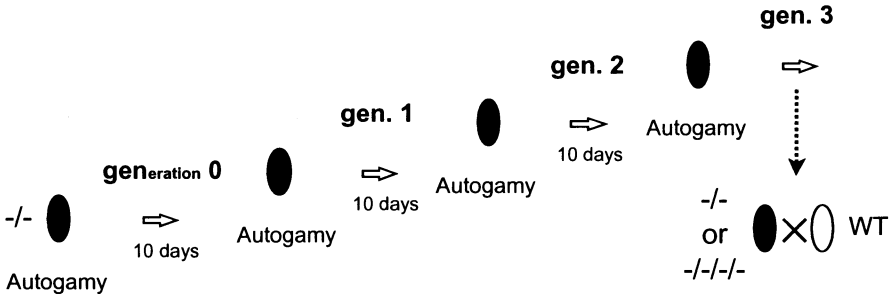

Fig. 3. Pawn segregants were cultured to examine the change in their genotypes during culturing. The nutritional condition of the segregants was controlled to induce autogamy every 10 days, corresponding to approximately every 30 cell divisions. Autogamous generations (gen.) of the segregants were counted from the F2 in the original cross between wild-type and d4-96. Cells were crossed with the wild-type, and then the phenotypic segregation in the F2 and non-mixed versus mixed in the F3 from wild-type F2 were examined (Fig. 2*A*). Wild-type segregants from the F3 were also examined in the same way and tested with d4-96.

Table 7. *Tests of tetrasomy and disomy in wild-type segregants after several autogamous generations*

Autogamous generation in which strains were crossed	Wild-type F3 or F4 segregants[a]	Survival in F2 (%)	Phenotype in F2 of this cross				Wild-type in F3 of this cross				Deduced genotype of wild-type
					χ^2				χ^2		
			Wild-type	Pawn	5:1[b]	1:1[c]	Non-mixed	Mixed	1:4[b]	1:2[c]	
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 number of autogamous generations (gen.; see Fig. 3). χ^2 values which indicate a probability (P) higher than 0.1 are indicated by an asterisk.

[a] Segregants derived from the F3 or F4 of original crosses (see Fig. 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.

(Fig. 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 Fig. 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 F2 from the crosses should be heterozygotes. Thus, the phenotype in the F3 from the autogamy of wild-type F2 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 first to the ninth autogamous generations (gen. 1 to gen. 9), no heterozygous wild-type F2s were found, indicating that all cells tested were still disomic (−/−). Thus, we 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 (Fig. 3). The genotypes of four wild-type F3 segregants (W1-3, W5-30, W12-10 and W12-36), which were isolated from the autogamous lineage of F3 (Table 3, Fig. 2*B*) and should thus be tetrasomic (+/+/+/+), were examined by crossing with the pawn d4-96. If the

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Table 8. *Comparison of cell division per day and percentage 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 ± SD	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 F2 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 F2 progeny examined			4	3	12	13	3
No. of cell lines examined				16		40	20

segregants maintain tetrasomic genotypes, the phenotype of F2 of the cross should segregate at a ratio of 5:1, while if the segregants lose half 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 first to the eigth 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 first generation (Table 7). Clones of four segregants were thought to be a mixture of tetrasomy and disomy (or trisomy) at the fourth and the eighth generations (Table 7). In Table 5, we 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 1 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 F2 of d4-96 × *nd7*; *ts111*, as well as two subclones of the original d4-96) from generation 3 to generation 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.

(iii) *Tetrasomy and disomy differ in their fission rate*

Some possible explanations can be drawn for the difference in 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, such as 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 ($t = 2{\cdot}09$, d.f. = 50, $P = 0{\cdot}04$, calculated from the 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 ($t = 0{\cdot}68$, d.f. = 16, $P = 0{\cdot}50$, +/+ 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 Section 4), 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. In the same way, the 1:2 segregation of non-mixed versus 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

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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 behavioural phenotype in F2				Segregation of wild-type in subsequent generation				
			Wild-type	Pawn	Expected	*P*	Non-mixed	Mixed	Expected	*P*
Wild-type × *pwB96*							Wild-type	Pawn			
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*			
Autogamous lineage	31	93.0					405	99	5:1	0.1	
Expected							*420*	*84*			

infer the preferable pairing among chromosomes in tetrasomy, pooled data of the phenotypic segregation of F2 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 is found.

4. 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 F2, we observed a repeated segregation ratio of 5:1, which is the theoretical ratio if the chromosomes make only bivalents (Fig. 2).

Although aneuploidy has been reported in intersyngenic crosses of *P. caudatum* (Tsukii & 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). We showed that the original wild-type strains, when genetically examined, did not contain cells harbouring four chromosomes carrying the *pwB* locus. Furthermore, our 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 we 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-

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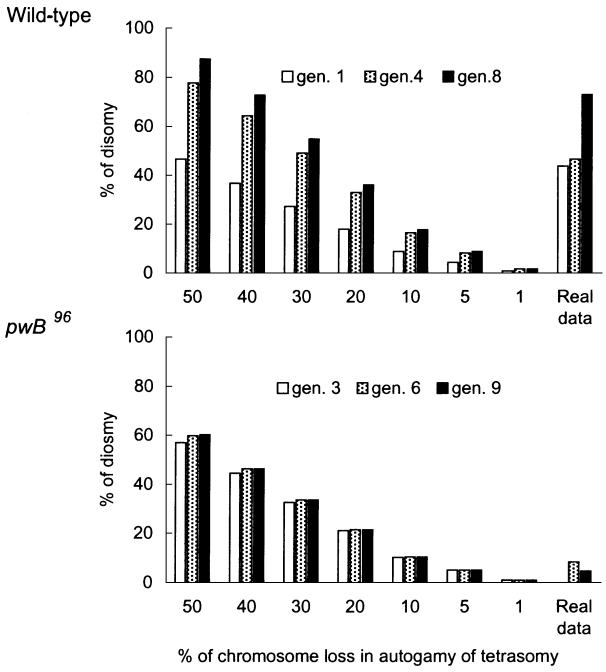

Fig. 4. Simulated and real appearance of disomy in the culture that was initially pure for homozygous tetrasomy through several autogamous generations (gen.; see Fig. 3). The percentage of disomy (ordinate) is presented individually as a function of the hypothetical parameter of chromosome loss in the 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%).

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 the causes of the maintenance of the tetrasomy of the *pwB* mutant. However, absence of selection by fission rate in the disomy and tetrasomy of the wild-type does not necessarily increase the frequency of disomic cells in 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 (Fig. 4, for calculation of the percentage of disomy, see Appendix). As shown in Fig. 4, the difference in percentage disomy of wild-type and that of pwB^{96} in the culture of homozygous tetrasomy can best be 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 (Fig. 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} (Fig. 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 (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 in chromosome loss was reported in autotetraploid maize cultivated for 10 years (Gills & Randolph, 1951). Although the exact time and cause of the chromosome duplication event that occurred in the micronucleus of strain d4-96 are 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. 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 on 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; $0 \leqslant f \leqslant 1$) 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 Fig. 3.

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(iv) Tetrasomy loses half its chromosomes in meiosis at a constant frequency, l ($0 \leqslant l \leqslant 1$), 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 $(1-l)f_tN_{t0}$.

The cells are allowed to grow for 10 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 $(1-l)f_tN_{t0}(2)^{10r_t}$ and $lf_tN_{t0}(2)^{10r_d}$, 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 $(1-l)f_tN_{t1}$, and that of disomic cells should be $lf_tN_{t1}+f_dN_{d1}$, where f_d is the survival from the autogamy of disomy. The cells are again allowed to grow for 10 days, and at the end of gen. 2, the numbers of tetrasomic and disomic cells are $(1-l)f_tN_{t1}(2)^{10r_t}$ and $(lf_tN_{t1}+f_dN_{d1})(2)^{10r_d}$, 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 2 days after autogamy. Thus, the numbers of tetrasomic and disomic cells at the period of testing in, for instance, gen. 3, are $(1-l)f_tN_{t2}(2)^{2r_t}$ and $(lf_tN_{t2}+f_dN_{d2})(2)^{2r_d}$, respectively. The percentage of disomy in gen. 3 is calculated as follows:

$$100\times(lf_tN_{t2}+f_dN_{d2})(2)^{2r_d}/\{(1-l)f_tN_{t2}(2)^{r_t}+(lf_tN_{t2}+f_dN_{d2})(2)^{2r_d}\}.$$

The percentage of disomy in the culture predicted from this model with various generations and l values (presented in %; i.e. $l\times100$) is given in Fig. 4.

However, the percentage 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 percentage of disomy in real data should be considered as a rough estimate.

As mentioned above, we have assumed that chromosome loss occurs in meiosis of tetrasomy (assumption (iv) above), probably through non-disjunction. Non-disjunction of +/+/+/+ (tetrasomy) should produce gametes with genotype (instead of the usual +/+) + and +/+/+, which results in progeny of 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 hexasomy (+/+/+/+/+/+) with tetrasomic *pwB*^{96} (−/−/−/−), the ratio of wild-type versus pawn in the F2 should be 19:1 (with possible genotypes 6 +/+/−/−, 6 +/+/+/+/−/−, 3 +/+/−/−/−/−, 3 +/+/+/+, 1 +/+/+/+/+/+ and 1 −/−/−/−) and that of non-mixed versus mixed in the F3 should be 15:4, while in the case of cross *pwB*^{96} of hexasomy (−/−/−/−/−/−) with ordinary wild-type (+/+), the ratio of wild-type versus pawn in the F2 should be 1:1 (with possible genotypes 1 +/+/−/− and 1 −/−/−/−) and these wild-type should be all mixed in the F3. Although we have some possible cases of the presence of hexasomy in the culture (data not shown), it was statistically difficult to distinguish the segregation ratio resulting 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 our model. Hexasomy was treated as tetrasomy in both the real data and the model; therefore what this model shows is the percentage of disomy (among other possible genotypes including tetrasomy and hexasomy). This gives 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. Additionally, hexasomy, if present, was rare compared with disomy in our 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.

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