Genet. Res., Camb. (1976), **27**, *pp.* 143–159
With 1 plate and 3 text-figures
Printed in Great Britain

Extra cytoproct mutant in *Paramecium tetraurelia*: a genetical study*

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(*Received* 11 *November* 1975)

SUMMARY

The basis of inheritance of the extra cytoproct (XP) character in *Paramecium tetraurelia*, stock d4-154, is shown to be nuclear and probably a single dominant gene, *Ec*, with reduced penetrance in heterozygotes. When the mutant gene is replaced by its wild-type allele, loss of the XP phenotype in some lines of descent occurs before 15 cell generations, but in more than half of the lines this occurs after 15–120 or more cell generations. The possibility is considered that these extremely long and variable 'lags' may be due to extranuclear (cortical) inheritance of cortical changes initially produced by gene action.

1. INTRODUCTION

This paper deals with the genetics of a morphological variant in *Paramecium tetraurelia* (formerly *Paramecium aurelia*, syngen 4, see Sonneborn, 1974), characterized by producing many animals with supernumerary cytoprocts (Plate 1, figs. 1–6). Details of the phenotype of this variant will be described elsewhere (Ng, 1976*a*). Evidence will be presented here which indicates that the variant character is due to the action of a single dominant gene. This genetic system has a most remarkable feature: after substitution of the mutant gene by its wild-type allele, the mutant character persists for an extraordinarily long time. Thus, this mutant offers a promising system for studying the effect of non-genic factors and their interaction with the genome in the expression of a morphological character.

2. MATERIALS AND METHODS

(i) *Stocks*

Stock 51S is a branch of stock 51, the standard stock used in *Paramecium tetraurelia* genetics. It lacks kappa – the endosymbiont which confers the well-known killer property on its host – and therefore is a non-killer and sensitive to killing.

Stock d4-154, a spontaneous mutant isolated from stock 51S, bears supernumerary cytoprocts. The frequency of expression, i.e. the percentage of animals in a sample

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possessing extra cytoproct(s), is about 22% (range: 8–58%). Most of these possess only one extra cytoproct, but two to six extra cytoprocts can be found in one animal. There are three classes of such extra cytoprocts according to their locations on the cortex: left ventral, right ventral, and dorsal. Some ventral ones are joined to the normal cytoproct (Plate 1, figs. 2, 4). The extra cytoproct character will be referred to as XP. For further details see Ng, 1976*a*.

Stock tsK (listed as stock d4-153K in the Sonneborn collection) is a derivative of stock 51 bearing kappa (therefore a killer) and the recessive temperature-sensitive marker *ts1001*. The animals usually die within 6 h at 35 °C (see Sonneborn, 1974, table 5, for further details about *ts1001* and other genes referred to below). Stock tsK was obtained by M. Ingle in Sonneborn's laboratory as an F2 segregant from the cross of stock d4-111 (a mutational derivative of stock 51, with *ts1001*, $nd3^b$ and kappa) to stock 51. This segregant lacked $nd3^b$.

Stock tsS (listed as stock d4-153S in the Sonneborn collection) has the same genotype as tsK but lacks kappa (and hence is a non-killer). It was obtained by M. Austin in Sonneborn's laboratory as an F2 segregant from a cross between stock tsK and stock 51.

Stock ptpw is a homozygous double recessive mutant carrying the genes *ptA* (pointless) and pwA^1 (pawn). Animals homozygous for pwA^1 cannot swim backwards. Animals homozygous for *ptA* have pointless trichocysts. The *ptA* gene also causes frequent mis-segregation of the macronucleus at cell division, yielding some animals lacking macronuclei. Stock ptpw was obtained as an F2 segregant from a cross between stock d4-94 (carrying pwA^1) and a derivative of stock d4-104 (carrying *ptA*). Both stocks d4-94 and d4-104 are mutational derivatives of stock 51.

(ii) *Culture methods*

Culture and handling of paramecia followed the methods of Sonneborn (1950, 1970). Baked lettuce medium inoculated with *Klebsiella aerogenes* buffered with $Ca(OH)_2$ to pH 6·5–7·5 was used. All experimental animals were kept at 27 °C. With excess food, they reproduced at the rate of 4–5 fissions per day. All heterozygotes from crosses were kept in depression slides by daily reisolation. All

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PLATE 1

Figs. 1–6. Silvered preparations showing different kinds of extra cytoprocts according to location on cortex and connection with other structures. *V* = posterior end of vestibule; *CVP* = posterior contractile vacuole pore; *N* = normal cytoproct; extra cytoprocts are indicated by arrows. Figs. 1–4. Posterior ventral views of *Paramecium*; Figs. 5–6: Posterior dorsal views of *Paramecium*. Magnification: × 1111.

Fig. 1. Extra cytoproct on the left of, and not joined to normal cytoproct.

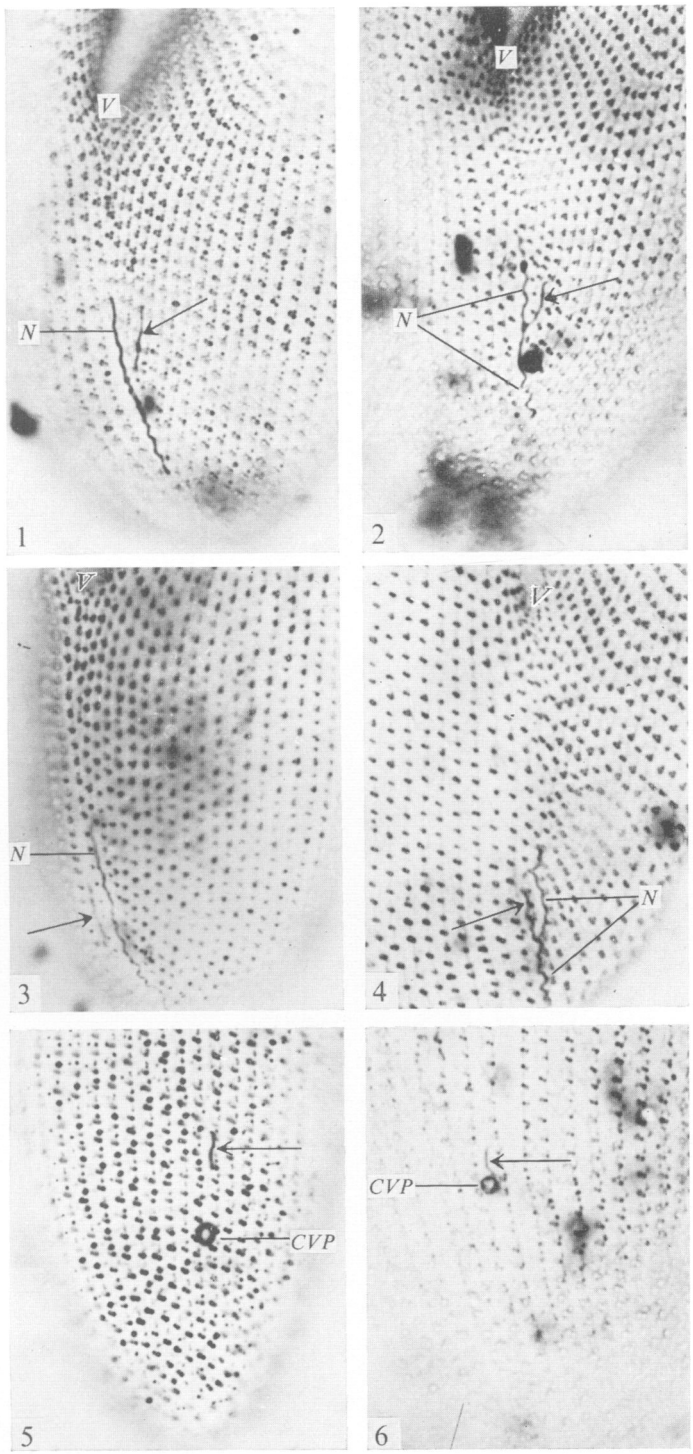
Fig. 2. Extra cytoproct on the left of, and joined to normal cytoproct.

Fig. 3. Extra cytoproct on the right of, and not joined to normal cytoproct.

Fig. 4. Extra cytoproct on the right of, and joined to normal cytoproct.

Fig. 5. Extra cytoproct on the dorsal surface, not joined to posterior contractile vacuole pore.

Fig. 6. Extra cytoproct on the dorsal surface, joined to posterior contractile vacuole pore.



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(*Facing p.* 144)

https://doi.org/10.1017/S0016672300016359 Published online by Cambridge University Press

homozygotes from crosses were kept in culture tubes: 24 ml of fresh medium were added daily to 0·5–1 ml of left-over culture in the tube. Asynchronous autogamy occurred frequently in tube cultures under such conditions. About every 50 fissions, tube cultures were left unfed for 2–3 days to induce autogamy (see below). A tube culture therefore eventually came to contain a group of exautogamous clones and will simply be referred to as a 'culture'.

(iii) *Methods used in genetic analysis*

Mating pairs were obtained following the method of Sonneborn (1950, 1970) by combining late log to early stationary phase cultures of complementary mating types. Usually mating pairs undergo reciprocal cross-fertilization. However, some may undergo (i) selfing, i.e. cross-fertilization between animals from the same culture (due to presence of a small number of animals of complementary mating types within the same culture), or (ii) cytogamy (usually in $< 5\%$ of the pairs), i.e. animals from the two cultures form pairs in which self-fertilization (like autogamy, see below) instead of cross-fertilization (true conjugation) occurs. Both selfing and cytogamous pairs need to be excluded from genetic analysis. They can be recognized by the use of an appropriate recessive gene marker and the endoplasmic marker, kappa, as set forth below.

(*a*) *Crossing homozygotes* (pw/pw × pw$^+$/pw$^+$ *or* ts/ts × ts$^+$/ts$^+$)

Cytogamous pairs can be identified in F1 by failure of the two exconjugant clones of a pair to become alike for the marker trait; they remain phenotypically diverse, like the two parents. Cross-fertilization results in both exconjugant clones of a pair being of identical genotype (*pw*/*pw*$^+$ or *ts*/*ts*$^+$) and phenotype (wild type). Selfing in the *pw*$^+$/*pw*$^+$ or *ts*$^+$/*ts*$^+$ parent gives the same F1 phenotypic result (wild type) as does cross-fertilization. However, F1 selfing pairs remain homozygous and so can be detected by their failure to segregate marker genes in F2.

F2 generations were obtained by starving F1 clones when more than 20 cell generations old to induce autogamy (i.e. self-fertilization in *unpaired* animals). To detect autogamy, a sample of more than 50 starved animals was stained with aceto-orcein (method of Beale and Jurand as modified by Yeung, 1965). When all of them possessed macronuclear fragments (from breakdown of the macronucleus, a sign of autogamy), unstained animals from the same culture were isolated. Each autogamous animal gave rise to a clone which was homozygous for one or the other of the two parental genotypes. When the F1 exconjugants are results of cross-fertilization, i.e. are heterozygous, the F2 genotypes segregate in a ratio of 1:1. For further details on the cytological and gentical aspects of autogamy, see Sonneborn, 1974.

(*b*) *Crossing heterozygotes* (ts/ts$^+$) *with homozygotes* (ts/ts)

Cross-fertilization yields 50% of the pairs (i.e. both exconjugant clones) being *ts*/*ts* and 50% of the pairs being *ts*/*ts*$^+$. Cytogamy gives similar results: 50% of the pairs being *ts*/*ts* in both clones and the other 50% having one clone *ts*/*ts* and

the other ts^+/ts^+. The latter pairs are clearly identifiable as cytogamous, but *ts/ts* pairs can result either from cross-fertilization or cytogamy. The number of *ts/ts* pairs due to cytogamy can be estimated because it should be equal to the number of pairs in which one exconjugant clone was *ts/ts* (temperature-sensitive) and the other ts^+/ts^+ (not temperature-sensitive). The results were therefore corrected accordingly, i.e. by subtracting from the number of *ts/ts* pairs a number equal to the number of pairs composed of one temperature-sensitive and one wild-type clone.

Selfing in the heterozygous parent culture yields *ts/ts*, ts^+/ts^+ and ts/ts^+ pairs. The wild-type ts^+/ts^+ pairs (which cannot arise by cross-fertilization) can be identifield in the next generation by failure to segregate temperature-sensitives and wild type. All three classes of selfing F1 pairs can be excluded from the F1 by marking one of the parents (the *ts/ts* parent) with the endoplasmic marker, kappa and selecting only F1 pairs in which one clone was a killer and the other was not. One has to pay for this exclusion of selfing pairs by being unable to use cross-fertilized F1 pairs in which endoplasmic transfer resulted in both clones being killers.

(c) *Identifying the genotypes*

Homozygous *ts/ts* animals were identified by comparing isolates at 27 and 35 °C. In 35 °C, *ts/ts* homozygotes die within 6 h, leaving brownish corpses, but ts/ts^+ and ts^+/ts^+ animals survive. Observations were usually made 20 h after transfer to 35 °C.

Homozygous *pw/pw* animals were identified by micropipetting the animals into a few drops of 8 mM of KCl in Dryl's solution (Kung, 1971) in a depression slide and observing immediately under a $\times 10$ dissecting microscope. Animals showing an avoiding reaction (backing) are pw^+/pw^+ or pw/pw^+ whereas animals not showing an avoiding reaction are *pw/pw*.

To identify the *Ec* genotype which will be shown to determine expression of the XP phenotype, the Chatton–Lwoff silver impregnation technique, as modified by Corliss (1953) and Ng (1976*a*), was used to reveal extra cytoprocts and their positions. In most cases, all animals in a sample were studied. In other specified cases, the scoring ended when two to twelve animals in a sample showed the XP phenotype. As noted in Table 1, some cultures were sampled twice or thrice when the first or second sample did not have animals showing the XP phenotype.

In three wild-type stocks studied (51S, d4-84, tsK), about 3–5% of the normal cytoprocts have a small 'branch' either on the left (1–2 μm long) or the right (1–3 μm long). In the present genetic analysis, classification of samples as showing the XP phenotype has been based on observing extra cytoprocts *unlike* those found in any wild-type stock to which it was crossed: extra cytoprocts on the dorsal surface, or extra cytoprocts on the ventral surface not joined to the normal cytoproct, or joined to the normal cytoproct on the left but longer than 4 μm, or in most cases, a combination of these. Only 0·2% of the animals in stock d4-154 possess extra cytoprocts, designated RNJ, which are on the right ventral surface but not joined to the normal cytoproct (Ng, 1976*a*). This very low frequency of occurrences in stock d4-154 makes it difficult to rule out their occurrence in stocks

51S and tsK although no RNJ has yet been found in them. Nevertheless, in crosses between these stocks and d4-154, animals with RNJ cytoproct, in the absence of any other extra cytoproct, have been scored as having the XP phenotype. Only a few such cases were encountered. Classifying them otherwise would not alter the interpretation of the segregation ratios obtained. On the other hand, the double mutant ptpw sometimes bears RNJ. Hence in crosses between this mutant and d4-154, RNJ could not be used to score the XP phenotype.

Unless specified, the frequency of expression of extra cytoproct was based on the total for *all forms* of extra cytoproct observed, including short branches (1–3 μm) of the normal cytoproct which were rarely found also in wild type. Obviously, classification of a sample as wild type does not imply that all of its animals lacked cytoproct with short branches.

(*d*) *Identifying the plasmatype*

The endosymbiont, kappa, was employed for identifying the exconjugants when a killer was mated with a non-killer. The non-killer mate could be identified even if it received a small number of kappa from its killer mate during conjugation. Reproduction at 4–5 fissions per day at 27 °C prevents low concentrations of kappa from rising to concentrations that result in resistance or killing by the time killing tests are made (15–20 cell generations after conjugation). Detection of resistance to kappa killing in both exconjugant clones of a pair (barring selfing of the killer parent) indicated massive transfer of endoplasm.

3. RESULTS

(i) *Genetic analysis*

The obvious way to attack the problem of the genetic basis of extra cytoproct in stock d4-154 is to carry out a standard Mendelian analysis. This was in fact done, as will be set forth below. Here we note only that usually the XP character was manifested by both clones from the two members of F1 conjugant pairs, indicating dominance. But the F2 results were so peculiar that other alternatives to genic inheritance had to be considered, particularly an extra-nuclear basis. This seemed all the more necessary since the cytoproct is a cortical organelle and examples of an extra-nuclear hereditary basis for cortical characters of *Paramecium*, including extra cytoproct, are well known (Sonneborn, 1963, 1970, and unpublished; Beisson & Sonneborn, 1965). Hence, the following experiments were carried out in an attempt to discover whether the basis of the XP character in stock d4-154 was nuclear or cytoplasmic.

Stock d4-154 was crossed to stock ptpw (Fig. 7). The key point about this cross is the postzygotic behaviour of the ptpw parent: it provides a decisive test of whether the XP character depends upon the d4-154 nucleus. The *pt* gene in the ptpw parent results in macronuclear misbehaviour of some exconjugants (from the ptpw parent) regardless of their new genotype: frequently both macronuclei developed from the fertilization nucleus pass at the first cell fission to one daughter

animal, the other animal receiving neither; but both animals receive some fragments of the prezygotic macronucleus. In the absence of a postzygotic macronucleus, the fragments of the prezygotic macronucleus regenerate into functional macronuclei. This is called MR, i.e. macronuclear regeneration (Sonneborn, 1940).

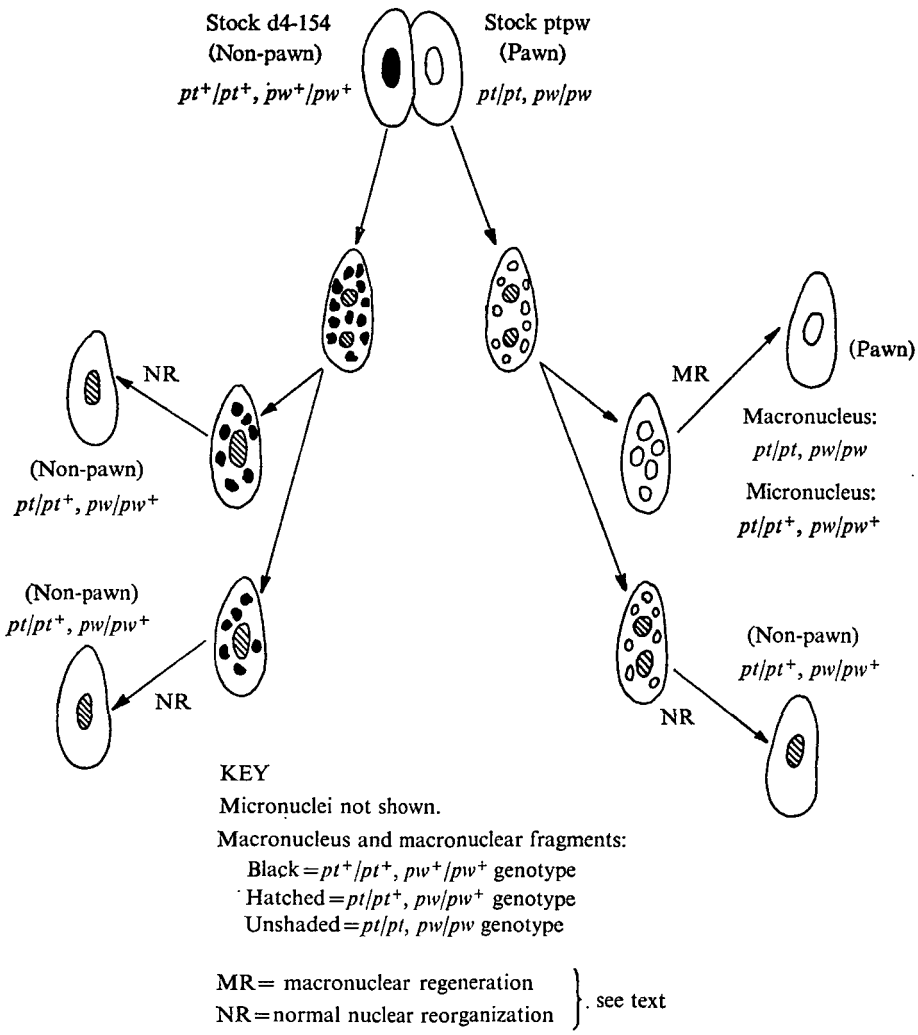


Fig. 7. Theoretical phenotypic results with respect to pawn character from cross between stocks d4–154 and ptpw.

In the presence of a postzygotic macronucleus, the fragments of the prezygotic macronucleus eventually disintegrate. This is NR, normal reorganization. Thus, the two products of the first cell fission differ in the genotypes of their macronuclei. In one – the MR animal – the parental genotype (*pt/pt, pw/pw*) is retained since its functional nucleus (macronucleus) develops from fragments of the prezygotic macronucleus. Its vegetative progeny will therefore retain the prezygotic pheno-

type and can be identified by the pawn character. In the other – the NR animal – the macronucleus has the hybrid genotype (*pt*/*pt*$^+$, *pw*/*pw*$^+$). Its progeny will therefore be wild type and can be identified by the non-pawn phenotype. Since both of these lines of descent arise from a single exconjugant animal and both kinds of macronuclei develop in a common cytoplasm containing identical micronuclei, any differences between them must be ascribed to the macronuclear difference. As shown in Fig. 7, the two lines of descent from the d4-154 parent (which lacked gene *pt*) undergo NR and should be heterozygous and phenotypically like the one NR line from the ptpw parent.

The question of importance is whether there is correlation between transmission of the XP character and the presence of hybrid (NR) macronuclei. In the present experiment, two F1 progeny animals from each exconjugant were isolated after the first postconjugation cell fission and their progenies were tested for the pawn phenotype at 3–4 and again at 7–8 fissions after conjugation. A search was made for pairs in which one exconjugant gave rise to one phenotypically pawn (MR) line of descent and the other phenotypically non-pawn (NR), while both lines of descent from the other exconjugant were phenotypically non-pawn (NR). Ten such pairs were found. After testing for pawn, only one of the two non-pawn lines from each d4-154 conjugant was held for further study. Silver impregnation was done at around 12–15 cell fissions after conjugation on samples of 40–128 (mean, 72) animals. Of the 10 lines from the 10 d4-154 parents, all showed the XP character. Of the 10 MR lines from the ptpw parents, none showed the XP character, but 8 of the 10 sister NR lines from the same parents did show the XP character. Thus the XP character was transmitted to the progeny of the mate only when the latter possessed hybrid macronuclei, never when it had macronuclei derived solely from that of the non-XP (i.e. ptpw) parent. This result proves that the development of the XP character depends on the genetic constitution of the macronucleus. Since the macronucleus in NR arises from a product of the fertilization nucleus (micronucleus), the ultimate source of hereditary transmission of the XP character to the progeny of the non-XP parent is the male gemate nucleus from the XP parent. Furthermore, the basis of inheritance of the XP character appears to be dominant, but with somewhat reduced penetrance in the hybrid (though apparently not in homozygotes), as only 18 of the 20 NR hybrid lines showed the XP phenotype. Penetrance was further investigated by another cross.

Stock d4-154 (= XP) was crossed to stock tsK (= non-XP). The 23 pairs of hybrid clones obtained were examined for XP 15–20 cell generations after conjugation (mean sample size = 50, range = 13–109). The ratio of the number of pairs in which *both*, *one*, or *neither* of the two exconjugant clones expressed XP is 14:8:1. The reduced penetrance in the hybrid is confirmed: of the 46 hybrid clones, 36 expressed XP, i.e. penetrance = 0·78. Also, this figure (0·78) permits calculating a decisive answer to the question of whether the two exconjugant clones of a pair are, or tend to be, alike with respect to expressing XP. The expected ratio of both, one, or neither of the two exconjugant clones of a pair expressing the XP phenotype is $23(0{\cdot}78)^2 : 23(0{\cdot}78 \times 0{\cdot}22) : 23(0{\cdot}22)^2$, i.e. 14:8:1 which is the observed ratio. Thus it

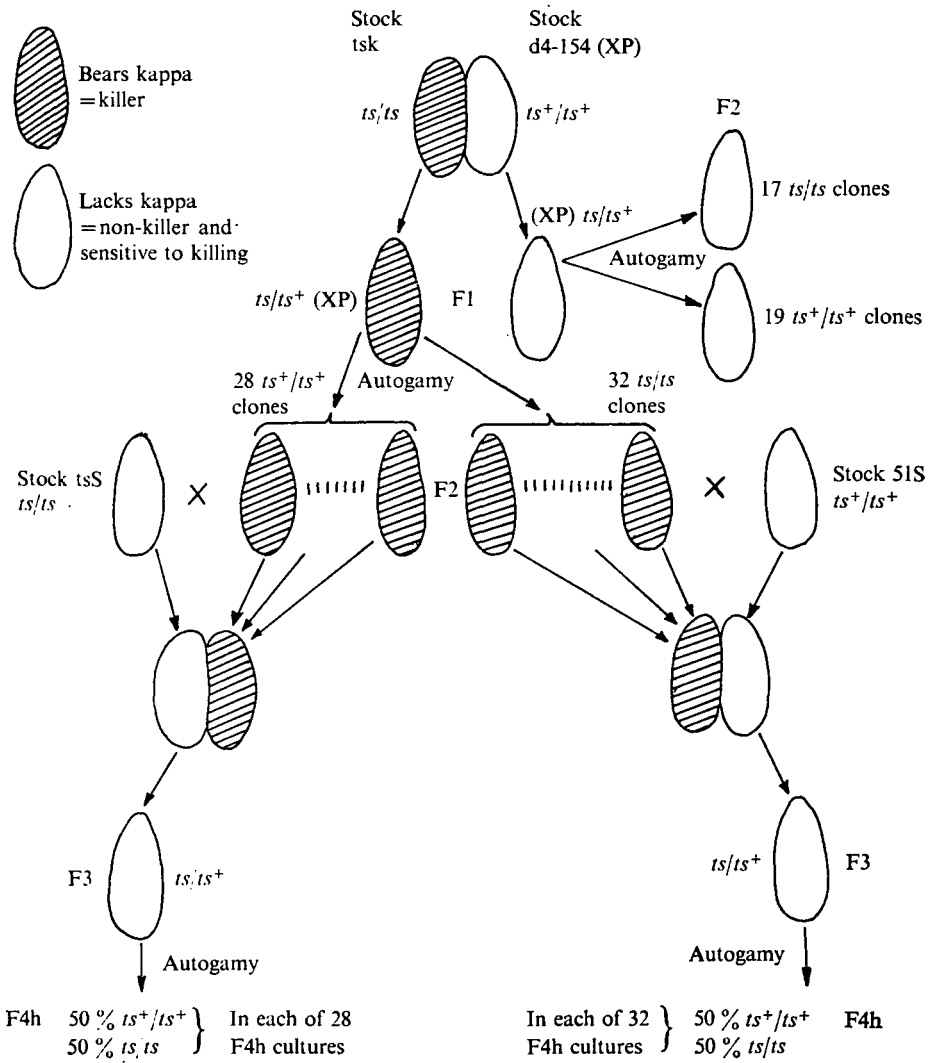
appears that the two exconjugant clones of a pair do not have a tendency to be alike in expressing or not expressing XP.

Further investigation of the nuclear basis of extra cytoprocts in stock d4-154, as mentioned above, yielded peculiar F2 results. In preliminary studies of crosses between stocks d4-154 and tsK, a great excess of F2 cultures showing XP (for culture composition, see Materials and Methods) instead of the expected 1:1 ratio for the segregation of a single gene was found. However, the excess decreased when the character was scored in these cultures at progressively later times: 61:7 (XP: wild type) at the 15th cell generation; 53:8 at the 50th and 50:11 at the 120th (five XP and two wild-type cultures were lost after the 15th generation). Six F2 cultures expressed XP at the 15th but not at the 120th generation. It is not known whether more F2 cultures would have changed eventually; apparently they would, as inferred from the result of another experiment (see below). The question thus arises as to whether such late-changing F2 cultures possessed the nuclear basis needed for expression of XP.

This question was answered by taking advantage of the fact, established above, that the XP character in stock d4-154 behaves as dominant. So F2 cultures, some expressing XP, and some not expressing, were crossed to wild type. The genotype of the F2 cultures could therefore be ascertained by whether they could or could not transmit to their wild-type mates the genetic basis for development of the XP character. However, the reduced penetrance in heterozygotes would yield F3 clones that failed to show XP in spite of having the genotype for it. It was therefore necessary to obtain homozygotes from the heterozygous F3 in order to overcome the penetrance problem. The homozygotes were obtained by inducing autogamy in the F3 clones. The resulting mass exautogamous animals from each F3 clone were grown together as an F4 culture. Both F3 and F4 were scored for the XP character.

The experiment is depicted in Fig. 8. From a single d4-154 (XP; ts^+) × tsK (non-XP; *ts*) pair in which both exconjugant F1 clones expressed XP, 60 F2 clones from the tsK parent and 36 F2 clones from the d4-154 parent were obtained by autogamy about 25 fissions after conjugation. The F1 was ts/ts^+, so half of the F2 cultures from each parent were temperature-sensitive (*ts*/*ts*). Stock tsK carries kappa; thus the 60 F2 cultures from the tsK parent were killers. The temperature-sensitive killer F2 cultures were crossed (when less than 100 fissions old) to stock 51S (non-temperature-sensitive; non-killer); those from non-temperature-sensitive killer F2 clones were crossed to stock tsS (temperature-sensitive; non-killer). In both cases, the *ts* marker permitted elimination of pairs that had not cross-fertilized (see section 3(*a*) and (*c*), Materials and Methods). The non-killer F3 exconjugants (i.e. from the tsS and 51S parents that had not become killers by receiving kappa from their mates) were retained for study. One such non-killer F3 exconjugant clone was obtained from each killer F2 culture; thus 60 non-killer F3 clones were obtained. These were (i) sampled for silver impregnation at about 15 fissions after conjugation and (ii) induced to undergo autogamy after about 25 fissions to give by autogamy F4 homozygotes. Autogamy was induced in depression slides and more than 200

autogamous animals from each F3 clone were transferred to a culture tube. Each F4 tube culture was genotypically heterogeneous because its parental F3 was heterozygous with respect to *ts* and also with respect to whatever gene(s) there may be for the XP character. To emphasize this heterogeneity, the F4 cultures are designated as F4h cultures.



Total: 60 F4h cultures

Fig. 8. Design of experiment to ascertain genotype of F2 from cross of stocks d4–154 and tsk.

The 60 F4h cultures were sampled for silver impregnation at about 30 fissions, and some again at about 150 fissions after their initiation. Samples of the cultures from the *killer* F2 clones were also silver-impregnated after about 50 fissions and

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again 300 fissions since their initiation. Those from the *non*-killer F2 clones were silver-impregnated after about 300 fissions.

The results on F2, F3 and F4 are summarized in Table 1, where cultures from the F2 clones are grouped according to expression of XP in F3 and F4 cultures. Groups A–D come from the killer (tsK) parent, groups E and F from the non-killer (d4-154) parent. Group A consists of 16 F2 cultures which had the XP character and whose mates produced F3 with the XP character. The character of the F3 thus shows

Table 1. *F2, F3 and F4 results from d4-154 × tsk cross (see experimental design, Fig. 8)*

			F2 cultures		F3 clones	F4h cultures
	Number of	Inferred F2	50 fissions	300 fissions	15 fissions	30,150 fissions
			F% (95% c.i.)†	F% (95% c.i.)	F% (95% c.i.)	F% (95% c.i.)
Groups	cultures	genotype	n (R)	n (R)	n (R)	n (R)
From tsk parent:						
A	16	*Ec/Ec*	10·1(7·1–13·4)	3·0(2·1–4·1)	11·6(7·9–13·9)	—
			51 (32–98)[a]	81 (32–156)[b]	53 (23–75)	
B	20	*Ec/Ec*	11·3(8·7–14·2)	3·8(3·0–4·9)	*	2·7(2·3–3·0)
			37 (21–81)[a]	63 (30–128)[b]	48 (12–82)	179 (55–403)[f]
C	12	*Ec*$^+$/*Ec*$^+$	10·7(6·7–15·5)	*	*	*
			38 (30–49)	197 (119–245)[c]	114 (46–166)[d]	364 (258–599)[e]
D	12	*Ec*$^+$/*Ec*$^+$	*	*	*	*
			88 (67–125)[a]	70 (33–102)	53 (30–102)	53 (30–100)
From d4–154 parent						
E	15	*Ec/Ec*	—	16·4(10·9–22·9)	—	—
				45 (7–137)[g]		
F	21	*Ec*$^+$/*Ec*$^+$	—	*	—	—
				95 (75–105)[g]		

† F% (95% c.i.) = mean frequency of expression % (95% confidence interval). n (R) = mean sample size (range).

[a] Includes samples taken at 80 fissions from 5 cultures of A, 1 of B and 10 of D.

[b] Includes samples taken at 360 fissions from 7 cultures of A and 6 of B.

[c] Includes samples taken at 360 and again at 400 fissions from all 12 cultures.

[d] The cross for each of the 12 cultures has been repeated, results from the first and second sets of crosses pooled.

[e] Results on F4h cultures obtained from both sets of crosses (see d above) pooled.

[f] One of the 20 cultures not sampled at 150 fissions.

[g] Sampling method for group E and F cultures: scoring ended when 2–12 animals in a sample showed XP phenotype.

* Classified as non-XP phenotype (see Materials and Methods).

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clearly that the F2 transmitted, and so possessed the XP genotype. Group B consists of 20 F2 cultures which also had the XP character, but the clones from their F3 mates did not. However, the F4h cultures from these F3 showed the XP character. Hence the F3 – and the F2 – must have had and transmitted the genotype for XP. Its failure to appear in F3 is assumed to be due to the previously demonstrated reduced penetrance in heterozygotes. Groups A and B together thus yield a total of 36 F2 cultures with the XP genotype. The other two groups, C and D, failed to show XP in either F3 or F4; hence, their 24 F2 cultures lacked the XP genotype. The inferred F2 segregation ratio is thus 36:24 (XP:wild type), which is not significantly different from a 1:1 segregation ratio (χ^2, $0{\cdot}2 > P > 0{\cdot}1$). This agrees with studies made directly on the cultures from *killer* F2 clones at 300 fissions: 36 XP to 24 wild type; each F2 culture falls into the same category by both the direct and indirect method of genotype ascertainment.

It is important to note that while the phenotypes of the F2 cultures, when 50 fissions old, do not reliably reveal their genotypes, their phenotypes at 300 fissions do. At 50 fissions, the 12 F2 cultures in group C still had the XP phenotype, but they had lost it by 300 fissions; whereas the 12 F2 cultures in group D had already lost the XP phenotype by the 50th fission. Thus, only about half of the F2 cultures that had lost the XP genotype had also lost the XP phenotype by the 50th fission; but all had by 300 fissions. Hence the phenotypes in F2 groups E and F, from the non-killer (d4-154) parent, which were scored at 300 fissions, are assumed to be reliable indices of their genotypes. There were 15 F2 cultures with the XP phenotype (group E) and 21 without (group F). The segregation ratio for the non-killer F2 cultures is therefore 15:21, again not significantly different from 1:1 (χ^2, $0{\cdot}5 > P > 0{\cdot}2$).

Combining all groups of F2 of like genotype, groups A, B and E (total 51) had the XP genotype, and groups C, D and F (total 45) did not. This is close to the theoretical 1:1 ratio for segregation of a pair of alleles (χ^2, $0{\cdot}9 > P > 0{\cdot}5$). We therefore conclude that there is a single allele difference involved and assign the symbol *Ec* to the dominant allele for the XP phenotype and Ec^+ for the recessive wild type.

Verification of this conclusion was sought by a method that would avoid the exceedingly laborious and time-consuming following of cultures for hundreds of cell generations, as had been required in the method used above. The plan, diagrammed in Fig. 9, is in principle to backcross F2 homozygotes (*Ec*/*Ec*) to wild type to get F3 heterozygotes (Ec/Ec^+). Instead of inducing the F3 heterozygotes to undergo autogamy to give the backcross segregation ratio in F4, the F3 heterozygotes were quickly backcrossed to wild type (Ec^+/Ec^+) to discover whether half of the wild-type mates (now F4) became Ec/Ec^+ and half of them Ec^+/Ec^+. However, as noted above, because of the reduced penetrance of *Ec* in heterozygotes, confining examination to the F4 backcross clones would not be expected to yield the 1:1 ratio expected for a fully penetrant dominant gene. Therefore, the F4 backcross clones were carried one step further; each one was induced to undergo 100% mass autogamy so as to yield F5 homozygotes and, as in the previous method, the multiple

autogamous progeny of each backcross clone were grown together in a tube culture and examined as a whole. In this way, an F4 backcross clone of genotype *Ec/Ec*$^+$ would yield an F5 mass autogamous culture containing 50% *Ec/Ec* clones that would be expected to be free of the limitation of heterozygotic reduced penetrance and therefore would exhibit the XP phenotype. Backcross clones of *Ec*$^+$/*Ec*$^+$ genotype would neither develop the XP phenotype nor yield any autogamous progeny that could develop it.

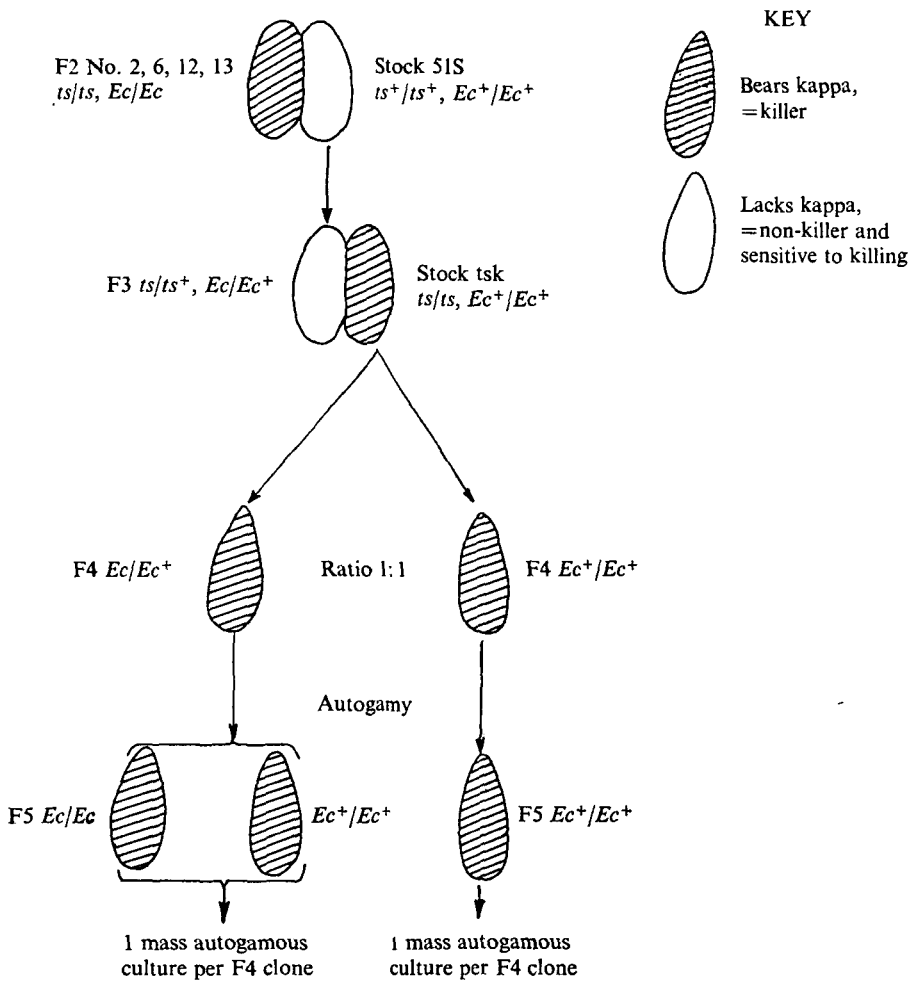

Fig. 9. Design of experiment to verify *Ec/Ec* genotype of four *F*2 cultures.

In this experiment, as in the preceding one, both the genic marker *ts* and the cytoplasmic marker kappa were employed to identify the parental source of each conjugant and to identify those that had cross-fertilized (Fig. 9). The F3 heterozygotes for backcrossing were obtained by crossing stock 51S (*ts*$^+$/*ts*$^+$, *Ec*$^+$/*Ec*$^+$, non-killer) to each of four F2 cultures (nos. 2, 6, 12 and 13) belonging to group A

https://doi.org/10.1017/S0016672300016359 Published online by Cambridge University Press

in the preceding experiment (Fig. 8). These four had been proved to be *ts*/*ts* killers and were inferred to be *Ec*/*Ec* because they still expressed XP at 300 fissions and had transmitted the genetic basis of it to their wild-type mates. The crosses were made when the F2 cultures were about 400 fissions old. The resulting non-killer F3 clones (*ts*/*ts*$^+$, presumably *Ec*/*Ec*$^+$), when 10–15 fissions old, were backcrossed to stock tsK (*ts*/*ts*, *Ec*$^+$/*Ec*$^+$, killer), giving F4. Mass autogamy was then induced in the F4 *killer* members of the backcross pairs, giving F5. Half of these F4 killer clones would be expected to be *Ec*/*Ec*$^+$; these would yield in F5 by mass autogamy 50% *Ec*/*Ec* clones that would have XP-animals, and 50% *Ec*$^+$/*Ec*$^+$ clones that may have XP-animals if observed soon after autogamy because of the previously elucidated (F2, group C) persistent expression of XP after loss of the *Ec* gene (see also following section). The other half should be *Ec*$^+$/*Ec*$^+$ and have no XP-animals. Observations for the XP phenotype were made when the F5 were about 20 fissions old; scoring ended when 2–12 animals in a sample showed the XP phenotype. The mean sample size of F5 cultures having XP was 80 (range = 3–180) and that of cultures not having XP was 182 (range = 110–300). The entire analysis involved only 50–60 cell generations instead of 300.

The results obtained with the descendants of two of the original four F2 cultures were in agreement with the genic expectations: F2 culture no. 2 yielded 38 F5 cultures containing XP-animals and 37 that did not; F2 culture no. 6 yielded 28:28. When corrected for cytogamy (see Materials and Methods, section 3(*b*)), the ratios were 38:34 and 28:26, respectively, which agree well (χ^2, $0{\cdot}9 > P > 0{\cdot}5$) with the theoretical 1:1 ratio and confirm the conclusion that the XP phenotype is due to a single gene, *Ec*.

On the other hand, the F5 from the other two F2 cultures yielded very poor corrected ratios for XP: wild type. F2 culture no. 12 gave 2:10 and F2 culture no. 13 gave 1:11. In the three F5 cultures that showed any XP, the frequencies of XP-animals were very low (0·6%, 1·5%, 3·8%). Because the sample sizes (150–300 animals) were probably too small to detect reliably such low levels of expression, these two cases remain indecisive.

(ii) *Persistent expression of XP after loss of* Ec *gene*

As pointed out earlier, 12 cultures from F2 clones of the preceding experiment (group C, Table 1) contained XP-animals after 50 cell generations, but were shown to be genotypically wild type (*Ec*$^+$/*Ec*$^+$). These 12 cultures had ceased to express XP by 300 generations. By analogy, the six F2 cultures in the preliminary experiment (p. 150) which expressed XP at 15 cell generations but not after 50 and 120 generations may also be considered to be of the *Ec*$^+$/*Ec*$^+$ genotype. All of the F1 *Ec*/*Ec*$^+$ clones from which these F2 cultures were derived expressed the XP phenotype. Thus it appears that after loss of the *Ec* gene at autogamy, i.e. when the genotype changes from *Ec*/*Ec*$^+$ to *Ec*$^+$/*Ec*$^+$, some of these *Ec*$^+$/*Ec*$^+$ cultures continued to express the XP phenotype for 15–120 cell generations, or even longer periods since in the preliminary experiment, the F2 segregation ratio at 120 fissions is significantly different from 1:1. Some lost the XP phenotype by the 15th generation

(seven cultures); some by the 50th generation (three cultures); others by the 120th generation (three cultures). The theoretical importance of such results will be dealt with in the discussion.

(iii) *Frequency of expression of extra cytoprocts*

Great variation in the frequency of expression, from 8% to 58%, was reported among samples of the homozygous XP mutant, stock d4-154 (Ng, 1976*a*). Among the 36 F1 *Ec*/*Ec*$^+$ clones derived from d4-154 × tsK crosses (Results, p. 150), the frequency of expression varied from 3% to 38% (mean = 12%). Preliminary studies suggested, but did not prove, that differences in frequency of expression might be clonal characters. Factors determining differences in frequency of expression remain unknown.

Cultures from F2 clones of groups A (*Ec*/*Ec*) and B (*Ec*/*Ec*) at 50 fissions had equal mean frequencies of expression (about 10%, Table 1). Although the F3 from group A had about the same frequency of expression, the F3 from group B – in spite of having the *Ec* gene, as proved by the F4h – did not express XP at all. This shows that whether the F3 *Ec*/*Ec*$^+$ heterozygotes expressed XP, or whether they did not (i.e. whether the *Ec* gene is penetrant or not), was independent of the frequencies of expression in their F2 *Ec*/*Ec* parent cultures.

As shown in Table 1, at 300 fissions the mean frequency of expression of both groups A and B dropped to a significantly lower value, 3–4% (t test, $0{\cdot}001 > P$). The basis for the consistent downward drift of frequency in these tube cultures remains unknown.

Remarkably, half of the F2 cultures that lost the *Ec* gene (Table 1, group C, genotype *Ec*$^+$/*Ec*$^+$) maintained the same frequency of expression at 50 fissions as F2 cultures that retained the *Ec* gene (groups A and B, genotype *Ec*/*Ec*). The other half of the F2 cultures that lost the *Ec* gene (group D, genotype *Ec*$^+$/*Ec*$^+$) had ceased to express XP by the 50th fission. This indicates that the frequency of expression after replacement of *Ec* by *Ec*$^+$ does not gradually decline during long 'lag' periods (at least until the 50th cell generation), but remains as high as in the *Ec*-bearing sister cultures and the parent culture. Although the kinetics of eventual loss remain unknown, decline of the frequency of expression remains inapparent for long periods in a high proportion of the cultures.

The mean frequency of expression in h (genotypically heterogeneous) cultures, consisting of both *Ec*$^+$/*Ec*$^+$ and *Ec*/*Ec* animals, was expectedly not much lower than in homogeneous cultures of *Ec*/*Ec* genotype: F4, group B, 3% (Table 1); F5, no. 2, 7% and 4% in two separate experiments; F5, no. 6, 10%. This is because the h cultures were studied at 20–30 fissions (some, again at 150 fissions) after replacement of the *Ec* gene by autogamy. Hence, many of the *Ec*$^+$/*Ec*$^+$ animals in those h cultures derived from *Ec*/*Ec*$^+$ clones are expected to express XP at 20–30 fissions, because of the above-mentioned long lag periods of expression.

4. DISCUSSION

The most interesting feature of the genetics of the extra cytoproct character is the long maintenance of the character after loss of the *Ec* gene in F2. Such long maintenance is not without precedent in Ciliate genetics. Sonneborn & Lynch (1934) demonstrated that when lines of *Paramecium aurelia* of different fission rates were crossed, the exconjugant clones attained intermediate fission rate after variable lag periods, up to 26 fissions after conjugation. De Garis (1935) crossed lines of *Paramecium caudatum* of different body lengths and reported that animals from the exconjugant clones attained their definitive lengths after variable transition periods, extending over 30 or more fissions after conjugation. Even longer lag periods have been reported in other systems: maintenance of the endosymbiont kappa ultimately depends on the presence of gene *K* in *Paramecium tetraurelia* (Sonneborn, 1943); but some *k*/*k* clones derived by autogamy from *K*/*k* killers can maintain kappa for more than 50 fissions (see review by Preer, 1974). Also in *Paramecium tetraurelia*, gene *dc* (Sonneborn, 1974; Maly's *ds* gene, 1958) is responsible for the monster character, but some *Dc*/*Dc* homozygotes can maintain the monster character for more than 100 cell generations after loss of gene *dc* (Maly, 1958). In all of the above cases, the mechanism underlying such long maintenance remains obscure.

Two other examples of long maintenance of morphological character after loss of the required genotype may offer some insight into the nature of this phenomenon. The first example concerns inheritance of the number of ciliary rows in *Euplotes minuta* (Heckmann & Frankel, 1968; Frankel, 1973, 1975). Crosses between two subspecies (arbitrarily designated as A and B) with different – though overlapping – numbers of ciliary rows result in all exconjugant F1 clones *eventually* having the row-number characteristic of the B parent; however, this only occurs after a 'lag' of 13–70 fissions after conjugation, during which the exconjugant clones from the A parent still include many animals with row-numbers characteristic of the A parent. Thus the row-number characteristic of the A parent may be conserved for a considerable period in the exconjugants from the A parent, in spite of change of genotype.

The other example concerns the inheritance of inverted kineties in *Paramecium tetraurelia* (T. M. Sonneborn, private communication). Gene *sn* causes misdivision of the animals resulting in chains of two (or more) animals. The posterior member of the chain may bend forward alongside the anterior member to yield a V-shaped pair of animals in heteropolar orientation. During subsequent growth and division some kineties at the junction between the two animals grow into the neighbouring animal. This results in a 'graft' of inverted kineties which persist in the progeny even after the *sn* gene is replaced (at conjugation and a following autogamy) by the wild-type allele sn^+.

Both the conservation of the number of ciliary rows (*Euplotes*) and the persistence of inverted kineties (*Paramecium*) may be understood in terms of the mode of growth of ciliary rows in Ciliates. New basal bodies (and cilia) develop within the

meridian of an existing ciliary row (in *Paramecium*, Dippell, 1968; in *Tetrahymena*, Allen, 1969). Thus, perpetuation of the parental ciliary row number and of inverted kineties after genotype change is apparently under the influence of preexisting ciliary rows.

What kind of mechanism would allow long maintenance of XP after loss of the *Ec* gene? The crucial question is whether a non-genic factor in the endoplasm or cortex affects expression of the *Ec* gene. In d4-154, non-genic factor(s) have been shown to affect the frequency of expression and location of extra cytoproct and the number of extra cytoproct per animal: proters differ from opisthes in these respects (Ng, 1976*a*). Such differences are *not* a result of differential gene action between proters and opisthes (Ng, 1976*b*).

Evidence of cortical determination of extra cytoproct comes from studies of cortical variants which are *not* results of gene mutation. The presence of extra cytoproct has been associated with experimental changes in the cortical pattern in animals that have the normal wild-type genome (Sonneborn, 1963; Ng, 1976*a*). Thus, without genomic change, the cortex can determine the presence of extra cytoproct.

However, in d4-154, the presence of extra cytoproct is not associated with any other visible cortical deviation from normal. Thus, an active participation of the cortex in contributing to the absence or presence of extra cytoproct in d4-154 has not been established. Nevertheless, the possibility has not been ruled out that the action of gene *Ec* has brought about changes in ultra-structural configurations of the cortex which play a role in formation of extra cytoproct. Like other cortical changes, such configurations might persist for a considerable period. If so, this could result in the presence of extra cytoproct for variable periods after the *Ec* gene has been replaced by Ec^+.

The author is indebted to Dr T. M. Sonneborn for his criticism on the present research and on the manuscript.

This work was supported by Sir Robert Black Fellowship (Hong Kong), Overseas Exchange Fellowship (Indiana University) and Carl Eigenmann Fellowship (Indiana University) to the author and by Grant PHS Rol GM 15410-08 from the Public Health Service to Dr T. M. Sonneborn.

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