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Coordination on timing of gene expression in *Paramecium*

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SUMMARY

In *Paramecium caudatum*, syngen 3, cells become mature (i.e. capable of undergoing conjugation) by about 50 fissions after conjugation. Mating-type-instability, another mating characteristic, begins to occur between 80 and 120 fissions after conjugation in heterozygotes for the mating type locus (Myohara & Hiwatashi, 1975). Mating-type instability also occurs in dominant homozygotes, earlier than in heterozygotes. In two different early maturing mutants the homozygotes become mature 20 fissions earlier than in wild-type clones, and mating-type instabiliiy also begins earlier than in wild-type clones. The number of fissions from maturity to the occurrence of the mating type change does not differ.

1. INTRODUCTION

In unicellular organisms such as the ciliated protozoa, some traits begin to appear at a certain time after fertilization. For example, the time of maturation is fixed in each species; in *Paramecium multimicronucleatum* after 40–90 fissions, in *P. bursaria* after about 50 fissions, and *Tetrahymena thermophila* after about 60 fissions (Bleyman, 1971). Thus, the ciliated protozoa can be used as model systems for analysing the mechanisms controlling the timing of expression of genes determining maturation. The ready isolation of mutants and the ease of genetic analysis make the ciliates more suitable material for this purpose than other systems. In *P. caudatum* most strains become mature after 50–60 fissions (Miwa & Hiwatashi, 1970; Takagi, 1970; Myohara & Hiwatashi, 1978). The appearance of mating activity is related to the number of fissions, and not absolute time (Miwa & Hiwatashi, 1970). The number of fissions is affected by some genetic factors (Myohara & Hiwatashi, 1978). In other systems – for example, determination of the imaginal disks in *Drosophila* (Garcia-Bellido, Ripoll & Morata 1973) – the number of cells divisions is also important.

In metazoan development, not only are some genes sequentially expressed, but also their expression is coordinated. In ciliated protozoa, the expression of some genes is determined sequentially. In *P. bursaria*, the two mating type loci are turned on sequentially in order for the final mature mating type to be expressed (Siegel & Cohen, 1963). In *P. caudatum* the cells become mature at about 50 fissions and mating-type instability, another mating characteristic, begins at about 100 fissions after conjugation. Mating-type instability means the changing of mating type without change in genotype. The clones of even mating type, both the homozygotes and the heterozygotes for the mating-type locus, express

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odd mating type in late stationary phase. The change of mating type is reversible (Hiwatashi, 1960); cells that changed to the odd mating type express the even mating type again after growth. In this paper I report coordination in time of the expression of mating activity and mating-type instability.

2. MATERIALS AND METHODS

(i) *Stocks and culture methods*

All strains used for this study belong to syngen 3 of *P. caudatum*, and are derived from strains Ky and Ksy. They are listed in Table 1. The culture conditions were the same as those described by Hiwatashi (1968). The culture medium was sterilized lettuce juice inoculated with *Klebsiella pneumoniae* 1 day before use. All cultures were maintained at 25 °C except during the time of the isolation procedures, which were performed at room temperature.

Table 1. *Stocks and derived strains used for this study*

Stock or strain	Mating type	Source
Ky	VI	Natural stocks collected in Kyoto
Ksy 1	V	
Ky-s 95a	VI	A selfing progeny of Ky
d-KK 2a	V	Descendants of a cross between Ky-s 95a and Ksy-1
d-KK 7a	VI	
d-KK 14a	V	
d-Ks	VI	Descendants of a cross between d-KK 7a and d-KK 14a
d-Ke	V	
Em III-2S	VI	Early mature strains. The original early mature stocks were obtained by treatment d-KK 7a with nitrosoguanidine (Myohara & Hiwatashi 1978).
Em IV-3 (2S)	VI	

(ii) *Crosses*

Cells of complementary mating types were mixed and about 6 h later conjugating pairs were isolated and transferred into exhausted culture medium or Dryl's solution (Dryl, 1959). To prepare exhausted culture medium, ordinary cultures of paramecia were kept without the addition of food for more than a month at 25 °C and the medium was then filtered through a Millipore filter (0·45 μm). Exconjugants were isolated into exhausted culture medium or Dryl's solution one day after conjugation. The next day each cell was transferred into fresh culture medium. These procedures were all performed in glass depression slides with 0·5 ml medium per depression. The cells were cultured in the same medium for two additional days, and were then transferred into test tubes that contained 2 ml fresh culture medium. When selfing pairs were isolated, the pairs were transferred into exhausted culture medium or Dryl's solution. The latter procedure was the same as that for isolating crossing pairs.

(iii) *Tube transfer culture*

The following method was used to estimate the number of fissions after conjugation. The cells cultured in each depression as described above were transferred into 2 ml fresh culture medium in a test tube and then 4 ml and 10 ml fresh medium were added to the tube on successive days. By the 4th day of this tube culture the cells had undergone about 20 fissions since the exconjugant stage. About 1000 cells were then transferred into a new test tube containing 2 ml fresh medium, and then 4 and 10 ml fresh medium were added to the tubes on successive days. At the 4th day of the tube culture about 1000 cells were transferred into a new test tube containing 2 ml fresh medium. This transfer was repeated until mating-type instability began to occur. The number of fissions between transfers was estimated from the number of cells in the first inoculum (1000 cells) and the total number of cells by the 4th day, and was found to be approximately five fissions. The total number of fissions until the appearance of mating reactivity and the initiation of mating-type instability was calculated by multiplying the number of tube-to-tube transfers by five, plus the 20 fissions that occurred before the first tube-to-tube transfer. Mating-type instability was tested by checking the mating type. The mating-types were tested at the 2nd and 3rd day after the final feeding of 10 ml culture medium by adding highly mating-reactive cells. In the case of d-KK $2a \times$ Ky-s $95a$ and Kys $1 \times$ Ky-s $95a$, mating type instability was tested by the appearance of selfing pairs in the culture. Because in unstable strains the mating-type does not change synchronously, two different mating types exist in the same culture for some time, and mating pairs are formed between changed and unchanged cells. Mating-type change almost leads to the appearance of selfing pairs.

3. RESULTS

(i) *Time of the first occurence of mating-type change in homozygotes and heterozygotes at the mating-type locus*

In *P. caudatum*, syngen 3, all clones from one conjugating pair express the same mating-type. This pattern implies that mating-type determination is under direct genetic control. Mating-type is determined by a pair of alleles at one locus, the *mt* locus. The recessive homozygote, *mt*/*mt*, determines mating-type V and the dominant + allele determines mating-type VI (Hiwatashi, 1968). When a homozygous dominant clone (+/+) is crossed to a mating-type V clone (*mt*/*mt*), all the progeny are mating-type VI and are heterozygous at the mating-type locus (+/*mt*). In the clones of mating-type V, the mating-type is usually stable and does not change to mating-type VI. In clones of mating-type VI, either homozygotes or heterozygotes, the mating-type is usually stable during the early period of maturity. Instability of mating-type occurs only after a certain number of fissions. I examined the number of fissions required for mating-type change to occur in mating-type VI clones. In the selfing progeny of d-Ks, homozygous at the mating-type locus, the mating-type change began to occur between 50 and 100 fissions after con-

jugation (Fig. 1). The mean was 77 fissions for the 51 clones examined. Once the change of mating type occurred in a line, it usually occurred later in every tube. Next, the time of the first occurrence of mating type change was examined in heterozygous clones. In d-Kl, the progeny of the cross between d-Ks (+/+) and d-Ke (*mt*/*mt*), the mean number of fissions to the beginning of the mating type change was 91 fissions ($N = 41$). The results from the heterozygotes agreed with

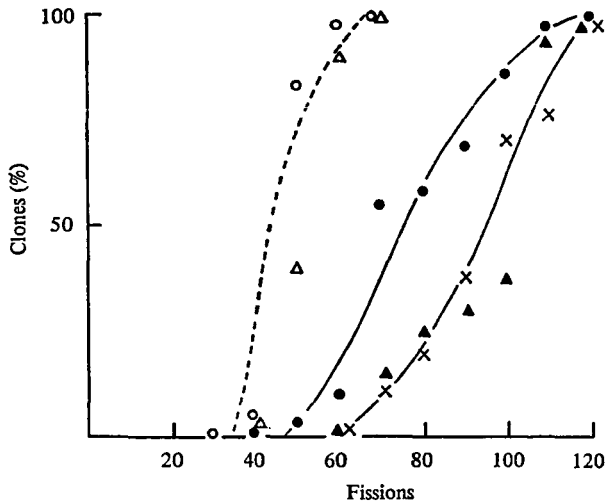

Fig. 1. Time of the first occurrence of mating type change in homozygotes and heterozygotes at the mating-type locus. ——, The percentage of clones in which mating-type change occurs; – – –, the percentage of mature clones. ○, ●, Selfing progeny of d-Ks (+/+); △, ▲ d-Ks × d-Ke (+/*mt*); ×, d-KK 2a × Ky-s 95a (+/*mt*). ○ and △, and ▲ and ×, are drawn as one curve.

those from heterozygotes from other crosses, Ksy 1 (*mt*/*mt*) × Ky-s 95a (+/*mt*) and d-KK 2a (*mt*/*mt*) × Ky-s 95a (+/*mt*) (Myohara & Hiwatashi, 1975). The difference between the heterozygotes (the progeny of d-Ks × d-Ke) and the homozygotes was statistically significant ($t = 3{\cdot}9$, $P < 0{\cdot}01$). The mating-type change occurs earlier in the homozygotes than in the heterozygotes.

(ii) *Time of the first occurrence of mating-type change in the early maturing mutants*

Is the time of the first appearance of mating-type instability determined by the number of fissions from conjugation or from maturation? To answer this question I examined the time of the first occurrence of mating-type change in two early-maturing mutants. If the time of the first appearance of mating-type instability does not change in the mutants, the time depends on the number of fissions from conjugation. Two independent mutants, obtained by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, were genetically analysed by crosses to wild-type stocks. Both mutants show incomplete dominance, i.e. the homozygotes become mature 20–25 fissions and the heterozygotes 15 fissions earlier than the wild-type clones. The genes are located at different loci and are named *Emt A* and *Emt B* (formerly *Em-1* and *Em-2* repectively) (Myohara & Hiwatashi,

1978). In the selfing progeny of Em III-2S the mating-type change began to occur between 30 and 80 fissions (Fig. 2). The mean was 57 fissions for the 24 clones examined. Compared with the selfing progeny of d-Ks, the difference in time of the first occurrence of mating-type change was significant ($t = 2{\cdot}0$, $P \fallingdotseq 0{\cdot}05$). In the early maturing clones, the mating-type change occurred about 20 fissions earlier than in the wild-type clones. This difference coincided with the difference in length of the immature period.

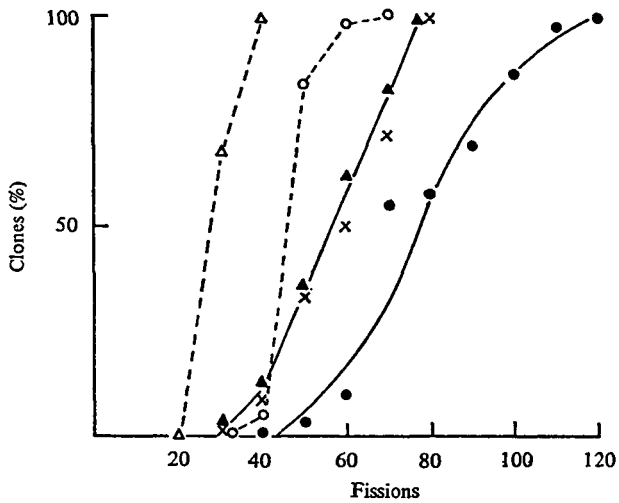

Fig. 2. Time of first occurrence of mating-type change in the early maturing mutants. ——, The percentage of clones in which mating-type change occurs; – – –, The percentage of mature clones. ○, ●, Selfing progeny of d-Ks (+/+, *Emt* A^+/*Emt* A^+, *Emt* B^+/*Emt* B^+); △, ▲, selfing progeny of Em III-2S (+/+, *Emt* A^+/*Emt* A^+, *Emt B*/*Emt B*); ×, selfing progeny of Em IV-3 (2S) (+/+, *Emt A*/*Emt A*, *Emt* B^+/*Emt* B^+). ▲ and × are drawn as one curve.

To determine whether this phenomenon is restricted to the *Emt B* locus, I examined the time of the first occurrence of mating-type change in another early mutant. In the selfing progeny of Em IV-3 (2S) (designated Em IV-3(3S)), the mating-type change began to occur between 50 and 80 fissions (Fig. 2). The mean was 66 fissions for the 21 clones examined. This result did not differ from that of Em III-2. In this mutant, compared with the selfing progeny of d-Ks, the difference in time of the first occurrence of mating-type change was significant ($t \fallingdotseq 2{\cdot}1$, $P \fallingdotseq 0{\cdot}04$). Mating-type instability occurred earlier in the two early maturing strains. So the early appearance of mating type instability is not restricted to a certain early maturing gene.

In one progeny of Em IV-3(3S) the mating-type change occurred exceptionally early, as soon as they became mature at 30 fissions. To determine if this early occurrence of the mating type change was inherited, I examined the selfing progeny of Em IV-3(3S), named Em IV-3(4S), to see when mating types first changed. The mating-type change began to occur between 40 and 80 fissions ($N = 22$). This result did not differ from that of Em IV-3(3S). So the difference

within the progeny from the same cross is not caused simply by some genetic differences.

DISCUSSION

In *Paramecium*, conjugation usually occurs when cells of complementary mating types are mixed together under appropriate conditions. For a long time, however, the occurrence of diverse mating types within a clonal culture which results in selfing conjugation has been known. Some selfing is caused by a temporary change of mating-type without a segregation of clones of different mating type (Kimball, 1939; Taub, 1966; Bleymann, 1967; Hiwatashi, 1969). In *P. caudatum*, syngen 3, selfing conjugation due to temporary change of mating-type is very common and is restricted to mating-type VI clones (Hiwatashi, 1958; Myohara & Hiwatashi, 1975). In the selfing clones of mating type VI, cells express mating-type VI in the early stationary phase but they express mating-type V in the late stationary phase. Thus, during the transient period from the early to late stationary phase, cells of both mating types exist in a culture and selfing conjugation occurs. This mating-type change is reversible (Hiwatashi, 1960); cells changed to mating-type V express mating-type VI again after growth. The mating-type is usually stable just after maturity. More fissions are needed before the instability of mating-type sets in. Though no extensive study on the onset of mating-type instability under different environmental conditions has been performed so far, conditions which cause fission rate to decline, such as low temperature, retard the onset of mating-type instability in actual time. This seems to show that the onset of mating-type instability depends upon the number of fissions.

To determine whether the time of onset of mating-type instability depends on the number of fissions since the time of conjugation or of maturity, I examined the timing in early maturing clones. In these clones, which became mature 20 fissions earlier than the wild-type clones, the mating-type change also began to occur 20 fissions earlier. Comparing the early maturing mutants and wild-type clones, there was no difference in number of fissions from maturity to the occurrence of mating-type change (Table 2). This result is clearer when the percentage of the mating-type unstable clones is plotted against the number of fissions afrer maturity of the clone. (Fig. 3). If both maturity and instability are proportionally displaced in the mutant homozygotes, instability would be expected at about 48 fissions ($33/53 \times 77$). This interpretation may be excluded at least in the case of Em IV-3(3S). The time of occurrence of mating-type change depends on the number of fissions from maturity rather than from conjugation. This is the first time the existance of coordination in the time of gene expression is ciliated protozoa has been suggested. Under all conditions, if the cells undergo the necessary numbers of fissions, maturity and mating-type instability occur. The changes are not triggered by the circumstances of growth but by some internal mechanism measuring biological time. In this respect the *Paramecium* system is different from that of the cellular slime mould or of any known prokaryote systems. In *Dictyo-*

Table 2. *The time of maturity and the onset of selfing in wild-type clones homozygous and heterozygous at the* mt *locus, and in early maturity mutants homozygous for different early maturing mutants and for the* mt *locus*

Strain	Genotype			Fissions		
	mt genotype	*em* genotype		To maturity	To initiation of mating type instability	From maturity to instability
d-Kl	+/*mt*	*Emt A*+/*Emt A*+ *Emt B*+/*Emt B*+	Mean	59 (N = 42)*	91 (N = 41)*	50
			$S_{\bar{y}}$†	0·90	2·8	2·5
d-Ks	+/+	*Emt A*+/*Emt A*+ *Emt B*+/*Emt B*+	Mean	52 (N = 52)	77 (N = 51)	31
			$S_{\bar{y}}$	0·82	2·7	2·1
Em IV-3 (3S)	+/+	*Emt A*/*Emt A* *Emt B*/+*Emt B*+	Mean	33 (N = 33)	66 (N = 21)	32
			$S_{\bar{y}}$	0·85	1·8	1·7
Em IV-3 (4S)	+/+	*Emt A*/*Emt A* *Rmt B*+/*Emt B*+	Mean	31 (N = 25)	60 (N = 22)	29
			$S_{\bar{y}}$	1·5	3·1	3·0
Em III-2 (2S)	+/+	*Emt A*+/*Emt A*+ *Emt B*/*Emt B*	Mean	32 (N = 33)	57 (N = 24)	25
			$S_{\bar{y}}$	0·66	2·4	2·2

* Differences in the number of clones reflect the death of some clones before the initiation of mating-type instability.

† Standard error of the mean.

stelium discoideum, for example, morphogenetic events and enzymic changes in a regular sequential pattern, although these changes depend on changing culture conditions and are to a large extent reversible (Soll & Waddill, 1975; Loomis, White & Dimond, 1976; Loomis, Morrissey & Lee, 1978). Moreover, in *D. discoideum* most of these developmental events occur after mitosis, unlike those I have

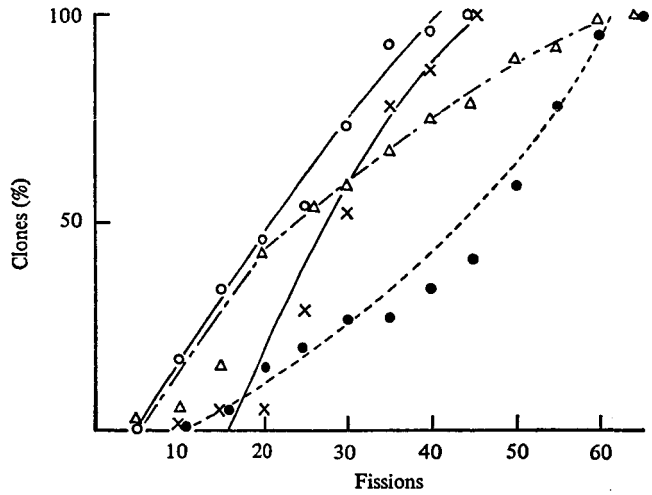

Fig. 3. Time of the first occurrence of mating-type change from the maturity. ——, Early maturing mutants; ○, selfing progeny of Em III-2S; ×, selfing progeny of Em IV-3(2S). –·–·–, Non-early mature clone, homozygotes at the mating-type locus; △ selfing progeny of d-Ks. – – –, Non-early-maturing clone, heterozygotes at the mating type locus; ●, d-KK 2*a* × Ky-2s 95a.

described here. In the previous paper (Myohara & Hiwatashi, 1975) we reported that selfing began to occur between 80 and 120 fissions after conjugation. Most of the clones used there were heterozygous at the mating-type locus. In this paper I report that mating-type change begins to occur between 50 and 100 fissions in dominant homozygotes at the mating-type locus. The difference between the homozygotes and the heterozygotes is significant.

In summary, (1) the time of the first occurrence of mating-type instability depends on the number of fissions from maturity rather than from conjugation; (2) in dominant homozygotes for the *mt* locus, mating-type instability begins earlier than in the heterozygotes. These results suggest that the expression of the dominant allele of the *mt* gene might influence the time of the first occurrence of mating-type change. Since isogenic lines at the mating-type locus were not used, the possibility that other loci affect the time of the mating-type change cannot be excluded.

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