MUTANTS OF SEXUAL MATURITY IN *PARAMECIUM CAUDATUM* SELECTED BY ERYTHROMYCIN RESISTANCE

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ABSTRACT

Cells of *Paramecium caudatum*, syngen 3 usually become sexually mature about 50 fissions after conjugation. In order to study the genetic mechanisms that control fission-dependent expression of maturity, an attempt was made to obtain early mature mutants by treatment with N-methyl-N'-nitro-N-nitrosoguanidine. A new cytoplasmic marker, erythromycin resistance, was used to eliminate nonconjugant and macronuclear regeneration clones. Twenty early mature clones were obtained from five different mutagenized cultures. Three of them were genetically analyzed by crosses to wild-type stocks. The results show all three mutants to be controlled by incompletely dominant genes, *i.e.*, the homozygotes became mature 20–25 fissions and the heterozygotes 15 fissions earlier than the wild-type clones. At least two different loci are suggested for the early maturity.

ONE of the basic concepts in metazoan development is sequential gene expression. In unicellular organisms such as the ciliated protozoa, it is known that expression of some genes is determined sequentially. For example, in *Paramecium bursaria*, the two mating-type loci are turned on sequentially in order for the final mature mating type to be expressed (SIEGEL and COHEN 1963). In Tetrahymena, esterase-1 and -2, serotypes H and T, phosphatase-1 and mating reactivity seem to appear in a predictable order (BLEYMAN 1971), although there are other interpretations of these data (DOERDER, LIEF and DEBAULT 1977). Thus, the ciliated protozoa may be useful as model systems for analysing the mechanisms controlling the timing of gene expression. The ready isolation of mutants and the ease of genetic analysis make the ciliates more suitable material for the above purpose than other systems, for example, the development of membrane antigens in mice (BOUBELÍK *et al.* 1975). Moreover, in unicellular organisms, there are few cellular interactions, which often make analysis more complicated in multicellular organisms.

In *Paramecium caudatum*, temporal regulation of gene expression has been observed in two traits, mating reactivity and mating-type instability. Mating reactivity appears about 50 fissions after conjugation (MIWA and HIWATASHI 1970; TAKAGI 1970), and mating-type instability begins about 100 fissions after conjugation (MYOHARA and HIWATASHI 1975). The appearance of mating reac-

tivity is known to be determined by the number of fissions, not by absolute time (MIWA and HIWATASHI 1970). In *Tetrahymena thermophila* (former *T. pyriformis*, syngen 1, NANNEY and MCCOY 1976), three early mature mutants, which express sexual maturity earlier than wild type, have been obtained by BLEYMAN (1971). These early mature mutants are dominant. At least two different loci of the early mature character are reported. This means that the length of the immature period in *T. thermophila* is in some way controlled by at least two different genes. In Paramecium, no early mature mutants have ever been reported. The purpose of this paper is to report the first early mature mutants discovered in *P. caudatum*. In *P. caudatum*, there are immaturity substances present in immature cells which, when injected into mature cells, repress their mating ability (MIWA, HAGA and HIWATASHI 1975). This technique, used with the early mature mutants, may help to elucidate the genetic control mechanisms of the immaturity period in ciliates.

MATERIALS AND METHODS

Stocks: All stocks and derived strains used for this study belong to syngen 3 of *Paramecium caudatum*. They are listed in Table 1.

Culture conditions: The culture conditions were the same as those of HIWATASHI (1968). The culture medium was sterlized lettuce juice inoculated with *Enterobacter aerogenes* one day before use. All cultures were maintained at 25° except during isolation procedures, which were done at room temperature.

Crosses: Cells of complementary mating types were mixed, and about six hours later conjugating pairs were isolated and transferred into exhausted culture medium. To prepare exhausted culture medium, ordinary cultures of paramecia were kept without the addition of food for more than a month at 25° and were then filtered through a millipore filter (0.45 μm). Exconjugants were isolated into exhausted culture medium one day after conjugation. The next day, each cell was transferred into fresh culture medium. These procedures were all done in

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 7a	VI	Descendants of a cross between Ky-s 95a and Ksy 1
d-KK 14a	V	
d-Kl 1	VI	
d-Kl 2	V	Descendants of a cross between d-KK 7a and d-KK 14a
d-Kl 3	VI	
d-Kl 4	V	Derived from a cross between d-KK 7a and d-KK 14a
MK 133	VI	Derived from crosses among four natural stocks; Ky, Ksy 1, Kok 1 and Koj

glass depression slides with 0.5 ml medium per depression. The cells were cultured in the same medium two additional days. Then these cells were transferred into test tubes that contained 2 ml fresh culture medium.

When the presumptive early mature clones were crossed, the exconjugants in the exhausted medium were placed in the culture medium for two days (as mentioned above), and then one cell from the eight to 16 descendant cells in a depression was transferred again into a depression containing fresh culture medium and cultured for two more days. Then, all the cells in a depression were transferred into a test tube. Thus, a test-tube culture consisted of a subcaryonidal clone, *i.e.*, a clone descended from the same developed macronucleus. This insured that a test-tube culture contained either exclusively normal exconjugant cells or cells that had undergone macronuclear regeneration (MR), not a mixture of the two. This is because MR cells are usually produced at the second or the third postzygotic division (MIKAMI and HIWATASHI 1975). MR clones usually are strongly mating reactive, while the early mature clones have only weak reactivity when it begins to appear. This makes it easy to distinguish true exconjugant clones, including the early mature clones, from MR clones. By this method, moreover, the percentage of MR clones is lower than is seen when all the cells from an exconjugant are inoculated into the same test tube because mixtures of MR and normal caryonides are classified as MR clones.

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 fourth day of this tube culture, the cells had undergone about 20 fissions since the exconjugant stage. About 1,000 cells were then transferred into a new test tube containing 2 ml fresh medium. This transfer was repeated until the appearance of mating reactivity. The number of fissions between transfers was estimated from the number of cells in the first inoculum (1,000 cells) and the total number of cells by the fourth day, and was found to be approximately five fissions. The total number of fissions until the appearance of mating reactivity 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.

Mutagenesis: Cells were treated with MNNG (N-methyl-N′-nitro-N-nitrosoguanidine, Nakarai, Inc.) for 20 minutes. MNNG was dissolved in Dryl's solution (DRYL 1959) and the final concentration was 20 μg/ml. About 2.4×10^4 cells were mutagenized. Mutagenized cells were washed twice with Dryl's solution by centrifugation and distributed into ten test tubes, each containing 4 ml of culture medium.

Theory of the screening for early mature mutants: Selection of early mature mutants involves selection of mating reactive cells soon after conjugation. However, these mating reactive cells also include MR cells and nonconjugant cells. Therefore, the early mature cells have to be selectively isolated. Before describing the screening method, it is necessary to describe the pattern of mating-type inheritance in *P. caudatum*, syngen 3. All clones from one conjugating pair are the same mating type. This implies that mating-type determination is under direct genic control. The recessive homozygote, *mt/mt*, determines mating type V and the dominant allele + permits the expression of mating type VI (HIWATASHI 1968). When a dominant homozygous clone +/+ is crossed to a mating type V clone, all the progeny are mating type VI and are heterozygous at the mating type locus (+/*mt*). If such progeny have a dominant early mature gene, the cells from the mating-type V cytoplasmic parent would show mating reactivity as mating type VI in a few fissions after conjugation, while the wild-type progeny would still be immature. MR or nonconjugant cells from the mating-type V parent would also be mating reactive but show mating-type V. If the cells from the mating type VI cytoplasmic parent can all be eliminated, the remaining cells would be vegetative or nonconjugant mating-type V cells, or those descended from V parent exconjugants including both immature cells and early mature mating-type VI cells. By this method, every early mature mutant is scored as a clone of mating-type VI. The same screening method can be applied when heterozygous mating-type VI is used. In this case, however, early mature clones among mating-type V segregants, which consist of half of the progeny, cannot be screened. The above method is accurate because there is no mating-

type change from V to VI during vegetative reproduction in *P. caudatum*. Mating-type instability occurs only in mating-type VI clones and never in mating-type V clones (MYOHARA and HIWATASHI 1975). To selection against the cells from the mating-type VI parent, we made use of the erythromycin sensitivity of wild type and erythromycin resistance in a mutant showing cytoplasmic inheritance.

Method for screening early mature mutants (Figure 1): The cells that were mating type VI and erythromycin sensitive (E^S) were treated with MNNG. Then, these cells were crossed to mating-type V and erythromycin resistant (E^R) cells. A few fissions after conjugation, cells

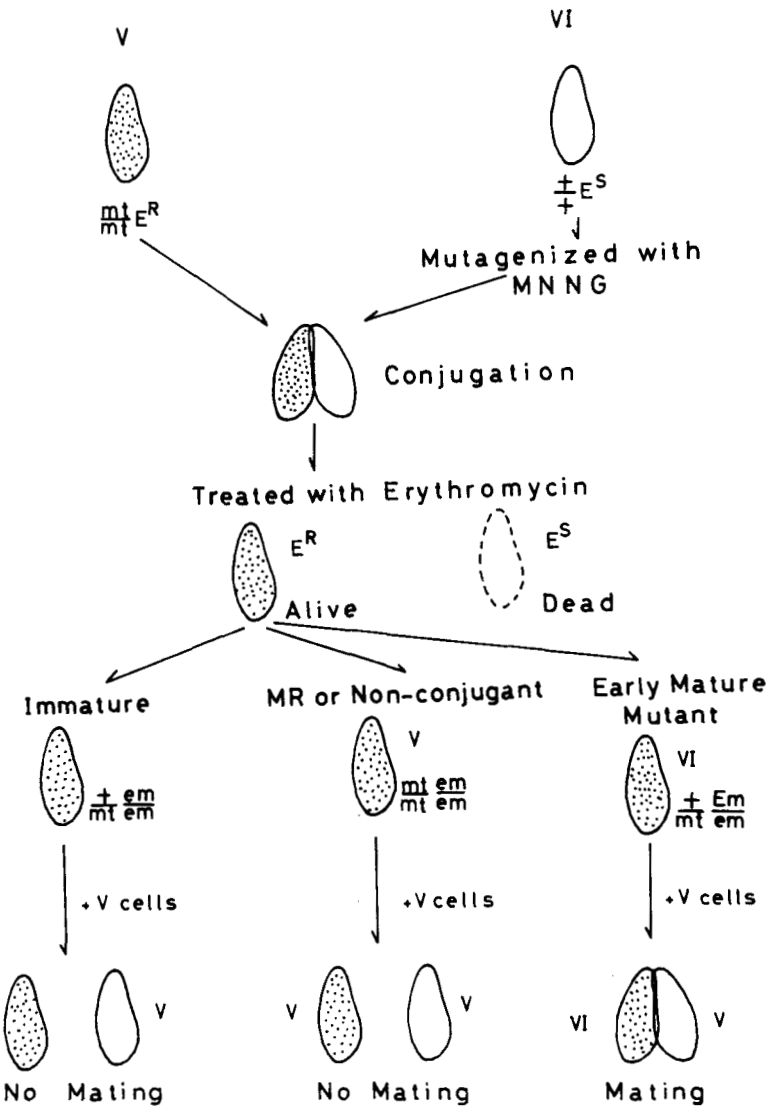

FIGURE 1.—The screening method for early mature mutants following mutagenization. E^R, erythromycin resistant; E^S, erythromycin sensitive; *mt*, mating type locus; *mt/mt*, mating-type V, +/+ and +/*mt*, mating-type VI.

were treated with 2 mg/ml erythromycin (erythromycin lactobionate, Abbott Lab.). After this treatment, all surviving cells were those from the E^R and mating-type V parent. The surviving cells were cultured for a few days by adding erythromycin-free fresh culture medium, and when they went into stationary phase, mating reactive cells of mating-type V were added. If clumps of two cells or holdfast union pairs were formed they were picked up. The pairs were split before union became firm and each cell was cultured to establish a clone. All such clones from split pairs were tested for mating type. If a clone was mating-type VI, it was judged to be an early mature mutant.

Isolation of E^R clone: The method of BEALE (1969) for isolation of E^R mutants in *P. aurelia* was used to establish the E^R clone. One ml of cell suspension (2,000 cells/ml) was added to 9 ml culture medium containing erythromycin to a final concentraiton of 2 mg/ml. Cell were kept in this medium for four to five days. When nearly all the cells were dead, 10 ml fresh culture medium was added. This process was repeated seven times.

Erythromycin resistance was tested as follows: A culture containing about 200 cells was divided and transferred into two different test tubes containing 10 ml culture medium, one with 0.2 mg/ml erythromycin and the other drug-free. The clones that divided normally in both media were judged to be the E^R clones, while the clones that divided only in the drug-free culture medium were the E^S clones. The E^R clones thus established continued to show the erythromycin resistance for at least 70 fissions in the drug-free culture medium.

RESULTS

Characterization of the E^R clone: The E^R clone was obtained from d-KK 14a, mating-type V, by the method described in the MATERIALS AND METHODS. This E^R clone can divide normally in culture medium containing 0.25 mg/ml, 0.5 mg/ml and 1.0 mg/ml erythromycin, while E^S clones stop dividing after one fission in the presence of 0.25 mg/ml erythromycin (Figure 2). In the presence of 2 mg/ml erythromycin, however, almost all E^R cells died by the fourth or fifth day of the drug treatment, although they could divide two or three times during this period (Figure 2). Death is delayed because of a difference in sensitivity between the cells in stationary and logarithmic growth phases. Cells enter the stationary phase after two or three fissions under the above conditions, and become more sensitive.

Inheritance of the E^R character: Erythromycin resistance in *P. aurelia* is caused by a mutation in the mitochondria, and the E^R character usually shows cytoplasmic inheritance (BEALE 1969; ADOUTTE and BEISSON 1970). To confirm this in *P. caudatum*, the following crosses were done. In the first cross, d-KK 14a (E^R) × d-Kl 1 (E^S), all 19 synclones (co-conjugant clones) showed cytoplasmic inheritance (Table 2). In the second cross, d-KK 14a (E^R) × d-KK 7a (E^S), eight of ten synclones had one E^R exconjugant and the other E^S. Two exceptional synclones that showed a coincidence of the character in the two exconjugants of a pair may have been selfing progeny, though cytoplasmic transfer between mates cannot be ruled out. Cytoplasmic exchange during conjugation occurs rarely as shown by other genetic analyses using the E^R clones (TAKAHASHI, personal communication). The E^R character in this clone was not lost even after 70 fissions in the erythromycin-free culture medium.

Isolation of early mature mutants using the E^R stock: Cells of d-KK 7a, mating-type VI and E^S, were mutagenized. d-KK 7a is heterozygous for the mating-type

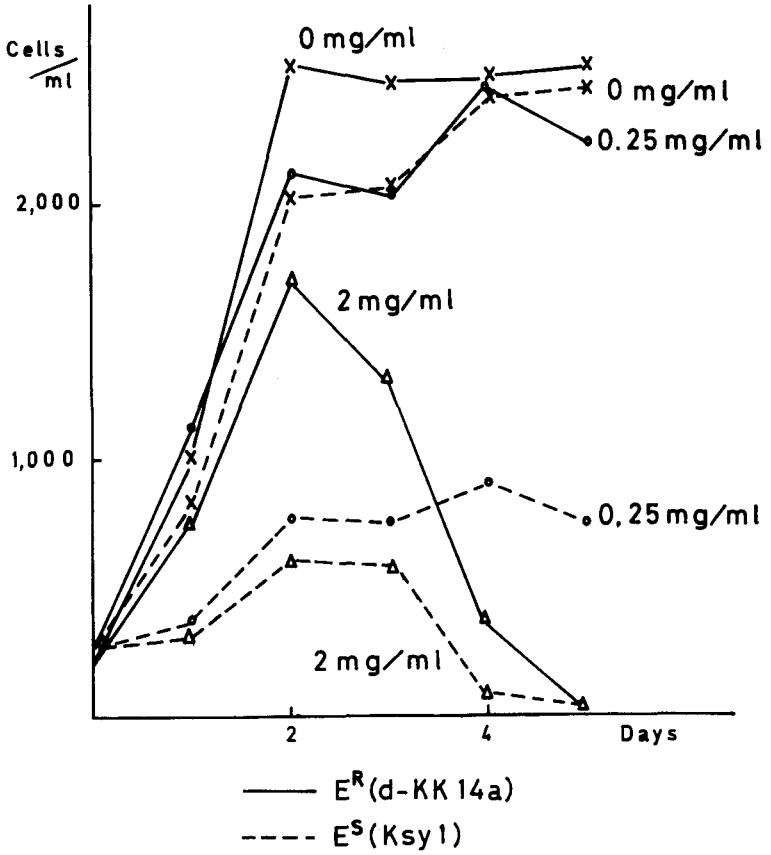

FIGURE 2.—Growth of cells in erythromycin media. Abscissa: time (days) after transfer to erythromycin media; ordinate: number of cells. Solid lines indicate the growth of E^R (d-KK 14a) cells. Dashed lines indicate the growth of E^S (Ksy 1) cells.

alleles. Each 4 ml culture of mutagenized cells was mixed with 2 ml mating reactive culture of d-KK 14a, mating-type V and E^R. After the occurrence of conjugation, the cells were left for three days in the same test tube, and then 10 ml drug-free culture medium was added and the cells were kept for two more

TABLE 2

Inheritance of erythromycin resistance

Cross	Mating type of two exconjugant progeny* V R/R	V S/R	V S/S	VI R/R	VI S/R	VI S/S
d-KK 14a (E^R, V) × d-Kl 1† (E^S, VI)	0	10	0	0	9	0
d-KK 14a (E^R, V) × d-KK 7a† (E^S, VI)	1	4	1	0	4	0

The figures indicate the number of F_1 synclones.

* R/R: both sister clones resistant; S/S: both sister clones sensitive; S/R: one of the sister clones resistant, the other sensitive.

† Heterozygous for mating type alleles.

days. This second step ensures that the remaining mutagenized cells conjugate. Then the cells were collected by centrifugation, transferred into 10 ml culture medium containing 2 mg/ml erythromycin, and left for three days without changing the medium. At the third day, the cells still alive were collected by centrifugation and transferred into newly prepared culture medium containing 2 mg/ml erythromycin. In the fresh medium containing erythromycin, only E^R cells could divide again (Figure 3). This shows that the effect of erythromycin on the cells in the stationary phase is nonspecific, but the effect on those in the

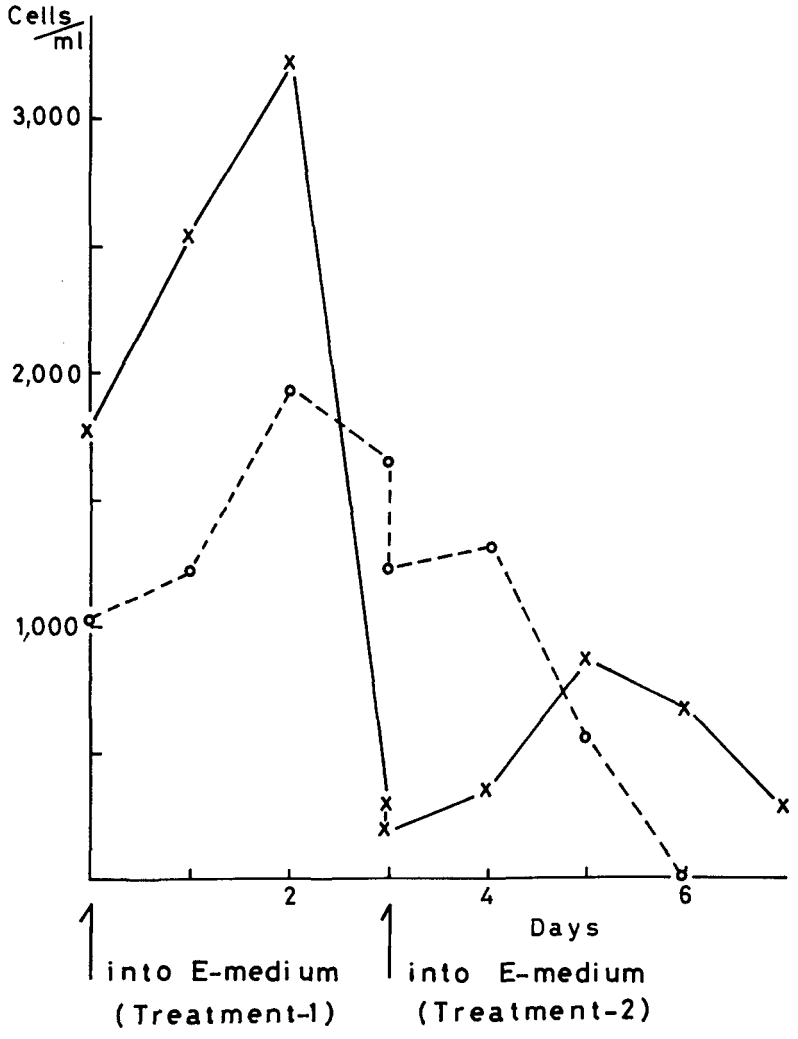

FIGURE 3.—Change of cell number when transferred to the fresh erythromycin medium (E-medium). Abscissa: time (days) in the erythromycin medium; ordinate: number of cells. Solid line is a mixture from mating $E^R \times E^S$ cells. Dashed line indicates the E^S cells of d-KK 7a (control).

growth phase is specific for E^S cells. The cells were cultured for four days in this medium. In order to confirm that the above treatment completely eliminated the E^S cells, E^S cells of d-KK 7a were similarly treated with erythromycin-containing culture medium. All E^S cells died at the sixth day, though in the experiment some E^R cells were still alive even at the eighth day of the treatment. There seems little possibility that E^S cells receive some E^R mitochondria during conjugation and survive, because massive exchange of cytoplasm does not occur during conjugation in this species (HIWATASHI 1967), and even if a few mitochondria are transferred from the E^R cells to the E^S cells, the latter will not survive in the high concentration of erythromycin used in this experiment. By the third day of treatment, about 90% of the cells from the mating mixture were dead (Figure 3). This may be due partially to low survival after conjugation in this cross. The viability of the cross between d-KK 14a and the MNNG treated d-KK 7a was about 17%, while that between d-KK 14a and nonmutagenized d-KK 7a was about 60%.

When the second erythromycin treatment was finished, 10 ml of erythromycin-free fresh culture medium was added. The next day, 10 ml of the culture was transferred to a new test tube and 10 ml of fresh culture medium was added (the second culture). This was repeated several times. Mating reactive d-KK 14a cells were added to the culture remaining in the previous tube (left over culture), and mating pairs were sought. If pairs were found, they were split by uptake into a micropipette and squirting back into the medium. Each cell of the split pairs were cultured in a test tube, and mating type tested. If a cell was mating-type VI, it was judged to be an early mature mutant (see MATERIALS AND METHODS). If mating pairs were not found, the second culture was checked in the same way. This test was repeated several times. Twenty clones that were judged to be early mature mutants were obtained from five different mutagenized cultures. All of them became sexually mature 10 to 15 fissions earlier than wild type. The number of mutagenized cells was about 2.4×10^4 and the viability of the cross after the mutagenesis was 17%. If we assume that all the above mutant clones were derived from different mutagenized cells, nearly 1/200 clones were early mature mutants. This estimation of the mutation rate may be a little too high because there is a possibility that some of the 20 early mature mutants are derived from single mutagenized cells.

Inheritance of early maturity: One of the early mature mutants that was obtained by the above mentioned methods, Em IV-3 (E^R and mating-type VI) was crossed to d-KK 14a (E^S and mating-type V). Forty-two pairs were isolated; 12 synclones were viable true progeny (29%) and 15 synclones were MR. Clones of MR were judged by erythromycin resistance, strength of the mating reaction and mating-type instability. MR clones show the same mating type as their cytoplasmic parent and usually have strong mating reactivity immediately after conjugation. If they are mating-type VI, they are also immediately unstable. In crosses between wild-type clones, very few progeny become mature earlier than 40 fissions. Therefore, 40 fissions was used as the criterion to distinguish

early mature and normal clones. The clones becoming mature earlier than 40 fissions are designated as "early", and those becoming mature later than 40 fissions as "late." In the true progeny from this cross, the ratio of early to late was 3:9. One of these early clones was testcrossed to d-KK 7a. Among 126 pairs isolated, 59 clones (47%) were viable true progeny and 13 were MR. The ratio of early to late was 29:30 (Table 3).

In the next experiment, a sibling cross was done to obtain the homozygote. Two early clones from the testcross progeny were crossed. One was mating-type VI and E^R, and the other was mating-type V and E^S. Among 126 pairs isolated, 33 clones were viable true progeny (26%) and 17 were MR. The ratio of early to late was 28:5. Six of these early clones became mature by 25 fissions. These very early clones may be homozygous for the Em gene. One of them, Em IV-3S 27a, was chosen to test this theory by isolation of progeny resulting from selfing in this clone. Out of 504 pairs isolated, 30 clones were MR and 86 (17%) were true progeny. The ratio of early to late was 48:38. This result showed that Em IV-3S 27a was not homozygous for the *Em* gene. It is most likely heterozygous despite the excess of late mature progeny. The reason for the excess of late mature progeny in this cross is probably technical. In the previous crosses one caryonidal

TABLE 3

Inheritance of early maturity in Em IV-3

Cross	Genotype		Number of synclones matured Early	Late	Mating type VI	: V	Survival* (%)
Em IV-3 × d-KK 14a	+/*mt*	*mt/mt*	3	9	6	6	29
	×		(6:6)				
	Em/em	*em/em*	χ^2=0.71	0.5>*P*>0.3			
F_1-Em × d-KK 7a	*mt/mt*	+/*mt*	29	30	30	29	47
	×		(29.5:29.5)				
	Em/em	*em/em*	χ^2=0.085	0.8>*P*>0.7			
(Testcross)							
Sibling cross between	+/*mt*	*mt/mt*	28	5	17	16	26
	×		(24.7:8.3)				
	Em/em	*Em/em*	χ^2=0.45	*P*=0.5			
F_1 progenies of the testcross							
Selfing of Em IV-3S 27a†	+/*mt*	+/*mt*	48	38	66	20	17
	×		(64.5:21.5)				
	Em/em	*Em/em*	χ^2=6.2	0.02>*P*>0.01			
Selfing of Em IV-3SS 74†	+/*mt*	+/*mt*	30	1	28	3	9
	×		(31:0)				
	Em/Em	*Em/Em*	χ^2=0				

For the calculation of χ^2 values, Yates' correction was applied.
* Percent of viable true progeny (excludes MR and nonconjugant clones).
† Only one clone from a synclone was expanded and tested.

clone each from the two exconjugants of a pair was isolated, and when at least one of them became mature, the synclone was judged to be mature. But in this cross, only one caryonide was isolated from one pair. This reduction in sampling may result in an apparent longer average immaturity than when two caryonidal clones are isolated. Hence, with this protocol there is much overlap between early and late clones at 40 fissions.

Homozygotes should be contained in the selfing progeny of Em IV-3S 27a. Among the progeny, Em IV-3SS 74 was chosen and its selfing pairs were isolated. Among 336 pairs isolated, 47 clones were MR and 31 were true progeny (9%). The ratio of early to late was 30:1. This result is clearly different from that shown by selfing Em IV-3S 27a, and suggests that Em IV-3SS 74 is homozygous.

A comparison of the onset of maturity between the Em homozygote, heterozygote and the wild type was done. The homozygote, Em IV-3SS 74, was crossed to two different wild-type clones, d-KK 14a and d-Kl 2 to produce heterozygous progeny. A cross between two wild-type clones, d-KK 14a and d-Kl 3, was also made. The appearance of maturity in progeny from the above crosses is shown, together with that of selfing progeny of the homozygote, in Figure 4. In all crosses, only one caryonide was isolated from one pair. As can be seen, homozygotes for the *Em* gene become mature about 20 fissions earlier than the wild type (50% maturity point). The heterozygotes become mature about 15 fissions

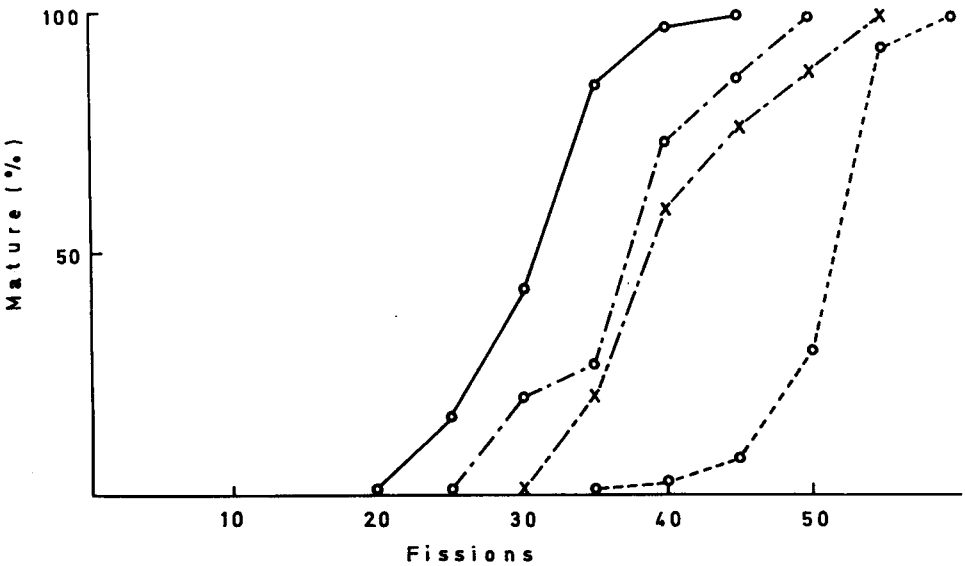

FIGURE 4.—Maturation curves of homozygotes and heterozygotes for the *Em-1* gene and of wild-type clones. Abscissa: fissions after conjugations; ordinate: percentage of mature clones. (O——————O) Selfing of Em IV-3SS 74 (*Em/Em*), *N* (total number of clones) = 31; (O— - — - — - —O) Em IV-3SS 74 × d-KK 14a (*Em/em*), *N* = 15; (X- - — - — - —X) Em IV-3SS 74 × d-Kl 2 (*Em/em*), *N* = 41; (O- - - - - - - - - - -O) d-Kl 3 × d-KK 14a (*em/em*), *N* = 30.

earlier than wild type and are intermediate in their behavior between wild-type and homozygous clones.

These results suggest that early maturity is controlled by a incompletely dominant gene, which is designated *Em-1*. Since early maturity appears independently of mating types, *Em-1* seems not linked to the mating-type locus. It is worthy of special mention that the early mature cells, even as early as 25 fissions after conjugation, underwent normal meiosis and produced normal progeny.

The same kind of genetic analysis was made with another early mature mutant, Em III-2. As seen in Table 4, the results were almost the same as those of Em IV-3 and again suggested control of early maturity by a dominant gene. In order to find out whether dominance is complete or incomplete, the homozygote, E mIII-2S 11, was crossed to a wild-type clone, d-Kl 4, to produce heterozygous progeny, and the appearance of maturity in the progeny was compared with those in progenies from the wild-type cross (d-Kl × d-KK 14a) and from selfing of the homozygote. Essentially the same pattern of maturation curves as observed in Figure 4 was obtained (Figure 5), suggesting that dominance is incomplete. Since phenotype and genetic control are so much alike in Em IV-3

TABLE 4

Inheritance of early maturity in Em III-2

Cross	Genotype		Number of synclones matured Early	Late	Mating type VI	V	Survival* (%)
Em III-2 × d-KK 14a	+/*mt*	*mt/mt*	9	30	22	17	31
	×		(19.5:19.5)				
	Em/em	*em/em*	χ^2=5.0	0.05>*P*>0.02			
F_1-Em × MK 133	*mt/mt*	+/+	10	11	21	0	50
	×		(10.5:10.5)				
	Em/em	*em/em*	χ^2=0.02	0.9>*P*>0.8			
(Testcross I)							
Progeny of testcross I ×	+/*mt*	*mt/mt*	10	5	5	10	36
	×		(7.5:7.5)				
	Em/em	*em/em*	χ^2=0.3	0.7>*P*>0.5			
d-Kl 4† (Testcross II)							
Selfing of testcross II	+/*mt*	+/*mt*	26	6	26	6	25
	×		(24:8)				
	Em/em	*Em/em*	χ^2=0.09	0.8>*P*>0.7			
progeny (= Em III-2S)†							
Selfing of Em III-2S 11†	+/+	+/+	33	0	33	0	11
	×		(33:0)				
	Em/Em	*Em/Em*	χ^2=0				

For the calculation of χ^2 values, Yates' correction was applied.
* Percent of viable true progeny (excludes MR and nonconjugant clones).
† Only one clone from a synclone was expanded and tested.

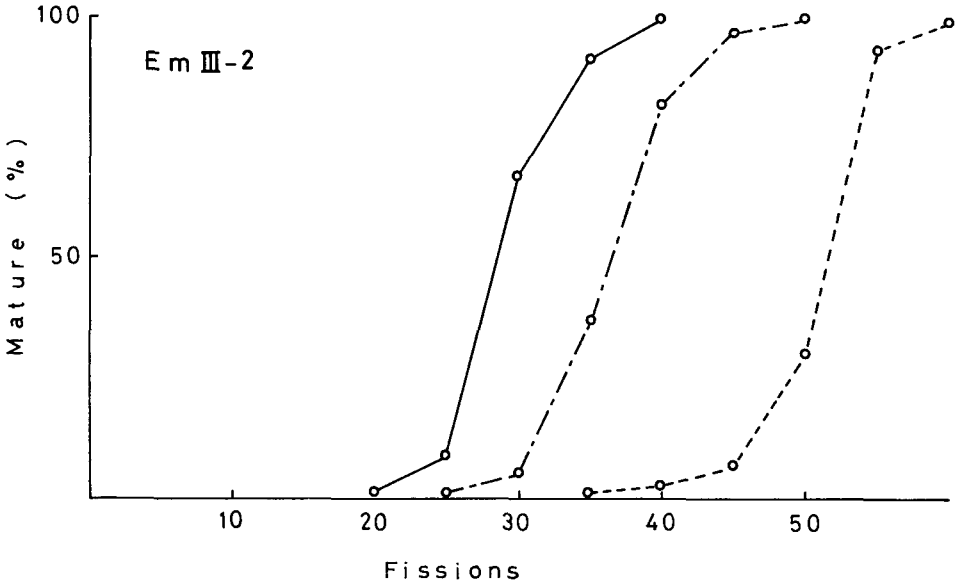

FIGURE 5.—Maturation curves of homozygotes and heterozygotes for the *Em-2* gene and of wild-type clones. Abscissa: fissions after conjugations; ordinate: percentage of mature clones. (O————O) Selfing of Em III-2S 11 (*Em/Em*), *N* (total number of clones) = 33; (O- - —- - - -O) Em III-2S 11 × d-Kl 4 (*Em/em*), *N* = 42; (O- - - - - - - - - -O) d-Kl 3 × d-KK 14a (*em/em*), *N* = 30.

TABLE 5

Inheritance of early maturity in Em I-2

Cross	Genotype		Number of synclones matured Early	Late	Mating type VI	: V	Survival* (%)
Em I-2 × d-KK 14a	+/*mt*	*mt/mt*	3	4	4	3	21
	×		(3.5:3.5)				
	Em/em	*em/em*	χ^2=0.07	0.8>*P*>0.7			
F_1-Em × d-KK 14a	+/*mt*	*mt/mt*	14	7	13	8	21
	×		(10.5:10.5)				
	Em/em	*em/em*	χ^2=0.61	0.5>*P*>0.3			
(Backcross)							
F_1-Em × d-KK 7a	*mt/mt*	+/*mt*	30	50	42	38	71
	×		(40:40)				
	Em/em	*em/em*	χ^2=2.1	0.2>*P*>0.1			
(Testcross)							
Sibling cross between	+/*mt*	*mt/mt*	17	7	16	8	22
	×		(16:8)				
	Em/em	*Em/em*	χ^2=0				
Em I-2 testcross							

For the calculation of χ^2 values, Yates' correction was applied.
* Percent of viable true progeny (excludes MR and nonconjugant clones).

and Em III-2, a series of crosses using homozygotes of both mutants has been performed to investigate the relation of the mutants to each other. Though the experiments are still going on, preliminary results showed that Em III-2 belongs to a locus different from *Em-1*. They also suggested that the effects of both mutants are additive, *i.e.*, double homozygotes mature earlier than either of the single homozygotes. The second early maturity gene is designated *Em-2*.

The third early mature mutant, Em I-2, was also genetically analyzed. The results of the crosses are shown in Table 5. They suggest that early maturity in this mutant is also controlled by a dominant gene. Homozygotes were looked for in the early clones from the sibling cross of heterozygotes (last line of Table 5). Every effort to find homozygotes failed. Every time we found some viable true progeny after selfing of the early clone, they contained both early and late clones. The low survival (22%) of the sibling cross of heterozygotes makes interpretation of the segregation ratio (17:7) difficult, though it is not significantly different from the ratio of 2:1, which is expected when homozygotes are lethal. In the second and third mutants, Em III-2 and Em I-2, early maturity appears independently of mating types. Thus, it suggests that these two mutants are also unlinked to the mating type locus, as was the first mutant, Em IV-3.

DISCUSSION

The first early mature mutants in ciliates were discovered in *Tetrahymena thermophila* (BLEYMAN and SIMON 1967; BLEYMAN 1971). This ciliate has an immature period of 50 to 100 fissions after conjugation. The early mature mutants discovered by BLEYMAN showed sexual maturity as early as 10 to 15 fissions after conjugation. Mutational alterations to early maturity in Tetrahymena are located in at least two different loci. All the mutations are dominant and three of them are lethal when homozygous. None of them is linked to the mating-type locus. Many of these features known in the early mature mutants in Tetrahymena were also seen in the early mature mutants in *Paramecium caudatum*. The early mature mutants in *P. caudatum* analyzed genetically in this study are also controlled by dominant genes and are not linked to the mating-type locus. Two of them are viable in homozygous states and in this respect resemble *Em-2* in *T. thermophila*. The third one appears to be lethal in the homozygous state like *Em-1*, *Em-3* and *Em-4* in *T. thermophila* (BLEYMAN 1971, 1972). BLEYMAN (1971) reported that the early mature mutants in *T. thermophila* showed markedly reduced growth rate and erratic division during the period immediately following conjugation. All early mature mutants isolated in *P. caudatum*, however, grow almost at the same fission rate as the wild-type clones from the time immediately after conjugation.

In this study, a new method for the isolation of early mature mutants using erythromycin resistance and a mating type marker was devised. This method should also select mutants that totally lack an immaturity period. As described in the RESULTS, 20 early mature clones were obtained from five different mutagenized cultures, but no immatureless mutants were discovered. The reason why

they were not obtained is unknown. However, the discovery of mutants having shorter immaturity periods suggests that there are at least two different stages in the period of immaturity, an early stage for which there are no mutants known as yet, and a later stage affected by the mutations described.

Recently, NANNEY and MEYER (1977) reported that phenocopies of early mature mutants in *T. thermophila* are readily obtained by treatment of late conjugants with high temperature or high concentration of $CaCl_2$. They called the phenomena traumatic induction of early maturity because adverse environmental conditions encountered during macronuclear development induce a precocious maturity. They consider that the genotypic early mature mutants have a certain association with the environmentally induced phenocopies from an evolutionary point of view. In *P. caudatum*, nongenetic early maturity is known to be brought about by treatment of cells in the immaturity period with mitomycin C (MIWA and HIWATASHI 1970) or bromodeoxyuridine (HAGA and HIWATASHI, unpublished). Both reagents are well-known for their traumatic effects on DNA. Whether the genotypically controlled and traumatically induced early maturity have any common mechanism will be an interesting problem for future study.

In *P. caudatum* syngen 3, MIWA and HIWATASHI (1970) reported that the length of the immaturity period is different in different mating types. In the early mature mutants, as well as in the wild types used in the present study, however, no difference in the length of immaturity period associated with mating type was observed. This can probably be attributed to genetic differences between stocks used. In *P. multimicronucleatum*, TAKAGI (1971) reported that during the development of sexual maturity, cells became capable of ciliary mating agglutination more than ten fissions earlier than they became capable of true conjugation union. As mentioned in RESULTS, the early mature mutants in *P. caudatum* became capable not only of mating agglutination but also of true conjugating union leading to meiosis and nuclear reorganization as early as 25 fissions after conjugation. This fact makes the early mature mutant a very convenient material for genetic studies in *P. caudatum* by shortening the length of one generation almost into a half.

Recently, MIWA, HAGA and HIWATASHI (1975) reported that cells of *P. caudatum* in the immaturity period contain substances that repress the expression of mating ability when introduced into mature cells by microinjection. If the early mature mutants are analyzed with this technique and compared with wild-type cells, the action of the genes controlling the immaturity period may be characterized. This line of analysis is now under way, together with genetic analyses of the other early mature mutants reported here.

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