Genet. Res., Camb. (1973), **22**, *pp.* 135–141
Printed in Great Britain

Further studies on the maintenance of mate-killer (mu) particles in Stock 540 *Paramecium aurelia*

By J. FRANKLIN

*School of Biological Sciences, University of East Anglia, Norwich, NOR 88C**

(*Received* 27 *December* 1972)

SUMMARY

1. Mu particles can be removed from stock 540 *Paramecium aurelia* by incubation in a solution of 1·0 mg/ml RNAase for 12 h. Only a relatively small percentage of cells become mu-free and it is suggested that this variation is due to the activity of the M genes producing metagons at different times.

2. Paramecia, made mu-free by growth at elevated temperatures, can be re-infected with mu by allowing the cells to swim in a brei of stock 540 paramecia. The re-infection is only permanently successful with cells that were made mu-free 10 days earlier.

1. INTRODUCTION

Mu particles, responsible for the phenomenon of mate-killing in *Paramecium aurelia* (Beale, 1957), are only maintained in stock 540 paramecia that contain at least one of two macronuclear genes denoted M1 and M2 (Gibson & Beale, 1961, 1962). A series of experiments showed that the actual maintenance of the mu depended on a gene product, denoted a metagon, that appeared to be stable, made entirely, or to a large degree, of RNA, and was able to replicate in another organism – *Didinium nasatum* (Gibson & Beale, 1963, 1964; Gibson & Sonneborn, 1964; Gibson, 1965). The possible importance of this postulated stable messenger RNA, with virus-like properties, led many authors to investigate other symbiont systems for metagon activity (Yeung, 1965; Widmayer, 1966; Beale & McPhail, 1967; Byrne, 1969). However, few of these results showed any similarity to those in the original experiments (Gibson & Beale, 1961, 1962, 1963) and, after an exhaustive enquiry, Byrne (1969) proposed that instead of making *ad hoc* assumptions other explanations should be sought to account for the variability that had been found.

During a series of experiments designed to investigate some general aspects of the biology of mu (Franklin, 1971), certain results showed that the situation regarding the metagon was not so defined as either Gibson and Beale (1961, 1962, 1963) or Byrne (1969) reported. Further experiments indicated that the metagon might

* Present address: Department of Science, Cambridgeshire College of Arts and Technology, Collier Road, Cambridge, CB1 2AJ.

not be the sole factor in the maintenance of mu particles, and so the whole concept of the maintenance of mu in paramecia may have to be re-examined.

2. MATERIALS AND METHODS

Paramecium aurelia, stock 540 containing M1 and M2 genes and mu particles, were used throughout these experiments. They were kindly supplied by Dr Ian Gibson, School of Biological Sciences, University of East Anglia, Norwich NOR 88C. The paramecia were cultured in depression slides, test-tubes, and Thompson bottles, containing a grass infusion broth inocculated 24 h before use with *Aerobacter aerogenes* (Sonneborn, 1950). The medium was adjusted to pH 6·8–7·0 with 1 N-NaOH before use. The cultures were grown at 27 °C.

(i) *Treatment with ribonuclease*

Paramecia were treated with RNAase (A) (Worthington) by placing them in a depression slide of Dryl's solution (Dryl, 1959) containing 1·0 mg/ml RNAase (the activity of the RNAase was checked by its ability to remove pyronin positive material from the cytoplasm of paramecia). The paramecia were left in the enzyme solution for 12 h at 27 °C. Single cells were then isolated into three spot depression slides of bacterised medium and incubated at 27 °C. Paramecia were checked for the presence of mu by staining with lacto-orcein following fixation by osmium tetroxide vapour (Beale & Jurand, 1966). This technique shows mu as purple rod-shaped inclusions in the cytoplasm of paramecia. It has been shown (Franklin, 1971) that mu are more easily detected by this staining method than when the squash technique (Gibson & Beale, 1961, 1962) is used.

(ii) *Infection of mu into mu-free paramecia*

Breis of mu-bearing stock 540 paramecia were made by concentrating 3 l of paramecia, grown for 1 week at 27 °C in Thompson bottles, to 10 ml of wet-packed cells using an Alfa-Laval Cream Separator and an M.S.E. Oil Testing centrifuge. The cells were broken by forcing them through a No. 1 hypodermic needle into a small beaker placed in an ice bath. Checks were made with a stereo-microscope to ensure that all of the cells were disrupted, and then the brei was stored at 4 °C. No brei was stored for longer than 2 h before the infection experiments were started.

Paramecia were made mu-free by incubating them at 37 °C for 60 h and then growing them at 3–4 fpd at 33 °C for at least 9 fissions (Beale, 1957; Franklin, 1971). The mu-free cells were then grown at 2–3 fpd at 27 °C in depression slides of bacterized medium. Fifty mu-free paramecia were placed, two in each depression, in depression slides containing various ratios of brei and Dryl's solution. After 24 h the paramecia were isolated and placed in a 1:1 mixture of Dryl's solution: bacterized medium. After a further 24 h, during which the paramecia completed 1 fission, some of the paramecia were examined for the presence of mu. This point was referred to as point A. Other cells were isolated from the culture and allowed

to grow in fresh bacterized medium for a further 48 h. Finally, each clone of cells was placed in separate test-tubes of bacterized medium and left for 1 weeks growth at 27 °C. This point was referred to as point B, and checks were again made on the paramecia for the presence of mu by randomly isolating samples of paramecia and staining them with lacto-orcein.

3. RESULTS

(i) *The effect of ribonuclease treatment on the maintenance of mu in paramecia*

Forty per cent of the treated paramecia survived. These cells began to divide 6–12 h after they were removed from the RNAase and no lag period, as noted by Gibson & Beale (1963), was noted. The isolated cells were allowed to divide twice and then the resulting clones were examined for mu by lacto-orcein staining. Of the 33 clones examined, 26 clones contained cells that were all mu-bearing, 3 contained 2 mu-bearing and 2 mu-free cells, and 4 contained cells that were all mu-free. Further checks, on freshly treated cells, showed that the paramecia that lost their mu did so soon after their first post-RNAase division. These mu-free cells did not regain mu after continued culture for 18 months at 27 °C in bacterized medium, so it can be concluded that the loss was permanent.

No morphological changes occurred in the mu during the RNAase treatment. Control paramecia, untreated by RNAase but otherwise exposed to the same conditions, suffered no loss of mu.

(ii) *The re-infection of mu into mu-free paramecia*

The results in Table 1 show the attempted re-infection of mu into paramecia that had been made mu-free 10, 17 and 24 days earlier by growing the cells at elevated temperatures. It is apparent that although mu entered the cytoplasm of the cells

Table 1. *The re-infection of mu into mu-free paramecia*

Time since loss of mu (days)	Ratio of brei:Dryl's	No. clones isolated	No. clones infected at	
			Point A	Point B
10	33:66	15	12	6
	66:33	15	9	3
	0:100	15	0	0
17	33:66	15	7	0
	66:33	15	6	0
24	33:66	15	6	0
	66:33	15	6	0

in each sample exposed to the brei (point A, Table 1), only those paramecia that had been made mu-free 10 days previously became permanently mu-bearing (point B, Table 1). Checks were made at regular intervals over the next 18 months on the cells that had been infected at point B (Table 1), and all of these clones

continued to maintain mu. Individual mu-bearing paramecia (obtained by allowing a cell at point B to divide, testing one of the daughter cells for mu, and, if mu were present, using the other cell) also gave rise to mu-bearing clones. Thus the infection appears to be permanent.

4. GENERAL DISCUSSION

The concentration, and types, of RNAase used in the experiments in section 1 above were chosen in order to repeat, as far as possible, the experiments of Gibson & Beale (1963). These authors had shown that such treatment removed a large amount of the cytoplasmic RNA from the host paramecium (Jurand, Gibson & Beale, 1962) and that the loss of mu was correlated to the breakdown of this RNA. Further experiments, concerning the isolation and characterization of a subcellular fraction from mu-bearing paramecia which contained metagon activity (Gibson & Beale, 1964), confirmed that metagon activity was sensitive to RNAase and it was concluded that the metagon represented a stable mRNA (Gibson & Beale, 1964). However, Byrne (1969) failed to repeat many of the results obtained by Gibson & Beale (1961, 1962, 1963), in particular failing to produce any loss of mu from paramecia following treatment with RNAase. She concluded that RNA was therefore not an essential component of the metagon.

It is apparent that a range of results, from 0% loss to 100% loss of mu can be obtained during the same experimental treatment of paramecia with RNAase. These results, and those of Gibson & Beale (1963) and Byrne (1969), can all be explained by a simple modification of the metagon hypothesis which assumes that the M genes can be switched on and off. If the M genes were inactive at the time of RNAase treatment, no new metagons could be produced and the existing metagons in the paramecia might be destroyed. Thus the mu particles would be lost before any new metagons could be produced. (Gibson & Beale (1963) reported that new metagons were produced in RNAase treated cells 2–3 fissions after RNAase digestion, but the mu were lost after the first post-RNAase fission.) However, if the M genes were active at the time the RNAase treatment occurred, although the existing metagons might be destroyed, sufficient new metagons might be produced between the end of the RNAase treatment and the division of the paramecia for the mu to survive. These (hypothetical) metagons would then be distributed to daughter paramecia and so the resulting clones of cells would all be mu-bearing. If these new metagons were only distributed to one of the daughter paramecia at the first post-RNAase division, the cell receiving the metagons would, presumably, give rise to a mu-bearing line of cells while the paramecium with no metagons would lose its mu. Thus this hypothesis also provides a possible mechanism for the production of the clones where half of the cells are mu-bearing and the other half mu-free. As noted above, this modification to the metagon hypothesis depends upon the method whereby the numbers of metagons are kept constant in any one paramecium (Gibson & Beale, 1962; Reeve & Ross, 1963). However, as the metagon is reputedly a gene product, it seems probable that such control would be linked to the activity of the M genes.

Another possible explanation for the range in results shown in section 1, also based on the metagon hypothesis, is that some of the metagons escape damage by the RNAase. It seems probable that if all the cellular RNA in the paramecia was destroyed by this treatment the cell would die, but, as many survive, it is reasonable to conclude that some cellular RNA escapes damage. Some of this RNA could be metagon RNA. Thus the results of Gibson & Beale (1963) could be explained by none of the metagons surviving, those of Byrne (1969) by all or some surviving, and those in section 1 by none, a few, or at least one, to be distributed to one daughter paramecium, surviving.

Although neither explanation can be conclusively verified at the present time, it is apparent that the metagon hypothesis need not necessarily be abandoned on the basis of the contradictory results obtained by Gibson & Beale (1963) and Byrne (1969).

The results in Table 1 show that paramecia, made mu-free by growth at elevated temperatures, can be re-infected with mu. It is apparent that the length of time the paramecia have been mu-free prior to the infection is important. Successful re-infection was only obtained with paramecia that had been made mu-free 10 days previously, and no infection was obtained with cells that had mu-free for longer periods. It appears that some factor(s), necessary for the establishment or maintenance of the mu, is lost from the host cells after they become mu-free. The obvious candidate for this role is the metagon. However, Gibson & Beale (1963) reported that metagons are produced in paramecia 2–3 fissions after they have lost their mu due to RNAase treatment. Furthermore, as the action of elevated growth temperatures in producing mu-free paramecia appears to be directed at the mu rather than the metagons (visual data and the kinetics of loss following such treatments as elevated temperatures strongly indicate that the metagons are undamaged; Franklin, 1971), it would appear that there are sufficient metagons in the cell to allow the survival of the mu. Therefore some additional factor is needed for the survival of the mu in paramecia. It seems that this postulated factor is involved with the establishment of the mu in the cytoplasm of the paramecia for, although some cells in each experiment initially took up mu (point A, table 1), the mu only became permanently established in paramecia made mu-free 10 days previously (and not when longer time periods had elapsed).

The maintenance of kappa particles in paramecia is known to be affected by at least 3 genes – K, S 1 and S 2 (Sonneborn, 1959; Balbinder, 1957, 1959). The mode of action of the S genes is unknown at the present time, but different permutations of K, S 1 and S 2, and their alleles k, s 1 and s 2 show differing abilities to influence the maintenance of the kappa. Whether these genes act directly on the kappa or indirectly on the host cell cannot be concluded from the available evidence. If similar genes are present in the mu system, it may explain why different kinetics of loss, of mu particles from paramecia, have been found by the various authors investigating these problems. Different combinations of genes might produce differing cytoplasmic states which vary in their tolerance to mu particles. Thus it is possible that the metagon is only one of a number of cytoplasmic factors needed for the survival of mu.

The possibility that a number of genes are necessary for the survival of mu in paramecia is consistent with the theory put forward by many authors that mu is on an 'evolutionary journey' from being a free-living prokaryote to becoming an integrated cell organelle (Ball, 1967; Preer, 1967; Gibson, Williams & Chance, 1971; Franklin, 1973). On such a postulated journey it would be conceivable that the mu would become increasingly dependant on the host cell's genome. Thus the appearance of an increasing number of host genes is consistent with this hypothesis.

The author wishes to thank Dr I. Gibson and Dr D. Wildon for their help during this work and the Medical Research Council for financial support.

REFERENCES

BALBINDER, E. (1957). Two loci controlling the maintenance and stability of the cytoplasmic factor 'kappa' in stock 51, var. 4, killers of *Paramecium aurelia*. *Genetics* **31**, 634.

BALBINDER, E. (1959). The genetic control of kappa in *Paramecium aurelia* syngen 4, stock 51. *Genetics* **44**, 1227–1241.

BALL, G. H. (1967). Organisms living on and in protozoa. In *Research in Protozoology*, Vol. 3 (ed. T. T. Chen), pp. 566–718. London: Pergamon.

BEALE, G. H. (1957). A mate-killing strain of *Paramecium aurelia*, variety 1, from Mexico. *Proceedings of the Royal Physical Society Edinburgh* **26**, 11–14.

BEALE, G. H. & JURAND, A. A. (1966). Three different types of mate-killer (mu) particles in *P. aurelia* (syngen 1). *Journal of Cell Science* **1**, 31–34.

BEALE, G. H. & MCPHAIL, S. (1967). Some additional results on the maintenance of kappa in stock 51 paramecia, after the loss of the K gene. *Genetical Research* **9**, 369–375.

BYRNE, B. J. (1969). Kappa, mu and the metagon hypothesis in *Paramecium aurelia*. *Genetical Research* **13**, 197–211.

DRYL, S. (1959). Antigenic transformation in *Paramecium aurelia* after homologous antiserum treatment during autogamy and conjugation. *Journal of Protozoology* **6** (supplement), 225.

FRANKLIN, J. (1971). Studies on the mu particle of *Paramecium aurelia*. Ph.D. Thesis, University of East Anglia.

FRANKLIN, J. (1973). The removal of mu particles from *Paramecium aurelia* (stock 540) by penicillin. *Journal of General Microbiology* **74**, 175–177.

GIBSON, I. (1965). The replication of metagons and mu particles from *Paramecium aurelia* in another cell – Didinium. *Genetical Research* **6**, 198–410.

GIBSON, I. & BEALE, G. H. (1961). Genetical basis of the mate-killer trait in *Paramecium aurelia*, stock 540. *Genetical Research* **2**, 82–91.

GIBSON, I. & BEALE, G. H. (1962). The mechanism whereby the genes M1 and M2 in *Paramecium aurelia*, stock 540, control growth of the mate-killer (mu) particles. *Genetical Research* **3**, 24–50.

GIBSON, I. & BEALE, G. H. (1963). The action of ribonuclease and 8-azaguanine on mate-killer paramecia. *Genetical Research* **4**, 42–54.

GIBSON, I. & BEALE, G. H. (1964). Infection into paramecia of metagons derived from other mate-killer paramecia. *Genetical Research* **5**, 85–106.

GIBSON, I. & SONNEBORN, T. M. (1964). Is the metagon an mRNA in Paramecia and a virus in Didinium? *Proceedings of the National Academy of Sciences, U.S.* **52**, 869–876.

GIBSON, I., WILLIAMS, J. & CHANCE, M. (1971). Extranuclear DNA and the endosymbionts of *Paramecium aurelia*. *Nature, New Biology, London* **234**, 75–77.

JURAND, A., GIBSON, I. & BEALE, G. H. (1962). The action of ribonuclease on living paramecia. *Experimental Cell Research* **26**, 598–600.

PREER, J. R. (1967). Genetics of protozoa. *In Research in Protozoology*, Vol. 3 (ed. T. T. Chen), pp. 133–278. London: Pergamon.

REEVE, E. C. R. & ROSS, G. J. S. (1963). Mate-killer (mu) particles in *P. aurelia*: the metagon division hypothesis. *Genetical Research* **4**, 158–161.

SONNEBORN, T. M. (1950). Methods in the general biology and genetics of *Paramecium aurelia*. *Journal of Experimental Zoology* **113**, 87–143.

SONNEBORN, T. M. (1959). Kappa and related particles in Paramecium. *Advances in Virus Research* **6**, 229–356.

WIDMAYER, D. J. (1966). Search for metagon activity for kappa's maintenance in stock 51m 43 of *Paramecium aurelia*. *American Zoologist* **6**, 589–594.

YEUNG, K. K. (1965). Maintenance of kappa particles in cells recently deprived of gene K (stock 51, syngen 4) of *Paramecium aurelia*. *Genetical Research* **6**, 411–418.