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Review

Mating Types in *Paramecium* and a Molecular Approach to Their Determination

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Mating types are expressed in ciliates for the duration of the mature period of their clonal cycle. During cell conjugation the reciprocal fertilization of complementary mating types takes place. Models of mating type determination in the *Paramecium aurelia* species complex based on classical genetics are reviewed including molecular aspects of the studies.

Key words: Conjugation, mating types, mating-type substances, macronuclear reorganization, mating type inheritance, *Paramecium aurelia* complex.

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Ciliates are microbial eukaryotes well suited for the genetic analysis of various cellular functions. They are the only unicellular organisms that separate somatic line functions and germ line in two different types of nuclei: one macronucleus, responsible for phenotypic traits, and two or more micronuclei, indispensable for sexual processes, respectively. Moreover, they show two alternative models of sexual reproduction of great benefit. Conjugation leads to reciprocal fertilization of cells with complementary mating types, while autogamy is a process of self-fertilization which leads to homozygosity. Moreover they show an amazing diversity of mating types. Species which have binary mating type systems are characterized as inbreeders, others with multiple mating type systems are outbreeders (BLEYMAN 1996). In ciliates, binary mating type systems occur, e.g. in *Blepharisma, Aspidisca* sp., *Glaucoma* sp., and *Paramecium* except *P. bursaria*. Multiple mating type systems have been described in *Tetrahymena*, *Euplotes* and also in *Pseudourostyla levis* and various species of *Stylontrichia* (BLEYMAN 1996).

Mating types discovered and described in ciliates, in *Paramecium aurelia and Paramecium bursaria* in 1938 by SONNEBORN and JENNINGS respectively, were the first demonstration of the presence of sex in a unicellular organism. This discovery also led to a new method of distinguishing species in the *Paramecium aurelia* complex. Moreover, it became possible to cross-breed stocks of diverse genetic ancestry, as in multicellular animals.

Physiological conditions essential for conjugation

Conjugation is a sexual process during which cells of complementary mating types mutually activate to acquire the capacity to unite and form conjugating pairs. Then the reciprocal exchange of the migratory pronuclei occurs. Reactive cells stick to each other and form two connections at their ventral surface at the oral region (HIWATASHI 1961), and then form large agglutinates. The anterior holdfast union is the first union between cells. It appears on the anterior-ventral surface. The paroral union is the second connection occurring posterior to the mouth. The mating reaction begins by accidental collision of the paramecia among the ventral cilia of the two complementary mating types. Specific substances inducing the mating reaction, termed mating-type substances, are present on the ventral cilia.

Mating activity appears when cells are sexually mature and moderately starved. Determination of mating type can be influenced by temperature. Furthermore, strains of *Paramecium bursaria*, *Paramecium biaurelia*, *Paramecium triaurelia* and

Paramecium undecaurelia show a circadian rhythm of mating activity (COHEN 1965; KARAKASHIAN 1968; SONNEBORN 1975). Moreover, *Paramecium multimicronucleatum* shows circadian rhythm involving mating type switches. The periodic oscillation continues for some time during continuous light or darkness. Nevertheless, the phenomenon seems to be initiated by regular cycles of light and darkness (BARNETT 1966).

After sexual reproduction *Paramecium* cells enter the immaturity period during which they cannot conjugate or enter autogamy (SONNEBORN 1975). The length of the immaturity period varies in different species and is measured in terms of number of cell divisions (SONNEBORN 1957; MIWA & HIWATASHI 1970). The next part of the clonal cycle is the maturity period; during this time paramecia can conjugate. If they lack a complementary mating type, starved cells undergo autogamy. Immaturity for conjugation is completely lacking in *Paramecium tetraurelia* and *Paramecium octaurelia*. However, according to SONNEBORN (1975) in clones of all species initiated with autogamy immaturity to conjugation is also missing. During senescence, the last period of the clonal cycle, anomalies in sexual processes and increased mortality take place (SONNEBORN 1954a).

Mating type substances mediate mating reaction

The term "mating-type substances" was introduced for the first time by METZ (1947) and defined as substances or molecular configurations on the cell surface that mediate the mating reaction. They exist on the ciliary membranes of the ventral surface of mating-reactive cells (COHEN & SIEGEL 1963; HIWATASHI 1961; TAKAHASHI *et al.* 1974), and are assumed to be proteins (KITAMURA 1988). According to the observations of XU *et al.* (2001), mating-type substances are key molecules in the sexual recognition of complementary mating type cells in *Paramecium caudatum*. They showed that mating-type substances exist in E (even) mating-type cells and strongly support the hypothesis that O (odd) mating type substances are the precursor molecules of the E substances. Theirs results confirm the hypothesis suggested by BUTZEL (1955) for *P. aurelia* species that the O mating-type substances are the basic substances, and the activity of the mating type (*Mt*) locus changes the O mating type substances into the E type. This hypothesis also explains the observation that unidirectional mating type change of selfing stocks occurs naturally only in E mating types (TAUB 1966). In selfing stocks, some E cells change into the O type by cytoplasmatic control or environmental factors which switch the *Mt* locus from an active to repressed state, and the mating reaction occurs during the sexually reactive phase (XU *et al.* 2001). Until 2008, no one has succeeded in identifying mating-type substances from cilia. OGNIBENE *et al.* (2008) identified a 38 kDa glycoprotein (gp38) that is functionally associated with mating activity in *P. primaurelia*. Gp38 is already present in non-mating competent cells but with a restricted localization at the level of their cell body surface and oral region. Nonetheless, gp38 is completely absent from the cell during the immaturity period (OGNIBENE *et al.* 2008).

Mating reaction can be induced by various chemical reagents

The mating reaction can be observed when killed cells (METZ 1947) or isolated cilia (COHEN & SIEGEL 1963) were applied or can be chemically induced. Different methods were used, depending on the *Paramecium* species, for inducing a mating reaction with killed cells (HIWATASHI 1981). For example treatment with formalin for *P. tetraurelia, P. calkinsi* and *P. caudatum,* picric acid and $HgCl_2$ for *P. calkinsi,* ammonium sulfate and glycerine for *P. caudatum,* $K_2Cr_2O_7$ for *P. multimikronucleatum* and *P. caudatum.* After mixing killed cells with reactive living cells of complementary mating types, a mating reaction is induced between both types of cells, and homotypic pairs consisting of living cells are formed (FUJISHIMA 1988). In the induced conjugation method, the following chemicals are used under Ca^{2+} poor conditions: K^+, Mg^{2+}, Rb^+, Cs^+, Na^+, Li^+, Mn^{2+}, heparin, acriflavine and EDTA (MIYAKE 1981), and methyl cellulose (YANAGI & HAGA 1998). When mating-reactive cells of a single mating type are mixed with a conjugation inducing chemical solution, they do not agglutinate into clumps as in the normal mating population. Instead, about one hour after mixing, some of the cells directly form pairs united as in conjugation and undergo the nuclear events characteristic for conjugation (CRONKIETE 1975).

Chemical induction by detached cilia proceeds via different pathways which can be inhibited independently of one another. Research conducted on *Paramecium octaurelia* by CRONKIETE (1974, 1975) revealed the presence of three genes involved in conjugation induced by chemical reagents. The existence of genes *kau-1* and *kau-2*, which block chemical induction (MIYAKE 1969, TAKAGI 1971), but not induction by detached cilia, supports the hypothesis that there are steps unique to chemical induction and unnecessary for induction by detached cilia. The third gene, *Su(kau-2)*, suppresses the phenotype of cells incapable of chemical induction so that they begin to resemble wild-type cells capable of chemical induction (CRONKIETE 1975).

The three genes known in *Paramecium aurelia* that are involved in chemical induction suggest that the differences between species in effective chemical induction solutions may be due to a few gene differences (CRONKITE 1974).

Reorganization of the macronucleus during sexual events

Conjugation, during which mating types are expressed, is preceded by a series of nuclear changes (MIYAKE 1981; HIWATASHI & MIKAMI 1989). During meiosis different numbers of haploid nuclei are produced depending on the species: 4 nuclei in *P. caudatum* and *P. bursaria*, 8 nuclei in *P. aurelia* complex and 16 nuclei in *P. multimicronucleatum.* Only one of these meiotic products survives and enters the paroral region where it divides. The others degenerate and are resorbed. Two haploid migratory gametic pronuclei are exchanged between mates and each of them migrates to the partner cell, where it fuses with the stationary pronucleus and forms a diploid synkaryon. Afterwards, the conjugating pairs separate and the synkaryon divides twice in the *P. aurelia* complex. Products of the division differentiate into two new micro- and two macronuclear anlagen. During the first division of exconjugants the mitotic division of micronuclei takes place and macronuclear anlagen are distributed to the sister cells without division. During conjugation the old macronucleus is fragmented. These fragments degenerate and are resorbed (HIWATASHI & MIKAMI 1989).

The development of a new somatic macronucleus involves macronuclear genome amplification and DNA elimination (YAO *et al.* 2002). There are two types of DNA elimination. One of these is the precise excision of the short (26-882 bp) non-coding internal eliminated sequences (IESs) found in the germline genome from coding and non-coding sequences (KLOBUTCHER & HERRICK 1997). The second type of elimination is imprecise elimination of repetitive germline sequences such as transposons or microsatellites which lead to internal deletions on chromosome fragments (LE MOUEL *et al.* 2003). Chromosome fragmentation results in shorter acentromeric macronuclear "chromosomes" healed by *de novo* telomere addition (LE MOUEL *et al.* 2003).

The massive and reproducible genome rearrangements that take place during zygote development are epigenetically controlled by homology – dependent maternal effects that score the presence or absence of a gene in the macronucleus (MEYER & CHALKER 2007). The latest research has revealed the regulatory roles of two classes of non-coding RNAs (ncRNAs) in these processes. These include scan RNAs (scnRNAs), which are produced from the entire germline genome and longer transcripts from the somatic genome which enable the selection of specific scnRNAs (DUHARCOURT *et al.* 2009).

The small scnRNAs formed from double stranded RNAs (dsRNAs) by a process related to RNA interference (RNAi), scan the macronuclear genome to identify IESs. ScnRNAs degrade sequences in the parental macronucleus which are homologous to macronuclear DNA. Only scnRNAs homologous to micronuclear-specific sequences such as IESs remain in the parental macronucleus. In later stages these IES-specific scnRNAs develop the macronucleus and sequences homologous to the scnRNAs are identified and targeted for elimination (MOCHIZUKI & GOROVSKY 2004; DUHARCOURT *et al.* 2009; LEPERE *et al.* 2009; MOCHIZUKI 2010).

Somatic ncRNAs protect homologous zygotic sequences against elimination while developing the macronucleus and also are necessary for the maintenance of somatic sequences across sexual generations (LEPERE *et al.* 2008).

The specificity of scnRNA-directed deletions can be explained by anticipation that germline-scnRNAs are selected from the initial, highly complex population of scnRNAs. The genome-scanning model shows that this is achieved by pairing interactions with the protective ncRNAs from the maternal macronucleus (DUHARCOURT *et al.* 2009). Some germline-specific elements produce such a large amount of scnRNAs that a fraction of them can escape inactivation by the limited amount of homologous maternal ncRNAs. The excision of some IESs might depend not on scnRNAs but on some other RNAs not submitted to the genome-scanning selection process in the maternal macronucleus (DUHARCOURT *et al.* 2009). The basic mechanism of the scnRNA/macronuclear RNA scanning model may describe all known epigenetic effects on genome rearrangements in *Paramecium* and *Tetrahymena,* including the maternal deletions and the induction of those deletions by small interfering RNAs (siRNAs) (GARNIER *et al.* 2004).

Genetic aspects of mating type diversity among the *Paramecium aurelia* species complex

Each of fifteen species of the *P. aurelia* complex has two mating types: odd (O) and even (E). Species of the *P. aurelia* complex have been classified by their mode of mating type inheritance. The species in group A show caryonidal inheritance and no cytoplasmic effect. The species of group B are characterized by cytoplasmic inheritance, whereas inheritance in group C is Mendelian (SONNEBORN 1966).

The caryonidal system A (SONNEBORN 1937, 1938; KIMBALL 1937) is found in *P. primaurelia, P. triaurelia, P. pentaurelia, P. novaurelia, P. undecaurelia, P. quadecaurelia.* This system was also observed in *P. sonneborni* (AUFDERHEIDE *et al.* 1983). As a rule, a caryonide is phenotypically pure for a single mating type, but the two sister caryonides from a single fertilized cell are randomly either alike or different in mating type (Fig. 1). The caryonidal rule of inheritance implicates the macronucleus as the place of mating-type determination. Moreover this system depends on the presence of the gene Mt^+. No wild stocks incapable of producing mating-type O have been found, nor has it been possible to obtain mutants of this type in the laboratory (SONNEBORN 1974). However, in species such as *P. primaurelia* and *P. pentaurelia* the amount of cells with different mating types depends on temperature. Caryonides with the E mating type are more sensitive to temperature in *P. primaurelia* and the percentage of them increases with higher temperature; in *P. pentaurelia* at 19°C 20% of caryonides are determined as type O (SONNEBORN 1974).

The B or cytoplastic (clonal) system of mating-type genetics is found in *P. biaurelia, P. tetraurelia*, *P. sexaurelia*, *P. septaurelia*, *P. octaurelia*, *P. decaurelia* and *P. dodecaurelia.* In this system differentiation of postzygotic macronuclei for mating-type determination is brought about by a cytoplasmatic agent which itself is under nuclear control (SONNEBORN 1954b; NANNEY 1957). The two new macronuclei that arise in the common cytoplasm of a fertilized cell are usually determined to control the same mating type. Due to the cytoplasmic factor that is present, produced under the action of the parental macronucleus, the mating type of the parental cell persists. It is again determined in the fertilized cell (Fig. 1) (SONNEBORN 1974). Several O-restricted mutations are known in *P. tetraurelia* and *P. septaurelia* (TAUB 1963; BYRNE 1973), and an E-restricted mutation is also known in *P. tetraurelia* (BRYGOO & KELLER 1981). These suggest that not only Mt^+ but also other genes are involved in the determination of mating type. With the exception of one mutation, none of the O-restricted mutations affect cytoplasmatic origin. This indicates that macronuclei are stably differentiated not only for mating type, but also for production of the corresponding cytoplasmatic factors (TSUKII 1988). SONNEBORN (1954b) suggested that the sensitive period for determining macronuclei for mating type is not the start of their development, but sometime later during the first cell cycle. Mating type E should be irreversibly determined at the end of the first cell cycle. Macronuclear anlagen at that stage have performed about four rounds of DNA synthesis. At the same time, the O-determining factor was also produced in an effective concentration and fully maintained its activity by the third cell cycle. The activity then gradually declines with subsequently repeated rounds of DNA synthesis and is finally lost by the eighth cell cycle (KOIZUMI *et al.* 1986). It has been suggested that micronuclei are determined for mating type before sexual reorganization and retain this determination through conjugation (SONNEBORN 1954c; BRYGOO 1977; BRYGOO *et al.* 1980). However, MIKAMI & KOZUMI (1982) suggest that it is unlikely that the micronucleus is determined for mating type in O or E cells during the

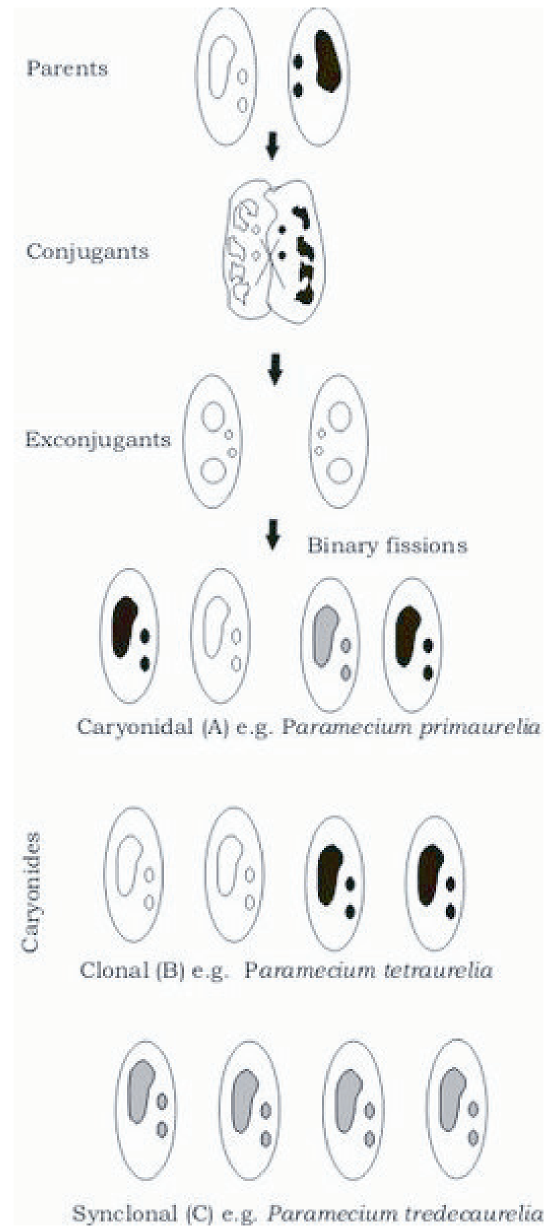

Fig. 1. Simplified scheme of life cycle and patterns of mating type inheritance. In ciliates genetic exchange occurs during conjugation between specimens of complementary mating types.

asexual cycle. KOIZUMI & KOBAYASHI (1984) showed that O-determining factors produced by the macronucleus operate both during the sensitive period of development of the new macronucleus and in determination of gametic nuclei. Moreover, these factors are produced or activated at the sexual stage and decrease in activity at subsequent fission after new macronuclear reorganization.

Normal cytoplasmatic inheritance is observed in crosses between O and E in stock 51 *P. tetraurelia* and by E and O in stock 32 *P. tetraurelia*. However, surprising results were observed in crosses of 32 E by 51 O because an appreciable proportion of the heterozygous F1 lines derived from the E cytoplasmatic member of the cross expresses a new cytoplasmatic state, which is called O^* (BRYGOO 1977; BRYGOO & KELLER 1981). When heterozygotes from F1 were carried through autogamy producing an F2, a ratio 1:1 of O: E was found. This result suggests that segregation at a single locus, mating type differentiation (*mtD*) exists. The two alleles at the *mtD* locus were segregated into the genotypes mtD^{51}/mtD^{51} which is type O and mtD^{32}/mtD^{32} – type E. It is thought that the cytoplasmatic factor O^* is different from the cytoplasmatic factor O found in stock 51. The weaker O^* cytoplasmatic state allows to see the segregation of the mating type genes in both stocks (BRYGOO 1977). In addition a mutant has been detected in *P. tetraurelia*, called mtF^E, restricted to mating type E rather than O (BRYGOO & KELLER 1981). This mutant does not produce the O mating type, but E cells. mtF^E is not allelic with mt^A, mt^B, mt^C, or mt^D. Homozygotes for the gene have a cytoplasm that is always E determining, irrespective of whether it comes from an O or an E cell. BRYGOO & KELLER (1981) found that mtF^E/mtF^E in the O^* cytoplasm is always the pure mating type O, but the mtF^E homozygotes prove to be pleiotropic and very weak and difficult to culture. Surprisingly, MAYER & KELLER (1996) showed that in the mtF^E mutant there was a failure to excise an IES found in the serotype G gene of stock 51.

The simplest model, Mendelian, of mating type inheritance in *Paramecium aurelia* complex occurs only in *Paramecium tredecaurelia* (SONNEBORN 1975). The O type is determined by a recessive gene, *mt,* and E type by its dominant allele, *Mt*. Heterozygotes are type E, except that a cell destined to be E may be type O for a few hours before it becomes E. This model of mating- type inheritance is also referred to as the synclonal system or C. In this system both clones of the synclone having the same genotype are of the same mating type (Fig. 1) (BEALE 1954; SONNEBORN 1974).

In *Paramecium bursaria* species, there are six syngens and each contains 4 or 8 mating types. In different syngens of *P. bursaria* conjugation can occur between any two cells belonging to a different mating type in the same syngen (SIEGEL & LARISON 1960). Syngen 1 has four complementary mating types which are determined by a specific combination of complementary genes at two unlinked loci. Cells whose macronuclei carry dominant genes at both loci are mating type A; mating type C is brought about by the combination of homozygous recessives at both loci. The two alternative combinations of homozygous recessive alleles at one locus and at least one dominant gene at the other locus determine in a specific way mating types B and D (SIEGEL & LARISON 1960). Moreover, genetic analysis of syngen 3 of *P. bursaria* shows direct genic determination of mating type in this syngen and no evidence of caryonidal or cytoplasmic determination (BOMFORD 1966). Genetic and physiological studies suggest that these multiple mating-type systems are produced by a duplication of the two mating-type system (NANNEY 1980).

Paramecium caudatum draws a parallel to mating type in species 13 of the *P. aurelia* group in which inheritance is Mendelian. In syngens 3 and 12 of *P. caudatum* there are two mating types, denoted as types O and E. TSUKII & HIWATASHI (1983) showed that mating type E is controlled by co-dominant multiple alleles at the *Mt* locus, and O mating type by co-dominant multiple alleles at two loci, *MA* and *MB*. Clones of heterozygotes express dual mating types. *Mt* is epistatic to *MA* and *MB,* and thus the O mating type can be expressed only in the recessive homozygote (*mt/mt*) at the *Mt* locus. In addition, at least one allele each at the *MA* and *MB* loci must have a common syngen specificity for the expression of O types. Thus, when *MA* is homozygous for one syngen and *MB* is homozygous for another syngen, no mating type is expressed.

Paramecium multimicronucleatum has two mating types per syngen, furthermore certain stocks show a circadian rhythm affecting mating type switches. This phenomenon occurs only in stocks bearing a certain dominant gene, *C*. In the presence of the recessive allele *c,* the cultures remain stable for one or the other mating type. In this condition, inheritance of mating type is by caryonidal system as in group A species of *P. aurelia* (BARNETT 1966).

Concluding remarks on the molecular basis of mating type determination

For years mating type determination was thought to be achieved during macronuclear differentiation through the stabilization of a master-switch gene under one or two alternative forms.

Activation of one of them can lead to the expression of E-specific functions during sexual reactivity, the other cannot, resulting in the O default state (SONNEBORN 1974; BRYGOO 1977). This master switch gene was identified as *mtD*, which appeared active in E cells and inactive in O cells, moreover one of its products was assumed to be responsible for the maternal inheritance of its expression pattern (BRYGOO 1977). However, mating type inheritance can be understood in terms of homology dependent maternal inheritance, as suggested MEYER & KELLER (1996). In this molecular model a strictly correlation between appearance of mating type O and excised version of G gene exist. At all stages of the mtF^E x mtF^+ crosses mating type E occur also in cells with non-excised version of G gene. Determination for O would be accomplished by developmental excision of the IES from the *mtD* gene, a process involving the *mtF*-encoded nuclear factor. The E-determining factors produced by the old macronucleus of the wild-type E cells would specifically prevent excision of the *mtD*-gene IES. The maternal inheritance of mating types would simply result from epigenetic self-maintenance of the *mtD*-gene IES, similar to that of the G-gene IES. This is also consistent with NANNEY'S (1957) conclusion that the same macronuclear quantities that control vegetative mating types are responsible for the effect of the old macronucleus on the determination of the differentiating macronucleus. Moreover, there can be another way of explaining this type of inheritance, by the epigenetic influence of the parental macronucleus on the development of the new macronucleus (KOIZUMI & KOBAYASHI 1989) possibly by an RNA interference-like mechanism (GARNIER *et al.* 2004; MOCHIZUKI & GOROVSKY 2004). Nevertheless there is no indisputably evidence showing that this hypothesis is correct, for this reason further investigations of mating type determination must be conducted.

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