General introduction

Sexuality is the diversification of biological units, such as the cell and the individual, into different types that are complementary for fertilization. The benefit of the sex or sexual reproduction is to give a competitive advantage to organisms in an unpredictable environment by the genetic recombination. It is striking that practically all-complex present-day organisms have evolved largely through sexual, rather than asexual reproduction.

Discovery of the mating type in *Paramecium* was the first demonstration of the sex in the unicellular organisms (Sonneborn 1937). Mating types in ciliates of protozoa are regarded as sexuality because mating reaction occurs only between the complementary mating types and the result of the mating reaction is conjugation that usually leads to the occurrence of fertilization. The fertilization and sexuality in ciliates are essentially the same as in other eukaryotes, but they have some remarkable features. First, in conjugation of many ciliates each partner fertilizes the other and is fertilized in turn thus behaving as a hermaphrodite. Second, diversification into mating types is often multiple in contrasts to the binary female-male diversification. The studies on mating type in

ciliates provide valuable contributions for understanding the evolution of species, one of the most important and fundamental problems of the biology.

The mating-type signal substances are named gamones in some ciliates and are secreted into the medium. They are called mating pheromones recently (Luporini and Micili 1986), following the practice in other microbiology. Since it is easy to isolate or purify gamones from the culture medium (Miyake 1968), mating-type signal substances have so far been isolated and well studied only in gamone excretors (Brunen-Nieweler et al. 1998; Miyake 1996; Ortenzi et al. 2000; Weischer et al. 1985). In many species, mating-type signals that are firmly bound to the cell membrane, as in *Paramecium*, were often referred to as mating-type substances. The term of mating-type substances was first introduced by Metz (1954) to define substances or molecular configurations on the cell surface that mediate the mating reaction (preconjugant interaction). The mating reaction in *Paramecium* can be instantaneously induced in a few seconds by mixing sexually mature cells of complementary mating types. Thus, *Paramecium* provides an excellent model for studying cell-to-cell interaction and sexual recognition.

The clonal life cycle of *Paramecium* begins with fertilization (conjugation and autogamy) and terminates in clonal

death (Sonneborn 1954). Takagi and Yoshida (1980) observed the fission-associated life spans but not calendar life span in *P. caudatum*. Although the number of the fissions spanning each stage is different among species, the clonal life stages consist of immature, mature, senescence, and death stages if the life cycle is not restarted by the fertilization (Seigel 1967; Smith-Sonneborn 1981; Takagi et al. 1987). Immaturity is the first life cycle stage, in which cells are unable to conjugate (Haga and Hiwatashi 1981; Miwa 1979; Miwa et al. 1975; Myohara and Hiwatashi 1975). Only these cells that are sexually mature and under a slightly starved condition, show mating reactivity.

No diffusible substance is known to participate in the mating reaction in *Paramecium* (Sonneborn 1937; Larison and Siegel 1961). Mating reactivity is known to be restricted to the ventral surface cilia (Hiwatashi 1961; Cohen and Siegel 1963). Takahashi et al. (1974) found that the mating-type substances are located only on the cilia and not on the cell surface. The inhibiting effect of protein and RNA synthesis inhibitors on the mating reactivity of paramecia suggests that *de novo* synthesis of RNA as well as protein is necessary for sexually mature paramecia to become mating reactive (Bleyman 1964; Kang and Taub 1968; Miwa and Hiwatashi 1970; Nobili 1963). *In vitro*, the results obtained by Kitamura (1988) using detached cilia of *P.*

caudatum were basically consistent with the previous data with mating-reactive killed cells of *P. tetraurelia, P. calkinsi,* and *P. caudatum* (Hiwatashi 1969; Metz 1954; Metz and Butterfield 1951). Both complementary mating-type substances in syngen 3 of *P. caudatum* are sensitive to digestion by proteolytic enzymes such as trypsin, chymotrypsin, and ficin, and to heating at 50°C, but insensitive to digestion by glycosidases or phospholipases. Neither high concentrations of salt nor EDTA had any effect on mating reactivity of cilia, but many kinds of detergents and alcohol destroyed the reactivity. These results suggest that the mating type substances are tightly membrane-bound intrinsic proteins of ciliary membranes and their active sites consist of simple proteins but not of sugar residues (Kitamura 1988). The extensive efforts had been paid; nevertheless, no one has succeeded to identify them as molecules.

Each morphological species of *Paramecium* is known to consist of a number of subgroups, designated syngens, which are reproductively isolated by the specificity of the mating types and other breeding relations (Sonneborn 1957). In all *Paramecium* species so far examined, except *P. bursaria* and *P. trichium*, each syngen consists of a pair of odd (O) and even (E) complementary mating types (Sonneborn 1975). In *P. bursaria* and *P. trichium*, each syngen contains four, eight, or more complementary mating

types. Genetic and physiological studies suggest that these multiple mating-type systems are produced by a duplication of the two mating-type systems (Nanney 1980). In the species with two mating types, it is generally accepted that a pair of alleles with simple dominance controls the expression of mating type. The recessive allele, *mt*, restricts homozygotes to type O while the dominant allele *Mt* permits the expression of type E (Sonneborn 1974). In the species with multiple mating types, the situation is essentially the same as those with two mating types except that two or more pairs of alleles with simple-dominance are involved in the determination of mating types (Siegel and Larison 1960; Cohen and Siegel 1963). In addition to the genotype, cytoplasmic and environmental factors, or action of other loci are involved in determining the expression of the dominant allele *mt* (Butzel 1955; Sonneborn 1974, 1977). The mating type in *P. caudatum* is the simplest system and is determined only by the genotypes. Three loci (i.e. *Mt*, *MA* and *MB*) are involved in these events (Tsukii 1988; Tsukii and Hiwatashi 1983). *Mt* is epstatic to *MA* and *MB* and determines E mating type. *MA* and *MB* are only expressed in the recessive homozygotes at *mt* locus and determine syngen specific O mating types. But the relationship between these three genes and the O and E mating type substances is still unknown. In *Paramecium*, Butzel (1953, 1955,

1973, 1974) proposed a hypothesis that the O mating-type substance is a precursor of the E mating-type substance, and the *Mt* gene controls the conversion of O substance to E substance.

Immunological methods are thought to be a useful way to identify and monitor the biosynthesis pathway of mating type substances. Immunological blocking of the mating reaction without mating-type specificity was reported by Hiwatashi and Takahashi (1967) and then by Barnet and Steers (1980). Mating-type specific antibodies were first obtained by Sasaki (1972) but no further information had bee reported. Monoclonal antibodies specifically inhibiting mating reactivity of O mating type without any ciliary immobilization effect had been obtained (Azuma et al. 1996). The antigen was only localized on the ventral surface cilia of mating-reactive O^3 cells. Although the above results suggest that the antigen molecules are mating-type substances, the direct identification of the antigens is not yet successful (Azuma 1999). To find best detergents to soluibilize the antigens from the cilia, Kaku (1997) tested more than 10 kinds of detergents and found that a non-ionic detergent, sucrose monolaurate (SM 1200), was the best one for both solubility and the maintenance of the antigenicities of the samples for dot-blot analysis. Using these antibodies, Kumakura (1997) examined the antigen in the cells in the different culture and clonal age, and

found that the detection of the antigen was completely associated with the mating reactivity of the cells. Unexpectedly, she discovered that there is substances recognized by these monoclonal antibodies in E^3 cells, but she did not examine the cellular localization of them. This result was inconsistent with the O-mating-type-specific inhibitory effects of these monoclonal antibodies.

I started my research from determining the localization of the substances recognized by these antibodies. In part I, it was clarified that the substances detected from the E^3 cells is contained in the cytoplasm, but not on its cilia. Furthermore, I proved directly that mating-reactive O^3 mating-type substances existing in the E^3 cell bodies and strongly supported the precursor hypothesis of mating-type substances.

In part II, in order to identify mating-type substance further, a new monoclonal antibody XomO was isolated. The inhibitory effects of antibody XomO on both mating types of syngen 3 were observed. The detection of the antigen was correlated with the mating reactivity of the cells. These results suggest that the antigen molecules are involved in mating reaction. The inhibitory effect of antibody XomO is syngen 3 specific. Therefore, the antigen molecules may be mating-type substances of syngen 3. The unusual localization of the antigen,

which exists only on the root side of the ventral surface cilia suggested that the antigen molecules are undergoing dynamic conformational change during the transporting process along the cilia membrane.