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Adolescence in *Tetrahymena thermophila*

(ciliata/development/cell interaction/outbreeding)

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ABSTRACT **The development of sexual maturity in *Tetrahymena thermophila* has been shown to include an intermediate stage, adolescence, during which cells are capable of mating with mature cells but not other adolescent cells. When the progeny of successfully mated cells are grown logarithmically and tested frequently for the ability to mate, they are unable to form mating pairs for about 65 generations. This period is known as immaturity. During the next stage, the progeny pair with mature cells but not with other adolescent cells despite the presence of complementary mating types. Adolescence persists for 20–25 generations before the cells attain maturity, which is defined as the ability to mate with any cell of different mating type. Once paired with mature cells, adolescents successfully complete conjugation. Cytological preparations show that both members of the pair undergo meiosis and form macronuclear anlagen. The proteins synthesized during a mating between adolescents and mature cells are similar to those synthesized during a mating between mature cells as determined by two-dimensional gel analysis. Both the adolescent cell and the mature partner contribute genetic markers to the progeny.**

The ciliate *Tetrahymena thermophila* contains a diploid micronucleus and a highly polyploid macronucleus. The macronucleus is responsible for most, if not all, transcriptional activity in the vegetative cell. *T. thermophila* can undergo sexual reproduction after pairing between two cells of different mating types. During conjugation, the micronucleus undergoes meiosis and the cells exchange haploid pronuclei that fuse. The mitotic products of the zygotic nucleus develop into new micronuclei and macronuclei, while the old macronucleus degenerates.

Immediately after successful conjugation, there is a period during which the progeny are unable to form mating pairs. In *T. thermophila*, this period of immaturity lasts between 40 and 100 generations (1). The absolute length of this period is both genetically (2) and environmentally (3, 4) controlled.

Mating type determination in *T. thermophila* occurs in the macronucleus. B-strain cells, which are homozygous at the *Mat2* locus, carry information for six different mating types in the micronucleus but only one is expressed in the macronucleus. A mature cell can mate with any cell that has a mating type different from its own. The progeny of conjugation between cells of any two complementary mating types may express any of the six possible mating types. Pedigree analysis indicates that mating type is determined in the four cells that result from the first division of the exconjugants. Selection of mating type among these four cells (caryonides) is independent of parent and sister caryonide mating type (5).

In this report we show that, following immaturity, exconjugant *T. thermophila* pass through a stage during which they may conjugate with mature cells but not with cells of a similar age. We call this stage "adolescence" by analogy to a previously described stage of restricted mating potential during the development of sexual maturity in *Paramecium bursaria* (6).

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MATERIALS AND METHODS

Strains. All strains were derived from inbred strain B1868 (7). Strains CU399 [*ChxA2/ChxA2* (cycloheximide sensitive, VI)] and CU401 [*Mpr/Mpr* (6-methylpurine sensitive, VII)] are functional heterokaryons kindly provided by P. Bruns. Following the previously suggested notation (8), we indicated the micronuclear genotype by a three-letter code with a capital first letter if the mutation is dominant and a superscript + for wild type. The macronuclear phenotype is indicated in parentheses.

Cell Culture. All cells were grown at 29°C with swirling at 90 rpm in 2.0% proteose peptone (Difco), 0.1% yeast extract, and 0.003% sequestrene (CIBA–Geigy) prepared as described by Gorovsky *et al.* (9). The fission time under these conditions is 2.5–3 hr.

Matings. Cells of different mating types were prepared for mating by a 24-hr starvation in 10 mM Tris·HCl (pH 7.4) as described by Bruns and Brussard (10). With the exception of strain F1-2, which was fed at 6 hr, all conjugating cultures were fed 24 hr after initiation of conjugation to maximize the number of diverse mating types (11).

Antibiotic Selection. Resistant cells were selected with 15 μg of 6-methylpurine or 25 μg of cycloheximide per ml of growth medium. Antibiotic was added to newly mated cells at 24 hr after refeeding.

Two-Dimensional Gel Electrophoresis. Cells were pulse-labeled *in vivo* with 10 μCi (1 Ci = 37 GBq) of [^{35}S]methionine per ml for 30 min, total cell proteins were solubilized, and nonequilibrium pH gradient electrophoresis (NEPHGE) was performed by the method of Guttman *et al.* (12).

RESULTS

Timing and Duration of Adolescence. Pure populations of immature cells were obtained by mating a functional heterokaryon that was homozygous for cycloheximide resistance in the micronucleus but sensitive in the macronucleus to cells that were cycloheximide sensitive and by selecting the successful exconjugants for cycloheximide resistance. Only cells that complete conjugation and develop a new macronucleus acquire resistance. Strain F1-2 was composed of the cycloheximide-resistant progeny of CU399 mated to CU401, and strain F1-3 was composed of the cycloheximide-resistant progeny of a cross between CU399 and BVII (wild type and mating type VII). Up to six different mating types could be expected in these populations of cells. The cultures were maintained in logarithmic phase by a daily dilution of 1:200 in fresh medium and tested frequently against mature mating testers for pairing. The results of two experiments are shown in Table 1. The cells were unable to pair with mature cells of any mating type for ≈65 generations. They were considered immature for this period. For a subsequent period of about 25 generations, they formed pairs with mature cells, but the cells within a maturing culture were unable to pair with each other. This stage is operationally defined as adolescence. After this period, cells acquired the ability to mate with their

Abbreviation: NEPHGE, nonequilibrium pH gradient electrophoresis.

Table 1. Development of mating reactivity in exconjugant *T. thermophila*

F1-2		F1-3	
Fissions after mating	Stage	Fissions after mating	Stage
6	I	20	I
29	I	35	I
42	I	41	I
		50	I
70	A	56	I
83	A	65	I
96	M	72	A
135	M	80	A
		87	A
		95	A
		110	M

F1-2 and F1-3 are exconjugant populations of cells consisting of more than one mating type. I, immature (no pair formation); A, adolescent (forms pairs with mature testers but not within the population); M, mature (forms pairs with mature testers and within the population).

siblings and were considered fully mature.

Lack of pair formation within the maturing population of cells could not be attributed to the presence of only a single mating type in the population. At least two different mating types were present because the cells formed pairs with mature cells of all six possible mating types (Table 2). Therefore, pairing within the population of maturing cells would be expected to occur at least to the level of the lowest pairing with mature cells of pure mating type.

Cells of Every Mating Type Undergo Adolescence. The preceding results show that at least two mating types display adolescent behavior. To determine whether cells of every mating type pass through adolescence, we established clonal cell lines from a newly mated strain (F2-1). Because the cells were isolated after the caryonidal stage, most lines were expected to be pure for one mating type. Each culture was tested with the six mating-type testers and with each other for the ability to pair. The mating type of each adolescent cell line could be assigned by comparison to the established mating testers (Table 3). While cell lines were adolescent, they

Table 2. Maximum pairing between exconjugant strain F1-3 and the six possible mating types and between the cells in F1-3 at 87, 95, and 110 fissions after its construction

	% of cells in pairs			
Mating type of cells added	Adolescent F1-3 at 87 fissions	Adolescent F1-3 at 95 fissions	Mature F1-3 at 110 fissions	CU399*
II	28.4	18.5	ND	90.5
III	25.0	10.3	ND	54.2
IV	4.3	9.3	ND	68.4
V	30.0	17.8	ND	70.0
VI	19.1	27.3	ND	0.0
VII	45.0	62.2	ND	87.9
None added	0.0	0.0	51.0	0.0

ND, not determined.
*CU399 is a mature control strain possessing mating type VI.

were unable to pair with other adolescent cell lines despite their having different mating types. When these same cell lines became mature, they retained the mating type expressed during adolescence and were able to pair with sibling cell lines of a different mating type.

Adolescent Cells Successfully Complete Conjugation. The previous experiments demonstrate that adolescent cells pair with mature cells, but they do not show that adolescents successfully complete conjugation. Conjugation events after pairing may be followed cytologically (13), biochemically (14, 15), and genetically.

The cytology of pairs formed in a mating culture containing adolescents and mature cells was observed in fixed preparations stained with Giemsa (16). Pairs formed between adolescents and mature cells were cytologically normal during meiotic prophase, meiosis, and macronuclear development (data not shown).

The production of genetically recombinant progeny constitutes proof of successful completion of conjugation. To test this, an adolescent cell line was constructed that was heterozygous for the dominant mutation *ChxA2*, which confers cycloheximide resistance. This strain, F1-3, was mated with a cycloheximide-sensitive strain (CU401). Successful exconjugants (F2-1) were selected for resistance to 6-methylpurine (Fig. 1) and examined for cycloheximide resistance. As a control, a mature strain that was homozygous for cyclo-

Table 3. Mating of clonal cell lines

Strain	Mating type	Strain								
		F2-1I	F2-1D	F2-1K	F2-1C	F2-1G	F2-1A	F2-1E	F2-1H	F2-1F
BII	II	−−	++	++	++	++	++	++	++	++
BIII	III	++	++	++	++	++	++	++	++	++
BIV	IV	++	−−	−−	++	++	++	++	++	++
BV	V	++	++	++	−−	−−	++	++	++	++
CU399	VI	++	++	++	++	++	−−	−−	−−	++
CU401	VII	++	++	++	++	++	++	++	++	−−
F2-1I	II	−−	−+	−+	−+	++	−+	−+	−+	−+
F2-1D	IV	−+	−−	−−	−+	−+	−+	−+	−+	−+
F2-1K	IV	−+	−−	−−	−+	−+	−+	−+	−+	−+
F2-1C	V	−+	−+	−+	−−	−−	−+	−+	−+	−+
F2-1G	V	++	−+	−+	−−	−−	−+	−+	−+	−+
F2-1A	VI	−+	−+	−+	−+	−+	−−	−−	−−	−+
F2-1E	VI	−+	−+	−+	−+	−+	−−	−−	−−	−+
F2-1H	VI	−+	−+	−+	−+	−+	−−	−−	−−	−+
F2-1F	VII	−+	−+	−+	−+	−+	−+	−+	−+	−−

BII, BIII, BIV, BV, CU399, and CU401 are strains with established mating types. The mating type of each experimental strain was determined by reference to these strains. In each data column, the symbol on the left indicates mating reactivity of the clonal cell line during adolescence. The symbol on the right indicates mating reactivity of the cell line after it attained sexual maturity. +, Mating; −, no mating.

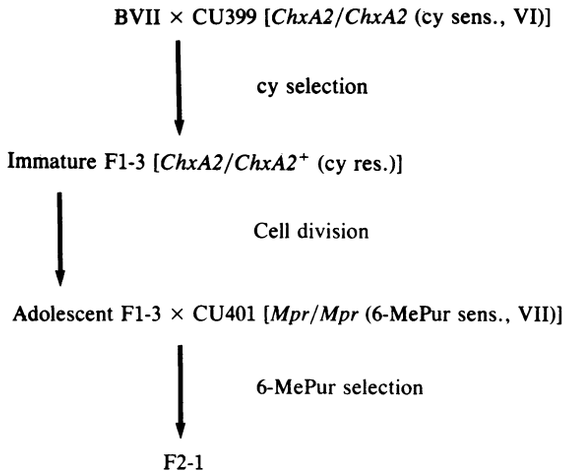

FIG. 1. Construction of strain F2-1. 6-MePur, 6-methylpurine; cy, cycloheximide; sens., sensitive; res., resistant.

heximide resistance (CU399) was mated with CU401.

Of 12 clonal cell lines established and tested for cycloheximide resistance from the control, 3 were cycloheximide resistant. Of 12 clonal cell lines established from the experimental cross, 2 were cycloheximide resistant. The presence of 2 cycloheximide-resistant cell lines from the F2-1 strain demonstrates the successful donation of genetic information from the adolescent cells. The predominance of cycloheximide-sensitive cell lines in the control and experimental crosses may be the result of parental self fertilization (cytogamy) (17) and phenotypic assortment of the heterozygotic macronuclear alleles (7). In fact, a subsequent mating of the F2-1 clonal cell lines uncovered the *ChxA2* allele in the micronucleus of 1 additional cell line from the experimental cross.

Comparison of Proteins Synthesized by Adolescent and Mature Cells Under Conjugation Conditions. During conjugation, the synthesis of a number of proteins is stimulated (14, 15). A comparison of the autoradiographic patterns of total proteins extracted from *in vivo* labeled starved cells and mature conjugating cells separated on two-dimensional NEPHGE gels (Fig. 2 *A* and *B*) reveals striking differences. In particular, synthesis of a number of basic proteins was strongly induced in conjugating cells. Patterns obtained from mating cultures containing adolescent and mature cells (Fig. 2*C*) are similar to those from mature mating cultures. Thus,

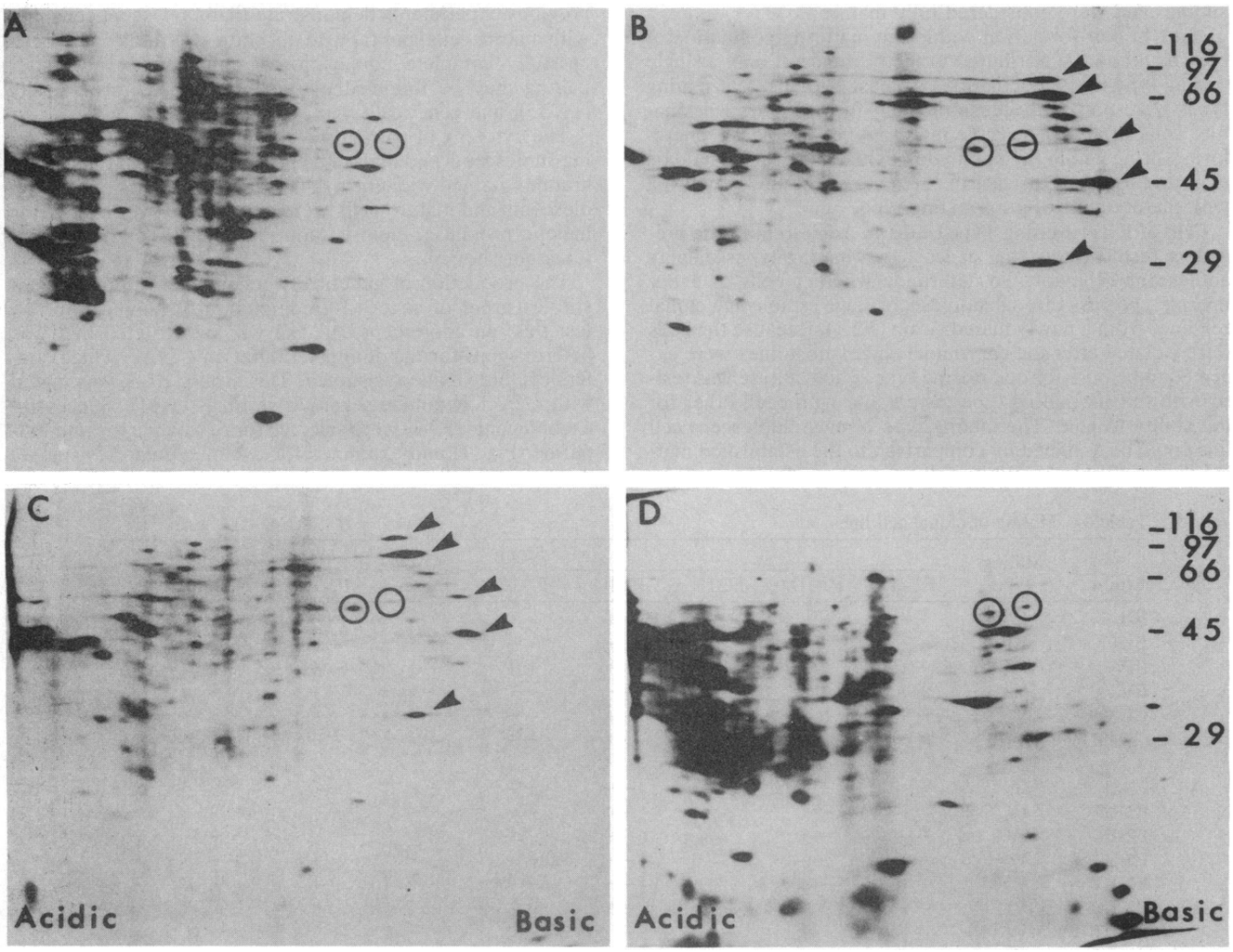

FIG. 2. Autoradiographs of two-dimensional NEPHGE gels of proteins extracted from [^{35}S]methionine pulse-labeled cells 2.5 hr after mixing under conjugation conditions. Cultures of mature CU399 and CU401 were starved separately and mixed with cultures of the same strain to simulate conjugation conditions without pairing (*A*) or mixed with the other strain to initiate pairing (*B*). Starved cultures of adolescent F1-2 were mixed with mature CU401 to initiate pairing (*C*). Starved cultures of adolescent F1-2, containing cells of more than one mating type, did not pair (*D*). Some of the proteins induced in conjugating cells are marked by arrowheads. Two proteins that are relatively constant in intensity between all starved cells are circled. Numbers indicate the molecular mass in kilodaltons of the protein standards (Sigma).

proteins characteristic of conjugation are induced when one member of a mating pair is adolescent. Patterns obtained from adolescent cells in populations that contained more than one mating type and were labeled under conjugation conditions are shown in Fig. 2*D* and resemble those from nonconjugating, starved mature cells.

DISCUSSION

Adolescence, a previously unrecognized stage in the long-term life cycle of *T. thermophila*, has been described. During this stage, which persists for about 25 fissions, an adolescent cell is able to form mating pairs with a mature cell but not with another adolescent cell.

Attainment of sexual maturity may require the completion of a number of cellular events of which adolescents have completed a subset. Because adolescents are capable of pairing and completing conjugation with a mature partner, one may postulate that the primary defect in adolescents is in a prepairing function, such as cell recognition or physical attachment. Two obligatory prepairing stages, initiation and costimulation, have been described (10). Initiation occurs over the first 2 hr of starvation and in the absence of cells of another mating type. Since adolescents pair with mature cells, it seems likely that adolescents complete initiation. The second preparing event, costimulation, requires cellular interaction between cells of different mating types. The fact that adolescents pair only when mature cells of another mating type are present may suggest that they are deficient in costimulation.

Although pedigree analysis indicates that mating type is determined at the caryonidal stage (5), it is not expressed in an immature cell. Mating type, as defined by the ability to form pairs with all but one of the six mating testers, is expressed by clonal adolescent cell lines in matings with mature cells. Yet adolescent cells cannot form mating pairs with each other. Therefore, expression of the molecules required to specify mating type is not sufficient for successful completion of the cellular interactions leading to pairing.

One anomaly was observed in the mating of clonal adolescent cell lines. Pair formation was noted between F2-1G (V) and F2-1I (II) when neither strain would pair with any other adolescent cell line. The observation was repeated in two wells of the microtiter plate. It is possible that one or both of these cell lines had progressed to a stage where it could pair with adolescent cells of only one of the five complementary mating types.

The evidence presented here suggests that pair formation is required for the specific induction of the proteins synthesized by conjugating cultures. We have shown that the proteins induced in mating cultures containing adolescent and mature cells are similar to those in cultures containing only mature cells. We cannot eliminate the possibility that these proteins are synthesized solely by the mature partner. An alternative hypothesis is that, once paired with a mature cell, an adolescent cell also synthesizes conjugation-specific proteins. We favor the latter view because cytological and genetic evidence show that the adolescent cells participate in conjugation. Adolescent populations containing more than one mating type and labeled under conjugation conditions do not synthesize detectable amounts of the proteins characteristic of conjugation. Thus, induction of these proteins requires something more than the presence of cells having complementary mating types.

A mutant (*Prc*) has been isolated in which the period of immaturity is brief (less than 20 fissions). Following immaturity, *Prc* behaves as an adolescent in that it will pair with mature cells but not with descendents of its sister caryonides (M. Katz, M. Baum, and E. Orias, personal communication). *Prc* demonstrates that the onset of observable adolescence may be genetically advanced.

Sonneborn (18) classified *T. thermophila* as an outbreeding species on the basis of its long immaturity period and the usual absence of selfing or autogamy. Immaturity in swimming cells prevents inbreeding by geographical separation of siblings. An adolescent period further commits *T. thermophila* to an outbreeding strategy by permitting conjugation only with mature cells that are likely to have arisen from a separate past mating. After adolescence, by which time the cells are even more unlikely to encounter a sibling, cells enter a long maturity period during which they may conjugate with any other competent cell. This strategy maximizes the probability of producing a new gene pool through outbreeding while permitting reassortment of an isolated gene pool through inbreeding.

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