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A Mutational Analysis of Conjugation

1. Phenotypes Affecting Early Development: Meiosis to Nuclear Selection

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Conjugation in the freshwater ciliate *Tetrahymena thermophila* involves a developmental program that models meiosis, fertilization, and early developmental events characteristic of multicellular eukaryotes. We describe a gallery of five early-acting conjugation mutations. These mutants, *cnj1–5,* exhibit phenotypes in which specific steps in the conjugal pathway have been altered or eliminated. Specifically, *cnj1* and *cnj2* fail to condense their micronuclear chromatin prior to each of the three prezygotic nuclear divisions. This results in nuclear division failure, failure to replicate DNA, and failure to initiate postzygotic development. The *cnj3* mutant appears to exhibit a defect in chromosome separation during anaphase of mitosis. *cnj4* mutants successfully carry out meiosis I, yet are unable to execute the second meiotic division and abort all further development. *cnj5* mutants are unable to initiate either meiosis I or meiosis II, yet proceed to execute all subsequent developmental events. These mutant phenotypes are used to draw inferences regarding developmental dependencies that exist within the conjugation program.

INTRODUCTION

Ciliates are unicellular organisms that execute many biological activities observed in multicellular eukaryotes. One feature that sets ciliates apart is the possession of two functionally distinct types of nuclei, a transcriptionally active ''macronucleus'' which performs the somatic functions of the cell and a transcriptionally silent ''micronucleus'' which serves as a germinal nucleus during mating. This nuclear duality allows for an extraordinary kind of genetic investigation. Due to the fact that the macronucleus undergoes an amitotic type of fission during cell division, it uses biochemical machinery distinctly different from the more conventional, mitotically dividing micronucleus (see Sweet and Allis, 1993; Sweet *et al.,* 1997). Furthermore, *Tetrahymena* cells are capable of surviving even extreme damage to the micronucleus up to and including complete loss of both diploid copies of one or more micronuclear chromosomes (Bruns and Brussard, 1981). For these reasons we can generate viable cell lines which are homozygous for nonconditional mutations which profoundly interfere with meiotic and mitotic cellular functions, mutations which would prove lethal in almost any other eukaryotic system. Finally, the ability to induce and visualize the nuclear events associated with sexual behavior in these cells provides us with an excellent assay for screening and characterizing novel conjugation mutations.

Conjugation in ciliated protists has been a subject of investigation since the 19th century (Maupas, 1889; Hertwig, 1889). More recently, Raikov (1972) compared conjugation of a number of ciliate species revealing a highly conserved developmental program typically consisting of three prezygotic nuclear divisions (meiosis I, meiosis II, and a third prezygotic mitosis), pronuclear exchange and fusion (karyogamy), and two to four postzygotic nuclear mitoses (depending upon the species).

Mating studies of *Tetrahymena* initially proved to be difficult in that wild strains were frequently sterile and often

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TABLE 1
Heterokaryon Genotypes and Phenotypes

Strain	Micronuclear genotype	Macronuclear phenotype	Mating type
CU427.2	*Chx/Chx* (cy resistant)	(cy sensitive)	VI
CU428.1	*Mpr/Mpr* (mp resistant)	(mp sensitive)	VII
CU438	*Pmr/Pmr* (pm resistant)	(pm sensitive)	IV

Note. cy, cycloheximide; mp, 6-methylpurine; pm, paromomycin.

gave rise to self-mating clones in which synchronous mating was impossible to achieve (see Elliot and Nanney, 1952). Once fertile cell lines which exhibited stable mating types were obtained (Elliot and Gruchy, 1952; Elliot and Hayes, 1953), detailed cytogenetic analysis became possible (Nanney, 1953; Ray, 1956; Doerder and DeBault, 1975; Martindale *et al.*, 1982; Orias, 1986). Thanks to these early efforts we are now able to mate *Tetrahymena* cells in high-density cultures with a high level of developmental synchrony. With immunofluorescence microscopy and the application of antisera generated against defined nuclear and cytoskeletal proteins, an ever more elegant picture of conjugation has emerged revealing a complex sequence of nuclear and cytoskeletal events (Numata *et al.*, 1985; Takagi *et al.*, 1991; Gaertig and Fleury, 1992; Madireddi *et al.*, 1994; Nelsen *et al.*, 1994; Hanyu *et al.*, 1995; Numata, 1996).

Using the complete developmental program as a mutational ''target'' we have isolated 10 novel conjugation mutants. Combined with 2 previously characterized mutants, *bcd* and *janA* (Cole, 1991; Cole and Frankel, 1991), these mutants collectively affect nearly every stage of nuclear behavior and highlight fundamental cellular processes. This paper describes mutations affecting early stages of conjugation leading up to pronuclear selection, which precedes the third prezygotic nuclear division. These mutations include defects in chromatin condensation, chromosome separation, and initiation of each of the two meiotic nuclear divisions. The accompanying paper (Cole and Soelter, 1997) describes mutations affecting middle and late stages of conjugal development beginning with the third prezygotic nuclear division and ending with separation of exconjugant partners.

MATERIALS AND METHODS

Stocks. Heterokaryons carrying the drug markers for cycloheximide resistance (*Chx*), 6-methylpurine resistance (*Mpr*), or paromomycin resistance (*Pmr*) in their micronuclei were of the inbred B strain of *Tetrahymena thermophila* (see Table 1). Defective ''star'' strains were of the A strain [A*(III), A*(V)].

Growth and induction of mating. The growth medium used throughout these experiments was an iron-supplemented proteose peptone medium (0.25% proteose peptone, 0.25% Difco yeast extract, 0.5% glucose, 0.033 m*M* $FeCl_3$). Stock cultures were maintained by loop transfer and stored at 15°C. All experiments were conducted at 30°C. Two starvation media were employed: Dryl's inorganic salts (Dryl, 1959) and 10 m*M* Tris (pH 7.4).

Matings were performed by prestarving cells of uniform mating type at 2.0×10^5 cells/ml for 18 hr in either Dryl's or Tris medium. Five milliliter samples of each mating type were adjusted to 2×10^5 cells/ml just prior to mixing (Time 0).

Mutagenesis and uniparental cytogamy. The mutagenesis and screening strategy we employed are described as follows. Either the 6-methylpurine-resistance heterokaryon CU428 or the paromomycin-resistance heterokaryon CU438 was grown to log phase (2×10^5 cells/ml), exposed to nitrosoguanidine (final concentration of 10 μg/ml) for 3 hr, pelleted by centrifugation, washed in Tris buffer, and starved overnight at 30°C. After 12–18 hr of starvation, mutagenized CU438 and starved, nonmutagenized A*(III) were mated at a final concentration of 2×10^5 cells/ml. After 5 hr of mating, the mating mixture was fed with growth medium (to prevent repairing) and pairs were manually isolated. [This initial pairing promotes meiosis within the heterokaryon and unilateral micronuclear transfer, hence the micronuclei of both exconjugants are homozygous and genetically identical, without bringing the mutation into macronuclear expression (Allen, 1967)]. Four thousand pairs were isolated into individual drops of growth medium, grown, and transferred to microtiter plates. After further growth, these plates were then replicated into microtiter plates containing prestarved A*(V) partners (in Dryl's medium) and allowed to mate again. A hyperosmotic shock was delivered to mating cells 6 hr after mixing by adding glucose to a final concentration of 1.4% in growth medium. [This hyperosmotic shock induces self-fertilization, creating whole-genome homozygotes with multiple mating types and exhibiting precocious sexual maturity (Cole and Bruns, 1991; Orias and Hamilton, 1979).] Homozygotes were selected in either paromomycin (100 μg/ml) or 6-methyl purine (15 μg/ml). After 4 days of incubation, survivors were brought to full maturity by serial replication.

Screening of conjugation mutants. Microtiter plates carrying individual mutant polyclones were replicated into flat-bottomed microtiter plates with 75 μl of Tris starvation medium per well. [Since each well contains multiple mating types of a genetically identical cell line, pairing will occur without addition of a mating partner.] After 12 to 15 hr, nuclear configurations and karyotypes were visualized by staining cell pairs. Seventy-five microliters of 4′,6-diamino-2-phenylindole dihydrochloride (DAPI; Sigma Chemical Co., St. Louis, MO) in fixative (32 ml of 10 m*M* sodium phosphate, pH 7.2, 3 ml of methanol, 1 ml glacial acetic acid, and DAPI to make 10 μg/ml) was added to each well. Cells were examined within their microtiter plate well using an Olympus inverted fluorescence microscope (Model IMT-2). Any wells that contained mating pairs whose nuclear configurations were abnormal were transferred from the master plate and subcloned for further analysis. Their respective parental heterokaryons were also subcloned for genetic analysis.

DAPI staining for photomicroscopy. One milliliter of cells in Tris starvation medium were pelleted in 1.5-ml Eppendorf tubes by gentle centrifugation in a benchtop centrifuge (setting 4 for 2 min in an IEC clinical centrifuge). The supernatant was aspirated, and the pellet was resuspended in 50% methanol. The cells were centrifuged, resuspended in 70% methanol, centrifuged once again,

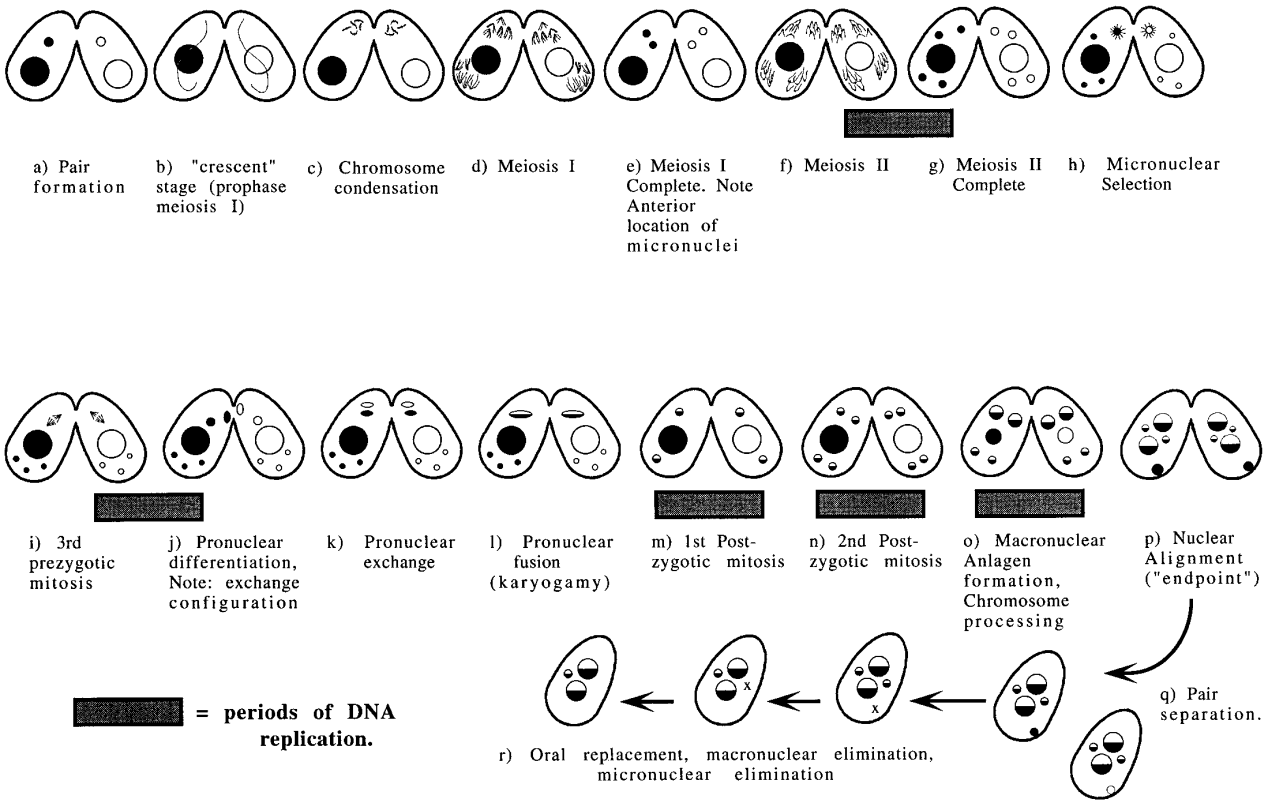


FIG. 1. Summary of nuclear behavior during (a–h) early, (i–l) middle, and (m–r) late stages of conjugation in *T. thermophila.*

and resuspended in a mixture of 70% methanol and 15% acetic acid. A final spin resulted in a pellet which was resuspended in 100 μl of the methanol–acetic acid fixative. This slurry of fixed, mating pairs was then dropped onto a microscope slide and air dried. To stain, the slides were dipped into 95% ethanol for 15 sec and then into a DAPI staining mixture: 0.3 *M* NaCl in 70% ethanol to which DAPI had been added making a final concentration of 0.1 μg/ml. (*Note.* Salt should be dissolved in water before alcohol is added.) Slides were stained for 1 min and then rinsed in 70% ethanol (10–15 sec) and 35% ethanol (30 sec). Slides were air dried and stored for later viewing or viewed immediately under a coverslip with a drop of 35% ethanol. *Note.* Over the next few minutes the background (cytoplasmic) staining diminishes and the nuclei become increasingly bright. Slides are best viewed several minutes after the coverslip has been added. Pairs were photographed using an Olympus PM-30 camera, Kodak Tech-Pan film at 320× magnification (using a 40× oil-immersion objective lens, a 1.6 optivar setting, and a 5× ocular lens on an Olympus B-Max fluorescence microscope).

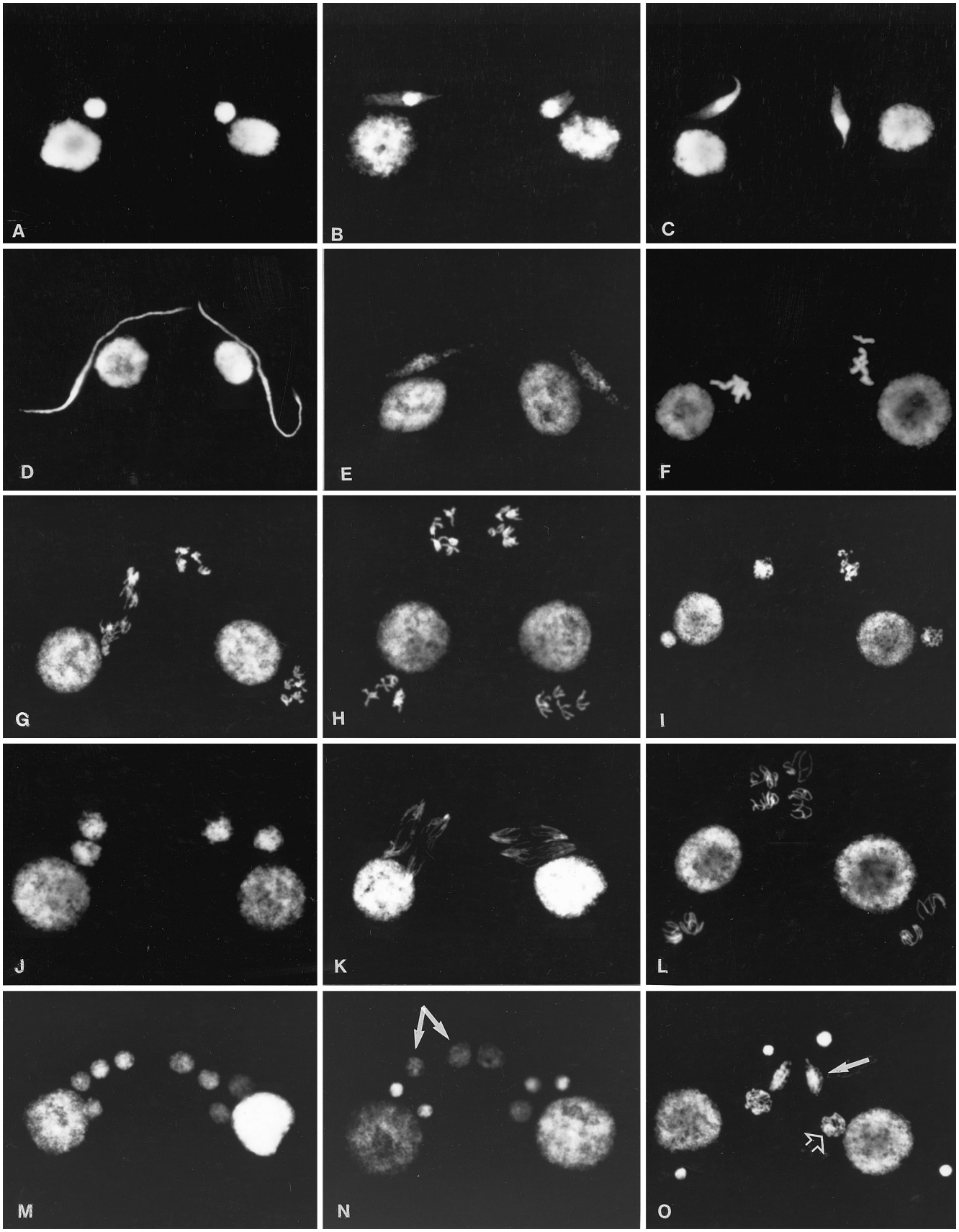
Indirect immunofluorescence microscopy. A monoclonal antibody to the ciliate protein ''fenestrin'' (Nelsen *et al.,* 1994) and an antiserum against β-tubulin were kindly supplied by Dr. Joseph Frankel (University of Iowa). Fenestrin has been shown to decorate *Tetrahymena* pronuclei just prior to nuclear exchange, and it is used here as a marker of pronuclear differentiation (Nelsen *et al.,* 1994). A polyclonal antiserum directed against dog-brain tubulin was kindly provided by Dr. Jacek Gaertig (University of Georgia). This antiserum has proven to be valuable in visualizing *Tetrahymena* microtubules (Gaertig and Fleury, 1992). Mating pairs (2×10^6) were pelleted by centrifugation, chilled on ice, and fixed in 0.5% Triton X-100 in 35% EtOH for 15 min. Cells were washed twice in 0.1% BSA in Tris-buffered saline [TBS: 0.9% NaCl, 10 m*M* Tris-hydrochloride (pH 7.4), 0.05% Nonidet-P40]. The pellet was suspended in primary antiserum [1:100 dilution for the fenestrin antiserum and 1:25 dilution for rabbit anti-dog-brain tubulin (see Gaertig and Fleury, 1992)] and incubated for 45 min. Cells were washed twice in 0.1% BSA in TBS and resuspended in FITC-labeled secondary antiserum (50 μl goat anti-rabbit or goat anti-mouse antiserum in 5 ml 1.0% BSA in TBS). Cells were washed after another 45 min in 0.1% BSA–TBS, pelleted, resuspended, and stored in 0.1% BSA–TBS. DABCO antiquenching agent was added just prior to viewing (Johnson and Araujo, 1981). Cells were viewed and photographed with an Olympus B-MAX fluorescence microscope.

Cytophotometry. Analysis of DNA content within specific nuclei was performed using Feulgen cytophotometry as described by Doerder and DeBault (1975).

RESULTS

Early Nuclear Events from Meiosis to Micronuclear Selection in T. thermophila

Figure 1 illustrates the chromosomal and nuclear behavior associated with conjugation in *T. thermophila.* [For a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more complete description see Martindale *et al.* (1982) and Gaertig and Fleury (1992).] Figure 2 provides DAPI-stained images of wild-type conjugation from pair formation through the third prezygotic division. Early developmental events include meiosis I, meiosis II, and ''nuclear selection,'' a process by which one of the four haploid meiotic products migrates into the paroral cortical cytoplasm and is protected from nuclear degradation. At the time of nuclear selection, the other three meiotic products are ear-marked for elimination. The selected nucleus initiates middevelopment by undergoing a third prezygotic nuclear division. Middevelopment includes this third prezygotic division, pronuclear differentiation, pronuclear exchange, and karyogamy (pronuclear fusion). Middevelopment and late developmental events are described in more detail in the accompanying paper (Cole and Soelter, 1997).

Phenotypic Profiles of Conjugation Mutations

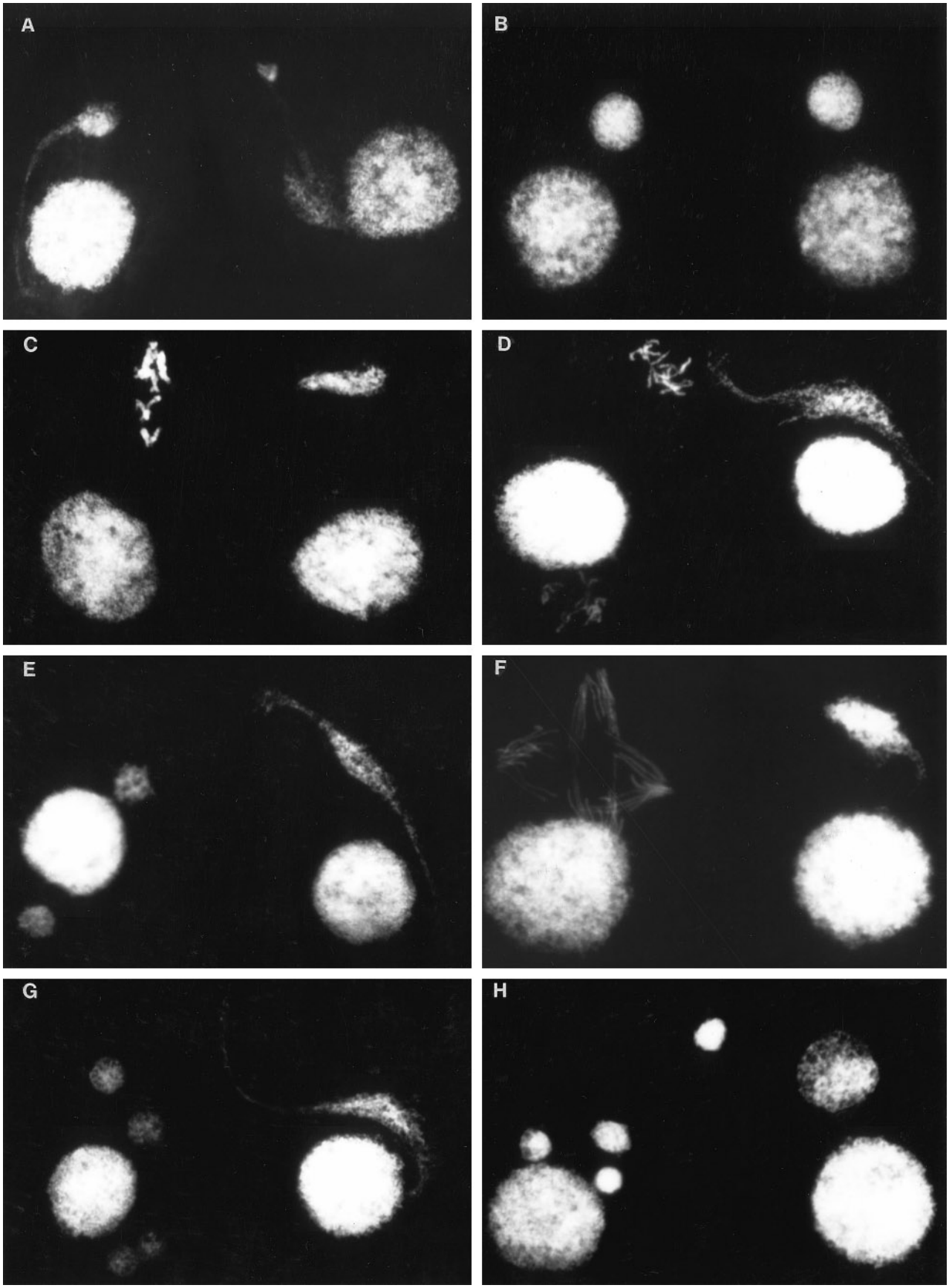
cnj1 and cnj2. Cloned *cnj1* or *cnj2* lines of differing mating types were grown and mated for a careful analysis of nuclear events. Figure 3 shows DAPI-stained preparations of both [*cnj1* × *cnj1*] and [*cnj1* × wildtype] matings (*cnj2* matings were indistinguishable from the *cnj1* phenotype). After 12 hr of mating, we saw pairs with elongated chromatin bundles (Fig. 3A), pairs with enlarged micronuclei (Fig. 3B), or pairs in which micronuclei were absent (not shown). The earliest defects made visible by DAPI staining were best seen in matings between *cnj1* and a wild-type partner (Figs. 3C–3H). In these heterotypic pairs, the wild-type partner serves as a developmental timepiece. There appeared to be a failure of chromatin condensation just following a normal looking crescent stage and just prior to meiosis I (Figs. 3C–3E) and meiosis II (Figs. 3F and 3G). Spindle-like nuclear elongations appeared to assemble and disassemble on schedule (Figs. 3D–3G), yet chromosome segregation and consequent nuclear divisions were suppressed. The *cnj1* and *cnj2* micronuclei appear to stretch out (presumably with microtubule assembly) and relax back into an undivided mass of decondensed chromatin (presumably with microtubule disassembly). Microtubule assembly was made apparent by staining [*cnj1* × wildtype] pairs with antisera against β-tubulin (see Figs. 4A and 4B). Clearly, tubulin assembly is occurring on schedule in the mutant cells. It should be noted that spindles were seen in [*cnj1* × *cnj1*] matings as well, although it was impossible to identify which nuclear division was occurring.

It also appeared that, in *cnj1* matings, the micronuclei were increasing in size throughout the course of development (Fig. 3H), hence the original phenotype. This led us to suspect that the schedule for DNA synthesis was proceeding normally despite nuclear division failure (see Fig. 1). To test this hypothesis, Feulgen cytophotometry was performed and DNA content was measured for individual mutant micronuclei in wild-type (premeiotic) and mutant cells (at the time of pronuclear exchange) (see Table 2). The *cnj1* micronuclei remained essentially 2N4C throughout prezygotic development leading up to and including the exchange configuration, suggesting that their enlarged appearance was simply the result of chromatin decondensation.

Figures 5 and 6 show developmental profiles respectively of mating [wildtype × wildtype] and [*cnj1* × *cnj1*] cells. *cnj2* matings showed a developmental profile remarkably similar to that of *cnj1*. Clearly, nuclear divisions are suppressed (probably due to a failure of chromatin condensation), though the cycle of cytoskeletal assembly/disassembly associated with spindle formation appears to proceed on schedule. DNA replication is also suppressed. These early defects occasionally resulted in failure of ''nuclear selection'' and subsequent elimination of all micronuclei via the abortive ''genomic exclusion'' pathway (Allen, 1967; Doerder and Shabatura, 1980). This is a common alternative developmental pathway seen in cells with defective micronuclei. It should be noted, however, that many *cnj1* pairs still possessed an unresorbed micronucleus even after 10–12 hr, suggesting that many cells do ''select'' and protect their single, undivided micronucleus against programmed nuclear elimination. Subsequently there is retention of the macronucleus and aborted postzygotic development which are both characteristics of the genomic exclusion pathway.

Another aspect of the *cnj1, cnj2* phenotypes is a vegetative macronuclear defect (see Figs. 7A and 7B). During vegetative growth, wild-type cells typically undergo an amitotic macronuclear division resulting in a random assortment of polyploid macronuclear material being distributed to each daughter cell (Orias and Flacks, 1979; Nanney and Prepar-

FIG. 2. DAPI-stained fluorescence micrographs of early stages in normal conjugal development. (A) Micronuclei migrate out from macronuclear proximity. (B) Chromatin begins to extend unidirectionally. (C) Chromatin extends out bidirectionally. (D) ''Crescent'' stage chromatin has elongated due to intranuclear microtubule polymerization. (E) Crescent has retracted, chromatin begins to condense into a diffuse, elongate cloud. (F) Chromosomal bivalents begin to condense. (G) Anaphase of first meiosis delivers five replicated chromosomes to each pole. (H) End of meiosis I. Five replicated chromosomes appear at each pole. (I) Chromatin diffuses into nuclear spheres. (J) Extension of first meiotic spindle delivers posterior nucleus to anterior cytoplasm where it joins the other. (K) Second meiosis (anaphase). (L) Unreplicated chromosomes appear in four discrete groups within each mating partner (nuclear membrane still intact). (M) Chromatin becomes decondensed. (N) In each cell, one (or occasionally two) meiotic product remains decondensed (see arrows). Other nuclei condense into brightly staining ''relics'' destined for elimination. (O) Spindle forms for third prezygotic (gametogenic) nuclear division (closed arrow). Occasionally, a second nucleus remains decondensed as well (open arrow), sometimes forming a secondary ''pseudospindle'' (not shown). The formation of the third prezygotic division spindle marks the end of early development (by our nomenclature).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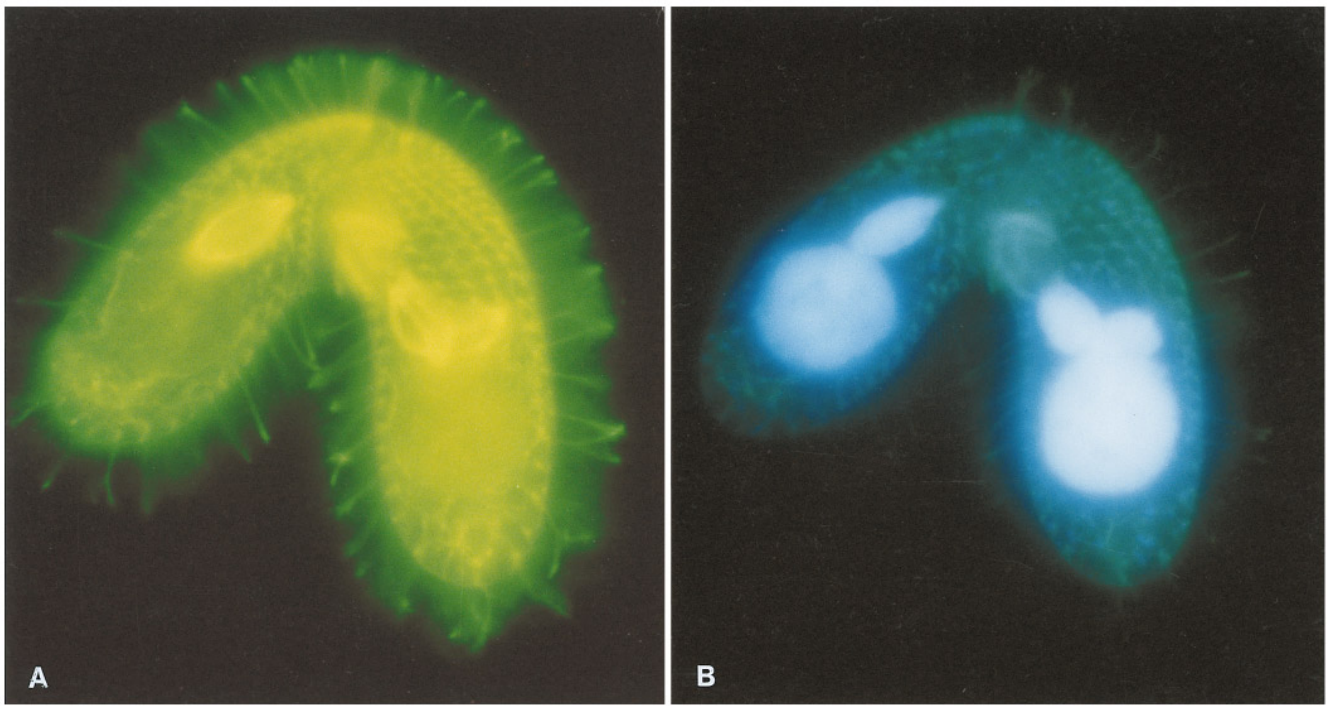

FIG. 4. (A) [*cnj1* × wildtype] mating stained with a polyclonal antiserum raised against dog-brain tubulin, visualized with FITC-conjugated secondary antiserum. (B) The same pair stained with DAPI to visualize nuclear configurations. The wild-type partner (viewer's right) is initiating the second meiotic nuclear division. Note two orthogonally oriented spindles in A.

ata, 1979; Cleffmann, 1980). In the *cnj1* and *cnj2* vegetative cells, a large body of macronuclear material is left behind at the midbody fission zone following macronuclear division (Fig. 7A, arrow). These ''chromatin extrusion bodies'' or CEBs (see Cleffmann, 1980), persist in the cytoplasm for some time as DAPI-staining satellites (Figs. 7A and 7B) and provide a fairly reliable vegetative marker of this mutant phenotype (they were far less prevalent in our wild-type controls). It should also be noted that during vegetative micronuclear divisions, chromatin condensation was successful and micronuclei appeared to divide normally (see Fig. 7B, arrow).

Finally, when mating [*cnj1* × wildtype] cells were stained with the antiserum 3A7 [a monoclonal antiserum raised against the *Tetrahymena* protein fenestrin (Nelsen *et al.*, 1994)], we saw that, although the wild-type partner showed antibody labeling of its two pronuclei (Figs. 8A and 8B), the *cnj1* partner showed no labeling, indicating that pronuclear differentiation doesn't occur in the *cnj1* partner. Fenestrin has been shown to decorate both migratory and stationary pronuclei exclusively just prior to nuclear exchange (Nelsen *et al.*, 1994). This makes fenestrin decoration a useful marker for pronuclear differentiation.

cnj3. *cnj 3* pairs showed a peculiar shortening of the crescent structure (not shown) and a striking phenotype in which instead of five chromosomal bivalents we saw a single apparently compound chromosomal bundle (Fig. 7C). Though meiosis I and meiosis II were frequently successful, not all nuclear divisions proceeded without problem. We frequently saw failures of meiosis I or II and abnormal looking anaphase figures in which ''bridging chromosomes'' were apparent (see Fig. 7D). Later defects involved sporadic

FIG. 3. DAPI-stained images of [*cnj1* × *cnj1*] and [*cnj1* × wildtype] matings. (A) [*cnj1* × *cnj1*] pair showing elongated masses of decondensed chromatin. (B) [*cnj1* × *cnj1*] pair showing enlarged micronuclei after 8 hr of pairing. In (C–H), a wild-type partner is on the left and the *cnj1* partner is on the right. (C) Chromatin condensation in wild-type partner, decondensed cloud of chromatin in mutant. (D) Anaphase of meiosis I in wild-type partner, elongate decondensed cloud of chromatin in mutant. (E) Completion of meiosis I in wild-type partner, elongate, decondensed cloud of chromatin in mutant. (F) Second meiosis in wild-type partner, elongate, decondensed cloud of chromatin in mutant. (G) Completion of meiosis II in wild-type partner, elongate, decondensed cloud of chromatin in mutant. (H) Completion of meiosis II in wild-type partner, enlarged, decondensed cloud of chromatin in mutant.

TABLE 2

DNA Content of *cnj1* Mutant Micronuclei at the Time of Pronuclear Exchange

Mating	[DNA] of postexchange micronuclei measured in relative absorbance units
CU427 × CU428 (3 hr into mating: diploid, 2N4C nuclei were measured as a standard for comparison)	18.3 ± 5.8
CNJ1 × CU427 (postexchange stage, enlarged *cnj1* micronuclei were measured)	18.5 ± 2.3

Note. Ten samples were measured for each mating; values were averaged. ±, standard deviation.

failures in postzygotic nuclear divisions resulting in abnormal numbers and sizes of micronuclei and macronuclear anlagen at the end of development (Fig. 7E).

The developmental profile of *cnj3* (Fig. 9) showed that although many pairs underwent an aborted ''genomic exclusion'' pathway, those pairs that were successful appeared to keep to the wild-type developmental schedule. We suspect that development was aborted when at least one partner was unable to complete the third prezygotic division, discarded all of its meiotic nuclei, and behaved as a ''star partner'' (Allen, 1967; Doerder and Shabatura, 1980).

cnj4. The terminal phenotype for *cnj4* matings was a pair of amicronucleate cells. DAPI analysis suggested that *cnj4* resulted in a phenotype in which meiosis I was normal, yet nuclei failed to enter meiosis II (Figs. 7F and 7G). Fenestrin labeling indicating pronuclear differentiation was never observed (Figs. 8C and 8D). Within *cnj4* pairs, both meiosis I products degenerated as ''relic micronuclei.'' This suggested either that meiosis II was necessary for ''nuclear selection'' to occur or that *cnj4* partners exhibited premature degradation of their micronuclear relics.

It has occurred to us that this phenotype is reminiscent of some of the star cell mating phenotypes (Pitts, 1979; Allen, 1967). These are cells which possess defective micronuclei [micronuclei which have become aneuploid or in some other way degenerate due to ''senescence'' (Nanney, 1974; Pitts, 1979)]. Such mating partners frequently complete meiosis I or meiosis II and yet fail to complete the third prezygotic division and abort development after discarding all of their nuclear remnants. In order to determine whether or not the *cnj4* cell line has simply become star-like, we performed a karyotype of *cnj4* cells by chromosomal squashing early in meiosis. Results from such a squash preparation (not shown) revealed cells with normal chromosome configurations as well as occasional incidences of tetraploidy suggesting mitosis problems during vegetative division. (Indeed, both amicronucleate and polyploid-appearing micronuclei are seen in *cnj4* vegetative stocks.) A developmental profile is not shown, but can be described as normal through meiosis I, followed by a rapid degeneration of both meiosis I nuclear products.

cnj5. We first isolated *cnj5* based upon the appearance of an exaggerated synkaryon stage with no micronuclear relics (Fig. 7I). Developmental analysis revealed that *cnj5* matings skip both meiosis I and meiosis II (despite forming normal looking crescent figures), yet the resident micronucleus successfully completed the third prezygotic (gametogenic) division, resulting in a pair of enlarged migratory and stationary pronuclei (Fig. 7H). Our conclusions regarding which nuclear divisions were skipped and which were successful were based largely upon the developmental profile (Fig. 10). *cnj5* anaphase configurations were not seen until 5 hr after mixing, and pairs with multiple nuclear products were not seen until well after this. These first anaphase configurations appeared precisely at the time that wild-type pairs initiated their third prezygotic division, the gametogenic division (compare Figs. 5 and 10).

Further evidence that *cnj5* pairs skipped meiosis and only underwent micronuclear division at the postmeiotic mitosis comes from anti-fenestrin labeling. When the mutant exchange configuration was assumed we found that fenestrin had indeed decorated the two nuclei, suggesting that they had differentiated into pronuclei (Figs. 8E and 8F). Fenestrin labeling of nuclei typically occurs only after the third prezygotic division (Nelsen *et al.,* 1994). Postzygotic divisions occurred, rarely producing some ''endpoints'' with macronuclear anlagen, yet these frequently possessed aberrant numbers of nuclear figures. More commonly, pairs were seen with enlarged (probably polyploid) micronuclei, yet retaining their parental macronuclei. It would seem that these pairs were behaving like genomic exclusion matings (described above).

cnj5 cells also exhibited a vegetative growth defect. Cell cultures expressing the homozygous *cnj5* mutation in their macronuclei have a very limited ''shelf life.'' Tube cultures appeared to terminate after several weeks of culture rather than after months of culture as do wild-type clones. This clonal deterioration was accompanied by the appearance of amicronucleate cells within the culture. The exact nature of this deterioration is uncertain and it makes the *cnj5* line difficult to work with. Nevertheless, fresh homokaryons can be regenerated from the heterokaryon stock.

DISCUSSION

Overview

We have successfully developed an efficient method of generating mutations that affect developmental processes occurring during conjugation in the ciliate *T. thermophila.* A summary of the mutant phenotypes affecting early developmental events appears in Fig. 11. This developmental program is valuable in that during a 12-hr period (at 30°C) one can witness 10^6 cells (or more) synchronously initiating

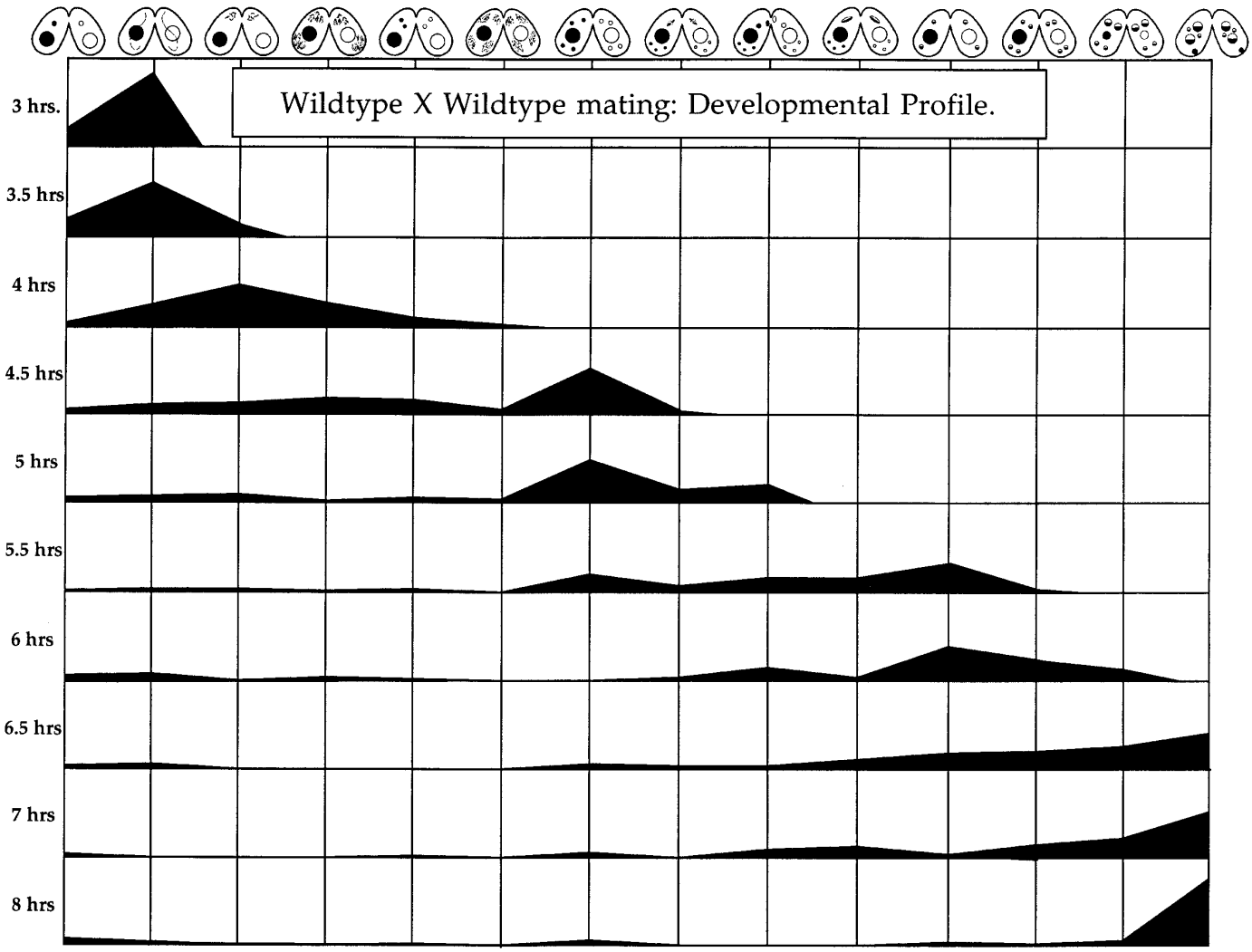

FIG. 5. Wild-type developmental profile. 100 pairs were scored for each time point. The *y* axis represents the percentage of the sample in each given stage.

such fundamental events as meiosis, mitosis, pronuclear differentiation, and fertilization. Several features make ciliates a unique and promising model system for the study of nuclear behaviors associated with mating. These cells can tolerate gross chromosomal damage within the micronucleus while remaining viable. The transcriptionally active macronucleus (which determines the cell's phenotype) utilizes an amitotic division pathway that is largely independent of the more conventional "mitotic" machinery driving micronuclear divisions. Hence, we can construct mutant homozygotes involving genes which govern the meiotic and mitotic behavior of the micronucleus without damaging the amitotic activities which maintain the macronucleus. This unique situation may allow us to generate a class of mutations which would be lethal in other eukaryotes and yet remain viable in *Tetrahymena.* Clearly, identification and characterization of genes whose products mediate such activities is of general interest. We are particularly interested, however, in what these phenotypes can tell us regarding developmental dependencies that exist during the conjugal pathway itself.

Developmental Dependencies during Conjugation

Developmental events can often be ordered into dependent pathways in which the initiation of late events depend upon the completion of early events. Control mechanisms enforcing such dependencies have been termed "checkpoints" and it has been noted that some such dependencies can be relieved by mutations which in turn serve to identify the genes that control those particular developmental decisions (Hartwell and Weinert, 1989). We are just beginning

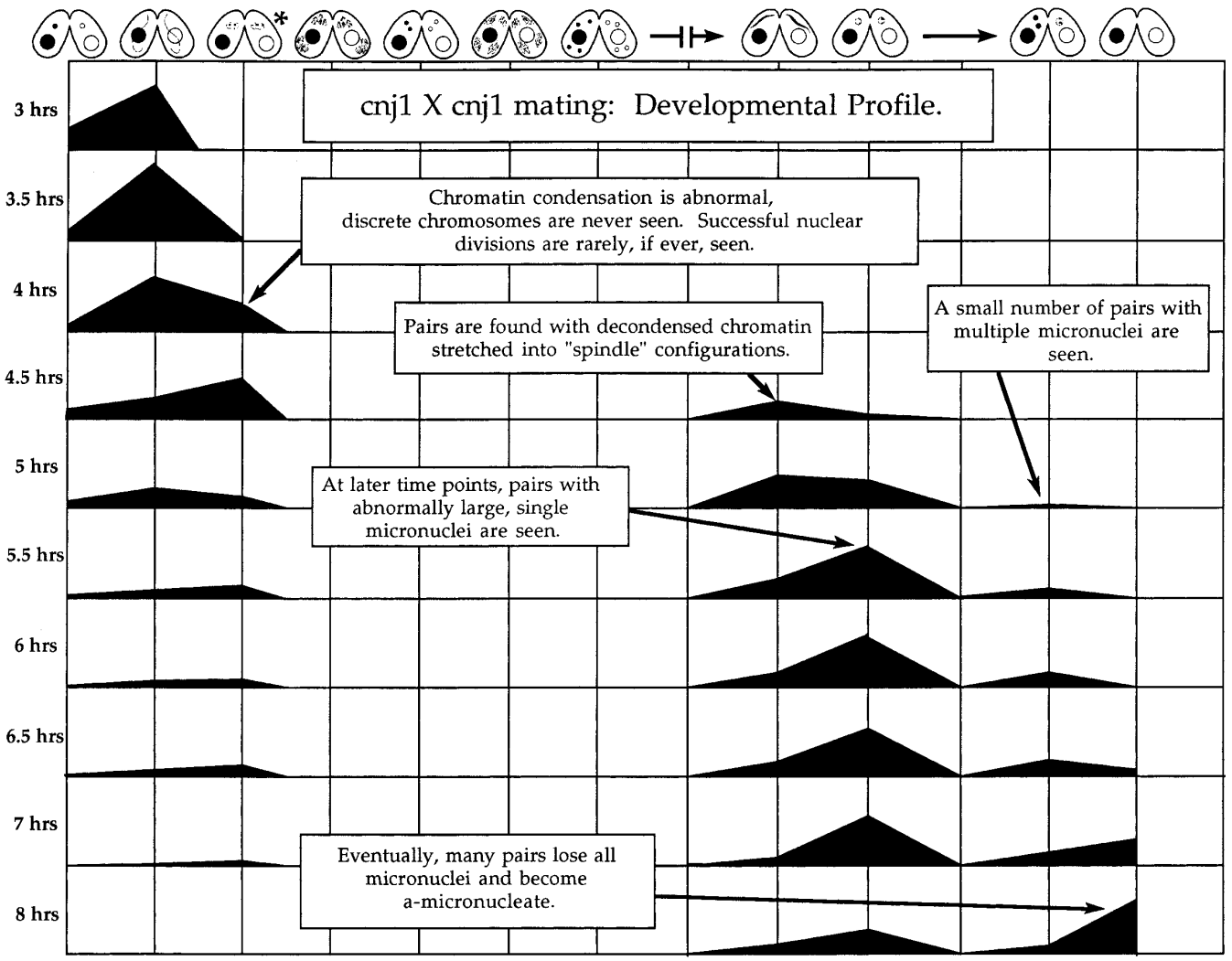

FIG. 6. Developmental profile of [*cnj1* × *cnj1*] matings. 100 pairs were scored for each time point. The *y* axis represents the percentage of the sample in given stages for each given time point.

to recognize developmental dependencies that occur during conjugation in *T. thermophila*. In particular, mutations which eliminate specific developmental steps are proving useful in dissecting the "developmental logic" of this particular program.

DNA synthesis, spindle assembly, and karyokinesis. From *cnj1* and *cnj2* we have learned that blocking nuclear division in *Tetrahymena* has no discernible effect upon the cycle of cytoskeletal assembly. Spindles assemble and disassemble on cue throughout the three prezygotic divisions despite failure of karyokinesis. This would appear to demonstrate that cytoskeletal regulation is independent of successful nuclear division.

In contrast, DNA synthesis appears to be dependent upon some activity associated with the successful completion of the second meiotic and third, prezygotic nuclear divisions. This is evident in that *cnj1* micronuclei remain at a 2N4C amount of DNA throughout early development even when cytoplasmically linked to a wild-type partner which has completed both the second meiotic and the third prezygotic division and hence two rounds of DNA synthesis (Fig. 1; see also Doerder and Shabatura, 1980). It would appear that DNA synthesis is dependent upon successful karyokinesis. Our observations at first seem contradicted by reports of DNA synthesis occurring in cells whose nuclear divisions have been blocked by drugs that affect spindle assembly (Kaczanowski *et al.*, 1985; Gaertig *et al.*, 1986). In these studies, nuclear divisions were effectively blocked by the anti-microtubule drug nocodazole, and yet subsequent rounds of DNA synthesis (associated with chromosome replication) were observed. These authors report one singular observation which sets their drug response apart from

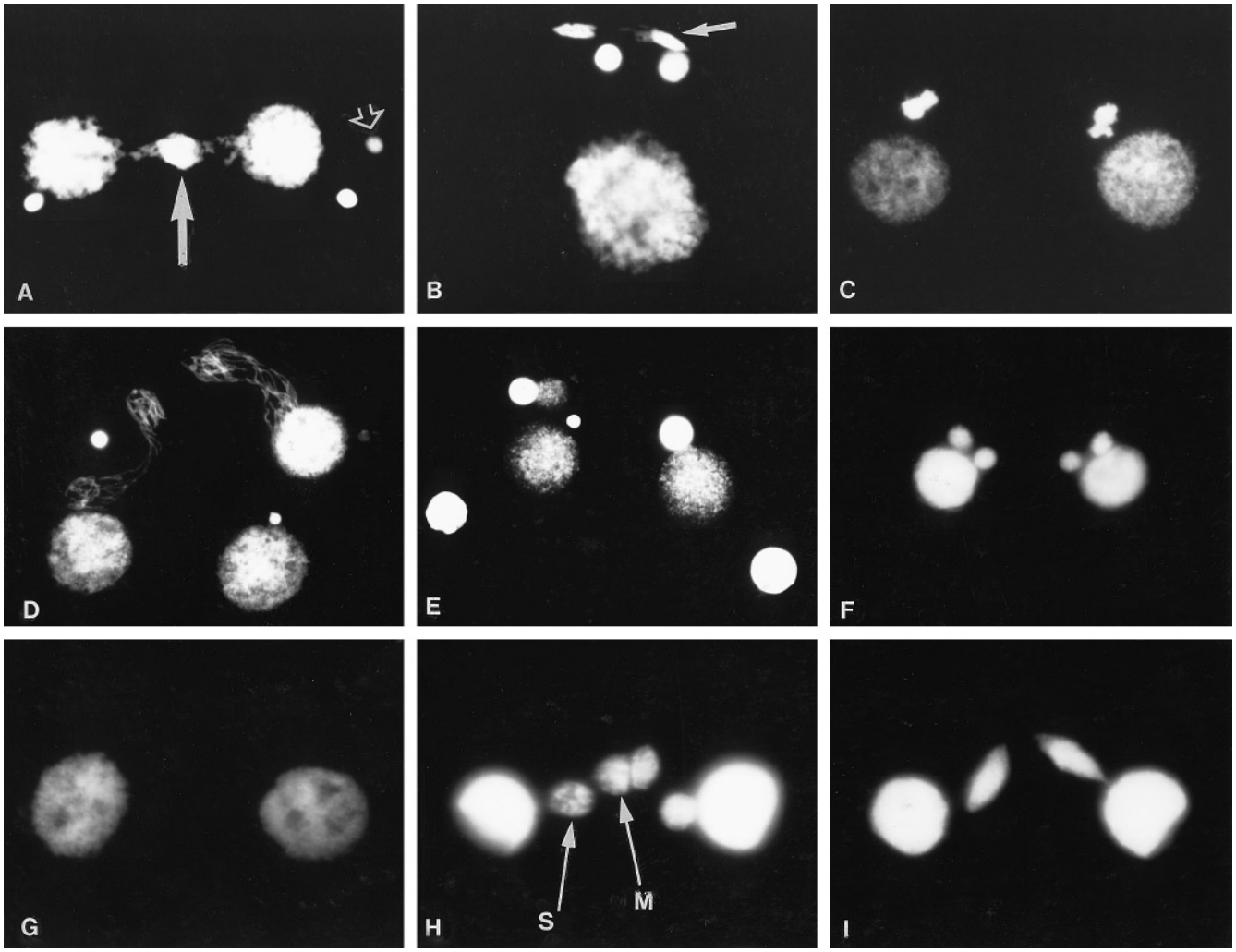

FIG. 7. A collection of mutant, DAPI-stained nuclear configurations. (A and B) Vegetatively growing *cnj1* cells. (A) A dividing cell showing the ''bleb'' of nuclear material being cast off at midbody (solid arrow). The two micronuclei are also visible as is a preexisting nuclear ''satellite'' (CEB) from a previous division (open arrow). (B) A single cell undergoing micronuclear mitosis (arrow indicates micronuclear mitotic figure). Chromosome strands are clearly visible. (Two satellites are also visible just below spindle.) (C–E) [*cnj3* × *cnj3*] pairs. (C) The unique chromosomal bundle at prophase of meiosis I. (D) Abnormal looking anaphase figures. (E) Abnormal endpoints with nuclei of varying sizes. (F and G) [*cnj4* × *cnj4*] *pairs. (F) cnj4* pair just after meiosis I. (G) Amicronucleate condition for *cnj4* pairs (7 hr after mating). (H and I) [*cnj5* × *cnj5*] mating pairs. (H) A *cnj5* pair just after its one and only prezygotic division. Note decondensed state of nuclei. Migratory (M) and stationary (S) pronuclei are clearly distinguishable, with migratory pronuclei aligned at the exchange junction. (I) A *cnj5* pair just after pronuclear exchange. Note enlarged synkarya.

our mutant phenotype; in nocodazole-treated cells, chromosomes condense and centromeres divide despite a failure of karyokinesis. We see no evidence for chromatin condensation or centromere division in either *cnj1* or *cnj2*. These results suggest that in *Tetrahymena* conjugation, DNA synthesis is dependent upon successful anaphase fission of the centromeres at meiosis II and the third prezygotic division, a conclusion reported earlier by Doerder and Shabatura (1980) (see our Fig. 12). This also suggests that nuclear divisions occurring during *Tetrahymena* conjugation are more similar to the highly regulated somatic nuclear divisions of higher eukaryotes than the relatively unregulated cleavage divisions of multicellular embryos (see Murray and Hunt, 1993).

Nuclear selection and pronuclear differentiation. It has long been recognized that there is something special about the nucleocytoplasmic interaction occurring at the nuclear exchange junction that functionally determines the different fates of the meiotic nuclear products. Sonneborn described this phenomenon in *Paramecium* (1951, 1954)

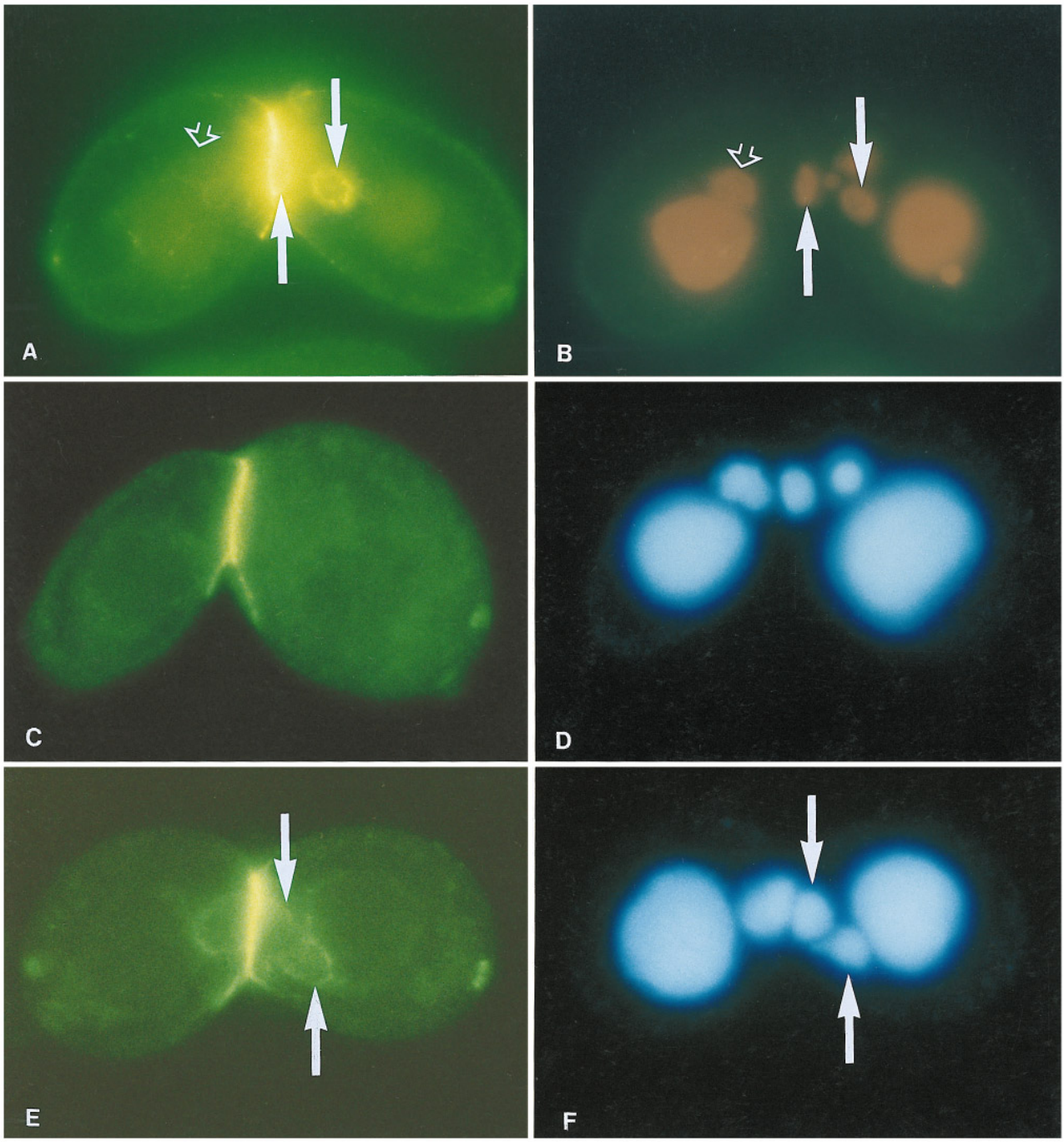

FIG. 8. Various mutant pairs stained with both 3A7, a monoclonal antiserum directed against the protein fenestrin visualized with an FITC-conjugated secondary antiserum (left), and DAPI nuclear stain (right). (A and B) [*cnj1* × wild-type]. The *cnj1* partner is on the left, the wild-type partner is on the right. Note absence of antibody labeling in *cnj1* partner. Open arrow indicates location of undivided *cnj1* micronucleus, solid arrows indicate fenestrin-staining wild-type pronuclei. (C and D) [*cnj4* × *cnj4*]. Note no antibody labeling of either partner at 5 $\frac{1}{2}$-hr time point. (E and F) [*cnj5* × *cnj5*]. Note fenestrin labeling of both *cnj5* nuclei (arrows).

though it was clearly known even to Hertwig (1889) and Maupas (1889). Nanney described the same event in *Tetrahymena* (1953) and suggested that once a single nucleus enters this special cytoplasmic region, all other nuclei migrate posteriorly, away from the exchange junction, thereby preventing more than one nucleus from being selected. In-

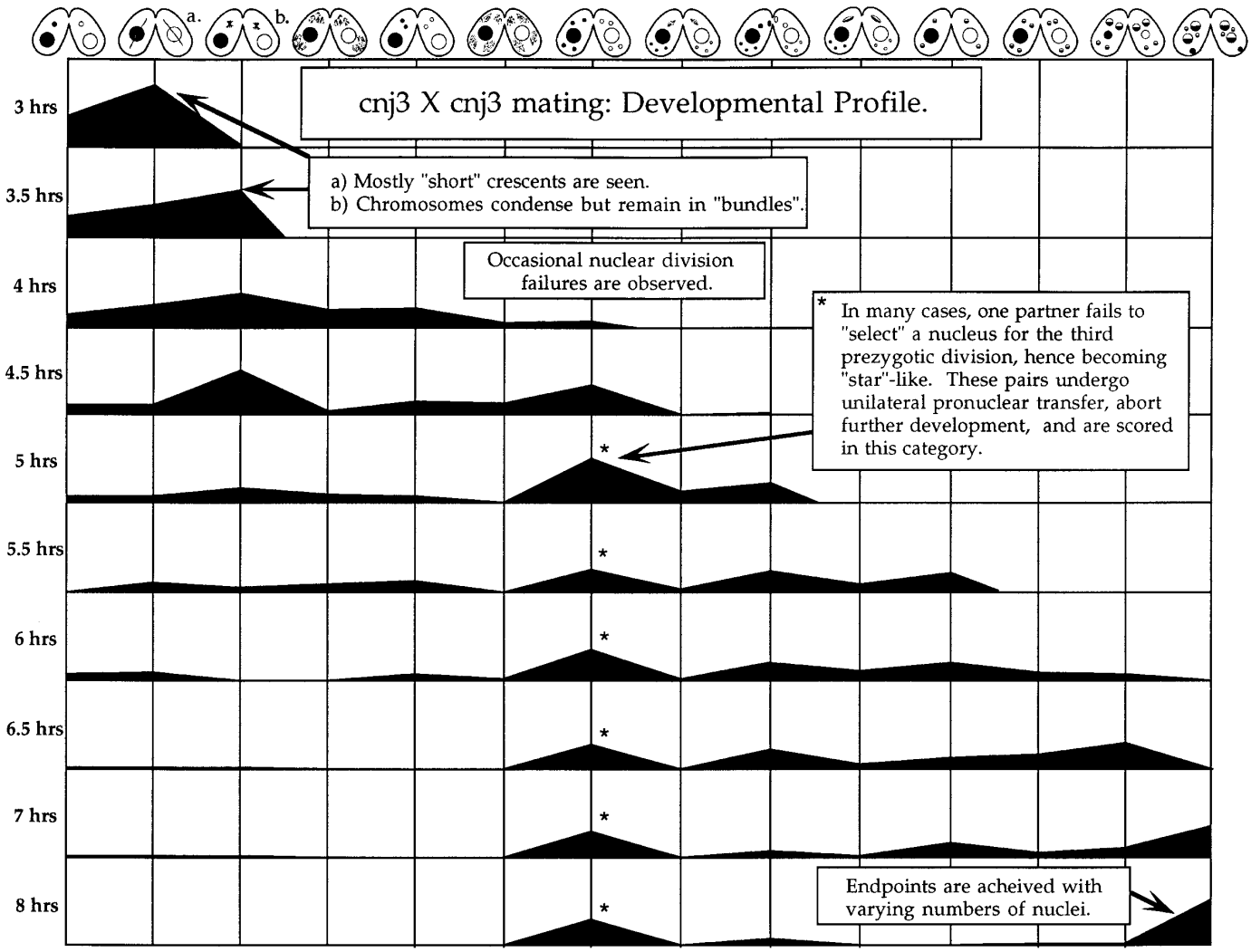

FIG. 9. Developmental profile for [*cnj3* × *cnj3*]. 100 pairs were scored for each time point. The *y* axis represents the percentage of the sample in given stages for each given time point.

deed, when a nucleus is removed from the paroral region in *Paramecium*, another micronucleus will migrate in to take its place (Yanagi, 1987). The special status of the selected nucleus is also made evident by its association with specific cytoskeletal proteins. Tubulin becomes associated with the selected nucleus (Gaertig and Fleury, 1992) as does a unique 49-kDa filament-forming protein (Numata, 1996).

In both *Tetrahymena* and *Paramecium*, the selected nucleus undergoes a third "gametogenic" division. The resulting pronuclei, variously described as the migratory and stationary pronuclei (Nanney, 1953) or the transfer and resident pronuclei (Nelsen *et al.*, 1994), are now ready to participate in the nuclear exchange event. They are distinguished biochemically by association with the 64-kDa protein fenestrin (Nelsen *et al.*, 1994) and a 25-kDa calcium-binding protein (Hanyu *et al.*, 1995). Once pronuclear exchange occurs, all such protein affinities disappear as the transfer pronuclei migrate and fuse (karyogamy) with the reciprocal resident pronuclei forming a zygotic nucleus within each mating partner.

Using the *cnj1–cnj5* mutants we can begin to ask which steps are necessary or sufficient for nuclear selection and pronuclear differentiation to occur. The persistence of a single, decondensed micronucleus in *cnj1* and *cnj2* mating cells and its apparent attempts to participate in all three prezygotic nuclear divisions suggests that this aberrant nucleus is, in fact, "selected." Further immunological studies should be conducted to confirm this, but the persistence of the micronucleus in a decondensed form for over 10 hr into the conjugation program argues that this nucleus has not become a "relic" nucleus. Nuclear selection is further evidenced by the observation that *cnj1,2* nuclei display a third

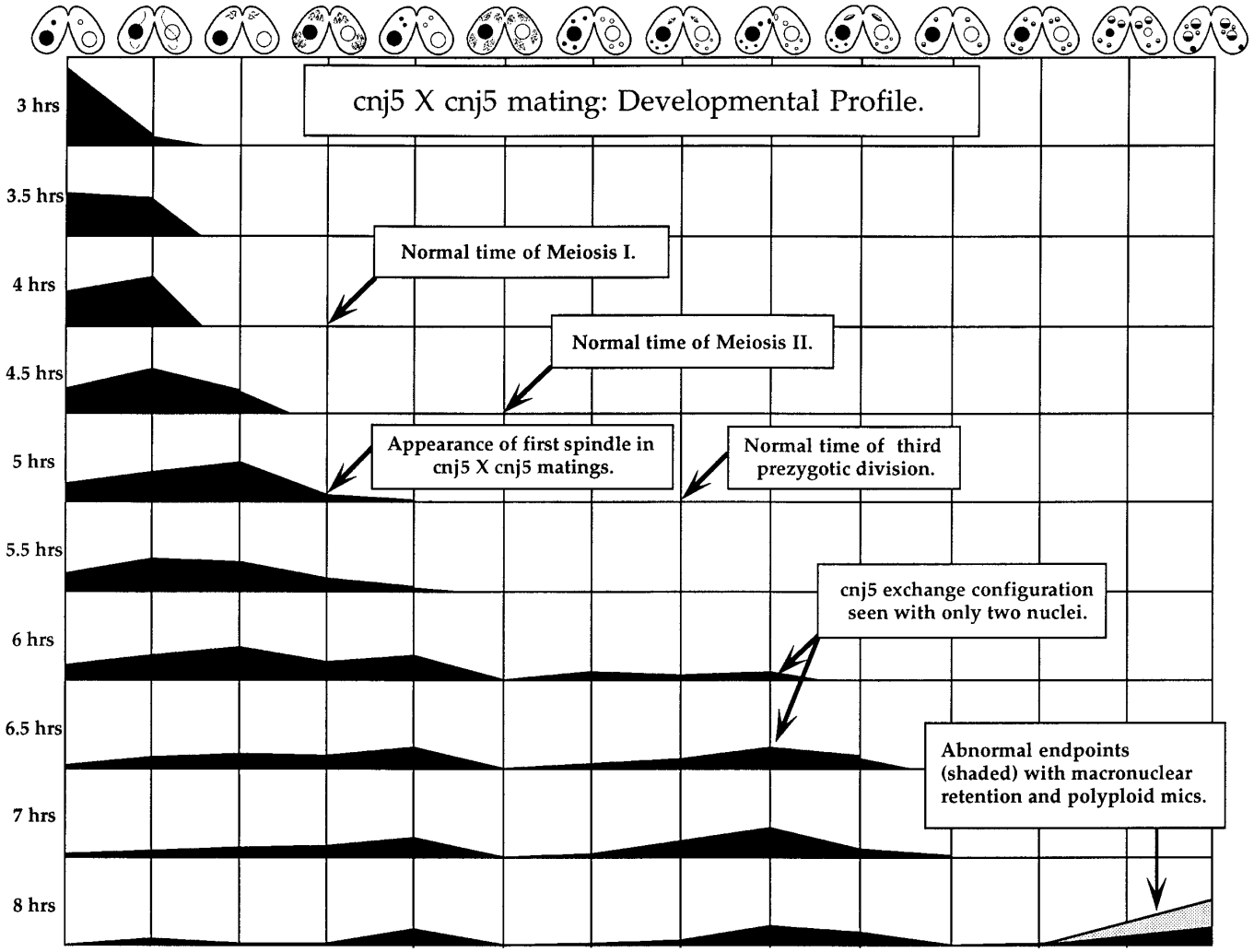

FIG. 10. Developmental profile for [*cnj5* × *cnj5*]. 100 pairs were scored for each time point. The *y* axis represents the percentage of the sample in given stages for each given time point.

division spindle (at least when paired with a wild-type partner) and often assume an extreme anterior localization within the cytoplasm. Nevertheless, these nuclei never appear anchored to the exchange junction and never acquire features associated with pronuclear differentiation (fenestrin decoration). Hence we suggest that nuclear selection and subsequent third-division spindle formation can be triggered by anterior localization of a nucleus at the appropriate time, but pronuclear differentiation (fenestrin/TCBP-25 decoration) may require actual physical docking of a nucleus at the exchange junction prior to the gametogenic nuclear division.

This is consistent with our observation of cells in which both a genuine third division spindle and an occasional pseudospindle in a second anterior meiotic product appear in wild-type cells, accompanied by failure of any but the ''true'' pronuclei to show fenestrin labeling (unpublished observations). This view is also consistent with Gaertig and Fleury's (1992) observations of microtubule ''decoration'' of both anteriormost meiotic products.

cnj5 matings prove that neither of the two meiotic divisions is essential for nuclear selection, the third prezygotic division, and pronuclear differentiation to occur. In the accompanying paper (Cole and Soelter, 1997), the mutants *cnj7* and *cnj8* are shown to complete meiosis but fail to complete the third prezygotic mitosis. Nevertheless, pronuclear differentiation does transform two of the meiotic products into migratory and stationary pronuclei, respectively. These phenotypes taken together suggest that pronuclear differentiation may in fact be independent of all three prezygotic divisions. (Pronuclear differentiation may fail in *cnj4* cells due to selection failure and subsequent nuclear elimination.)

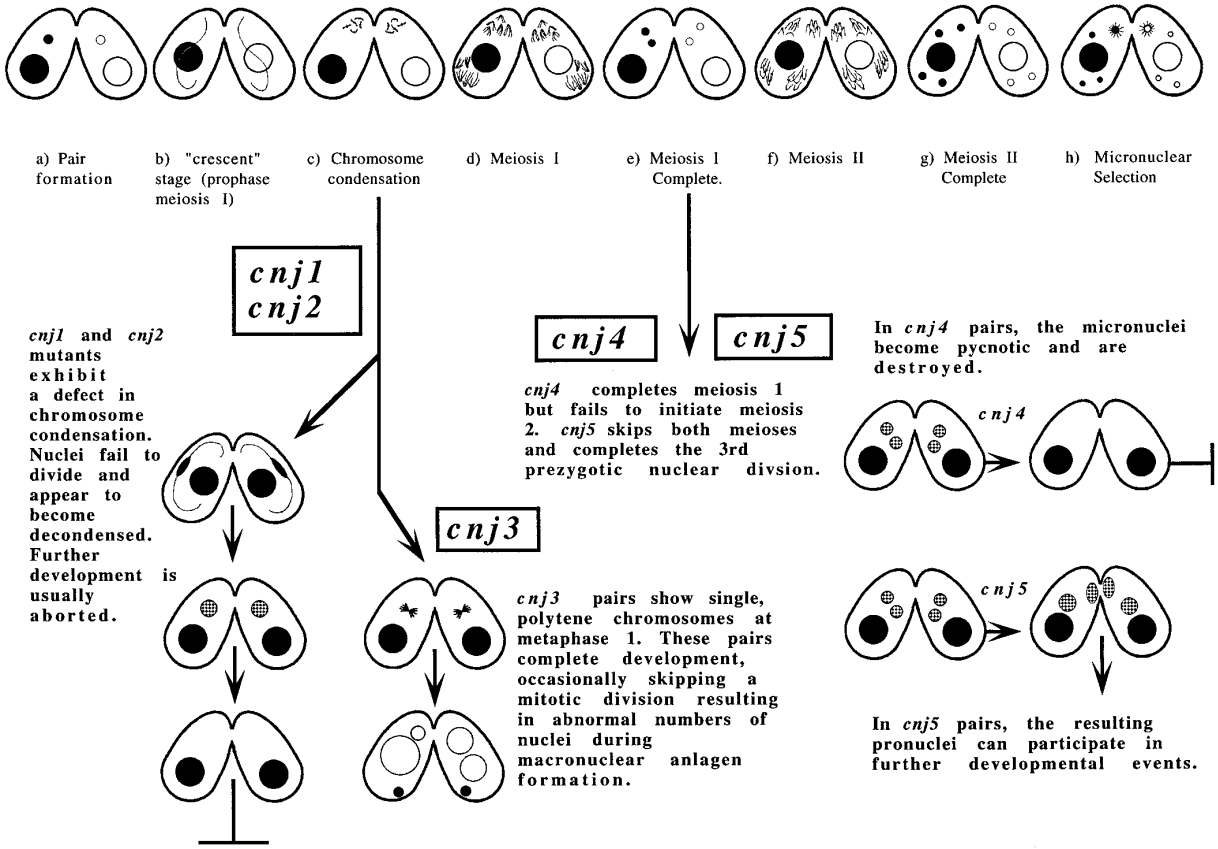

FIG. 11. Summary of nuclear behavior during early stages of conjugation in *T. thermophila* and phenotypes of some conjugation mutants. Lines ending in a crossbar indicate lethal endpoints.

The emerging model of developmental dependencies governing early conjugation in *Tetrahymena* can be summarized as follows (Fig. 12). Pair formation sets in motion an internal developmental clock which triggers each of the three prezygotic nuclear divisions independently. Therefore any one of the early nuclear divisions can be eliminated, and subsequent nuclear divisions are initiated more or less on schedule. Three other developmental events also appear to be triggered at a reasonably ''fixed'' time interval after pair formation: nuclear selection, nuclear ''relic'' condensation leading to nuclear resorption, and pronuclear differentiation. Pronuclear differentiation is dependent upon nuclear selection but does not appear to be dependent upon any of the three prezygotic nuclear divisions. Finally, the two rounds of DNA synthesis appear to require some signal (centromere fission?) associated with successful nuclear division at prezygotic nuclear divisions 2 and 3.

Potential Candidates for the Early Acting cnj Genes

cnj1 and *cnj2* mutants appear to result in abnormal chromatin condensation. Gene products that affect micronuclear chromatin condensation include those that regulate histone phosphorylation (Sweet and Allis, 1993) and topoisomerase II, which has been implicated in chromatin condensation (Rose *et al.*, 1990). More recently, a new class of molecules (the SMC motor proteins) has been implicated in chromatin condensation (see Peterson, 1994). Of considerable interest here is an elegant cytological study using drugs which produce the ''opposite'' effect, that is, an overcondensation of chromatin during meiosis (Kaczanowski and Kaczanowska, 1996). These researchers have demonstrated that cycloheximide and actinomycin D (agents that block translation and transcription, respectively) result in an inability of mating cells to regulate chromatin condensation. Such cells appear to lack some gene product which normally arrests and reverses the chromatin condensation process. It will be interesting to see whether or not *cnj1* and *cnj2* mutants will exhibit chromatin condensation when treated with these agents.

cnj3, which produces a single chromosome ''bundle'' at prophase/metaphase of meiosis I, is less easily interpreted. Some candidates include defects in recombination or defects in chromosome congression to the metaphase plate. A more interesting possibility is that *cnj3* micronuclei may

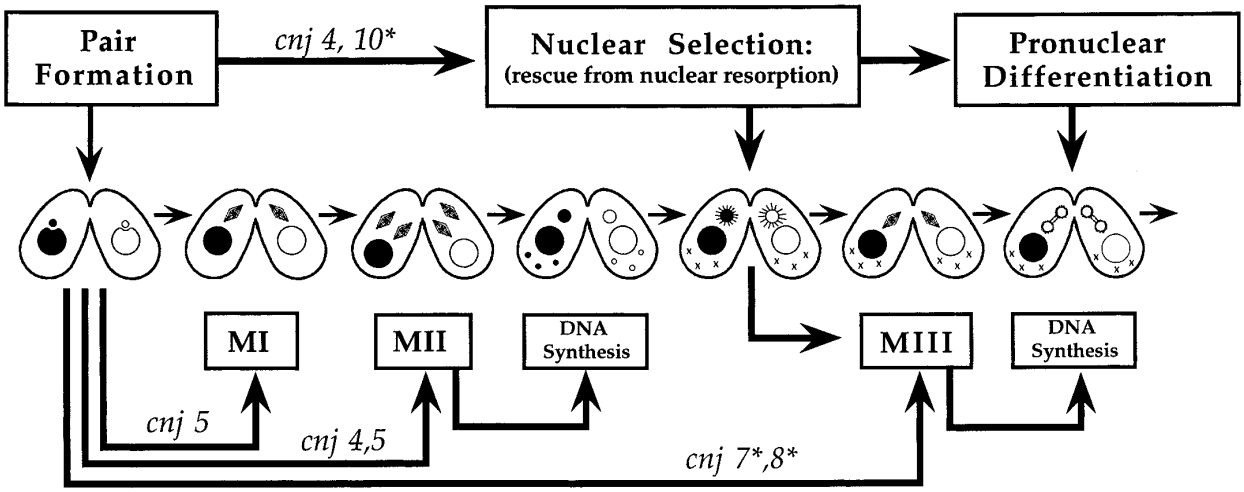

FIG. 12. Developmental dependencies affecting micronuclear behavior during early prezygotic conjugation in *Tetrahymena*. Bold lines with arrows indicate proposed developmental dependencies. Mutant designations refer to those genes whose wild-type activities appear necessary for a given step. Asterisks (*) refer to mutants identified in the accompanying manuscript (Cole and Soelter, 1997). Pair formation appears to be the principle event triggering the program of nuclear behavior associated with conjugation. Four independent programs (at least) are initiated by this event. To the right of the ''pair formation'' block, we see events (requiring wild-type *cnj4* and *cnj10** gene products) which lead to nuclear selection. Results from our mutant analysis suggest that nuclear selection can occur independently of all three prezygotic nuclear divisions. Each nuclear division (MI, first meiosis; MII, second meiosis; and MIII, third ''gametogenic'' nuclear mitosis) can be initiated despite failure of the preceding nuclear division(s). MI requires functional *cnj5* gene product, MII requires functional *cnj4* and *cnj5* gene products, and MIII requires functional *cnj7** and *cnj8** gene products. All nuclear divisions require functional *cnj1* and *cnj2* gene products to be successful, though initiation of nuclear divisions is independent of either gene product. Each of the three nuclear divisions has its own arrow, indicating its ability to occur on schedule without successful completion of the other two nuclear divisions. DNA synthesis occurs following MII and MIII and appears dependent upon some event associated with successful completion of those divisions (centromere fission?). Finally, nuclear selection is necessary for two subsequent events: initiation of MIII and pronuclear differentiation (association with fenestrin). These latter dependencies may be apparent for the trivial reason that without nuclear selection, all micronuclei are targeted for destruction; consequently, no nuclei remain in a competent condition to respond to signals driving either MIII or pronuclear differentiation.

have become both monosomic and polytene (possessing a single highly replicated chromosome) during vegetative growth. This could result from a defect in centromere separation at anaphase (comparable to the *pimple* mutation in *Drosophila;* Stratmann and Lehner, 1996) or a transformation of mitotic vegetative divisions into meiotic reductional divisions. In either case, this hypothesis predicts a progressive loss of chromosomes and endoreduplication of surviving chromosomes during vegetative growth. Recent tests demonstrate that *cnj3* cells are indeed ''polytene unisomics'' and become so as a result of progressive chromosome loss sustained during vegetative cell division (D. Cassidy-Hanley *et al.*, in preparation).

cnj4 pairs complete meiosis I and subsequently destroy all of their micronuclear products. These pairs resemble some of the star cell lines, despite apparently ''healthy'' micronuclear chromosomes (Pitts, 1979). Two possible causes for the *cnj4* defect are a failure to enter the second meiotic division and a consequent nuclear elimination due to failed nuclear selection or early activation of the micronuclear elimination pathway. An analogous phenotype is seen in yeast cells bearing mutations in the *spo14* gene in which first meiosis is completed but meiosis II is arrested (Honigberg *et al.*, 1992; Honigberg and Esposito, 1994). It will be interesting to see if *cnj5* (in which pronuclear differentiation occurs despite a failure in both meioses) is epistatic to *cnj4*.

As mentioned, *cnj5* pairs skip both meiotic divisions and yet apparently proceed with the third prezygotic nuclear division. This mutation offers the exciting possibility of identifying a gene that regulates entry into the meiotic, but not mitotic, nuclear division ''subprograms.''

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