# CYTOGENETICS OF GENOMIC EXCLUSION IN TETRAHYMENA[1]

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GENOMIC exclusion is an abnormal form of conjugation that has been observed in syngen 1 of *Tetrahymena pyriformis* (ALLEN 1963; NANNEY 1963). In crosses of certain clones of a strain to normal clones of other strains, only the genes derived from the normal parent appeared in the progeny, although both exconjugants were recovered, and both were diploid and identical in phenotype. Partial insight into the nuclear basis of genomic exclusion came from the results of a cross of C*, an abnormal clone of the inbred C strain, and a normal clone from the AB strain, heterozygous for *H* serotype alleles distinct from those found in strain C (ALLEN 1963). Only alleles derived from strain AB were recovered in the progeny, and the progeny were distributed among three phenotypic classes in an approximate 1:2:1 ratio. This observation suggested that two meiotic products from the AB parent united to form the syncaryon.

Strain AB is also heterozygous at the phosphatase-1 locus, and three classes of *P-1* progeny appeared in the cross to C*. However, when *H* homozygotes were selected from this cross and scored for their phosphatases, a departure from a 1:2:1 ratio was noted, an increase in the frequency of *P-1* homozygotes being observed (ALLEN 1963). The increase in homozygotes was interpreted as support for one of three hypotheses concerned with the derivation of the two meiotic products contributed by the AB parent. According to that hypothesis, all four meiotic products were elaborated, one of these replicated and the replica fused randomly with any one of the four products to form the syncaryon.

This line of reasoning was based on the erroneous assumption that genomic exclusion included only one round of conjugation. Instead, genomic exclusion includes *two consecutive* rounds of conjugation, and only *one* meiotic product is usually contributed by the AB conjugant. Heterozygotes arise when remating occurs between exconjugants derived from different first round pairs containing different meiotic products. This discovery depended on the adoption of timed matings and on a comparison of the results of "stopped" and "unstopped" matings in a combined genetic and cytological approach. This paper documents the evidence for this conclusion and presents the sequence of nuclear events that occurs during genomic exclusion. A synopsis of this work has appeared elsewhere (ALLEN 1967).

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## MATERIALS AND METHODS

*Materials:* In the analysis of genomic exclusion crosses were made between AB-7a, a clone of the heterozygous AB strain (derived from a cross of A-11613 and B-1214d) and C* (C-5573), a clone belonging to the inbred C strain. AB-7a is mating-type VII, and C* is mating-type III. Cells of the AB clone had normal diploid micronuclei. The cells of C* had defective micronuclei, which were largely hypodiploid (with nuclei smaller than diploid nuclei) or amicronucleate. Control crosses were made between micronucleate clones A-13636b (from inbred strain A) and B-14634b (from inbred strain B). The clone designation, i.e., B-14 63 4b, refers to the B strain, 14th generation of inbreeding, inbred in 1963, and mating-type IV; the letter "b" distinguishes this mating-type IV clone from other clones of similar mating type.

The genotypes of the strains referred to above are, as follows:

| Strain | Genotype |
|---|---|
| AB | $H^A/H^D$; $P\text{-}1^A/P\text{-}1^B$; $mt^A/mt^B$; $E\text{-}1^B/E\text{-}1^B$; $E\text{-}2^B/E\text{-}2^B$ |
| C | $H^E/H^E$; $P\text{-}1^B/P\text{-}1^B$; $mt^C/mt^C$; $E\text{-}1^C/E\text{-}1^C$; $E\text{-}2^C/E\text{-}2^C$ |
| A | $H^A/H^A$; $P\text{-}1^A/P\text{-}1^A$; $mt^A/mt^A$; $E\text{-}1^B/E\text{-}1^B$; $E\text{-}2^B/E\text{-}2^B$ |
| B | $H^D/H^D$; $P\text{-}1^B/P\text{-}1^B$; $mt^B/mt^B$; $E\text{-}1^B/E\text{-}1^B$; $E\text{-}2^B/E\text{-}2^B$ |

The phenotypes are denoted by the following symbols, exemplified by the phenotype of strain AB: Had; P-1ab; mt-ab; E-1b; E-2b. The phosphatase cell types are designated by the symbols $P_1$, $P_3$, $P_5$ etc. and specify which of the five phosphatase isozymes are present (ALLEN 1965). By this criterion strains B and C are $P_1$, strain AB is $P_3$, and strain A is $P_5$.

The genotypes of progeny produced by genomic exclusion were tested in outcrosses to inbred strains A, A1, B, and C1. The genes of these strains pertinent to the outcrosses are listed in Table 9.

*Timed matings:* All operations were carried out at 23°C. For cytological work plateau cultures (2 to 3 days old) grown in 1% proteose-peptone were used, and sterile technique was employed throughout all steps. For genetic work either peptone-grown cultures or Cerophyl-Aerobacter cultures were used, and sterile technique was employed only for the growth of the peptone cultures.

The cultures were washed by centrifugation at approximately 500 $\times$ *g* for 3 minutes in three changes of Dryl's physiological salt solution (DRYL 1959). They were permitted to sit in a fourth wash for 12 to 24 hours, recentrifuged, and a small amount of fresh Dryl's solution was added to achieve a particular concentration of organisms. The crosses were then made with mixtures of the appropriate parental cultures. Mating usually occurred between 1 and 2 hours after mixing. Time 0 was set as the beginning of mating.

When matings were stopped, this was accomplished by the addition of fresh medium to the cultures. In genetic work, Cerophyl-Aerobacter was added, and single pairs were isolated into depression slides containing the same medium. In some crosses exconjugants were placed in separate depressions after the pair came apart. In cytological work, proteose-peptone was added to the depressions 4 hours after time 0. After appropriate time intervals samples containing many thousands of conjugating cells (or only single cells) were collected from several depressions, washed with Dryl's solution, and processed as outlined below.

*Genetic techniques:* Three days after the isolation of pairs, all cultures were examined, and the progeny were classified into three types: mature, dead and immature. Mature cultures contained pairs (when exconjugants were not separated) and reacted sexually with samples of a tester culture of non-parental mating type in a "maturity test." These cultures contained either Round 1 exconjugants (to be described in RESULTS) or nonconjugants, which are parental cells which prematurely separated without undergoing conjugation. Dead cultures included those which were dead as well as those unable to undergo more than two or three fissions. Immature cultures did not contain pairs and were sexually unreactive in the maturity test. These are produced by normal conjugation and include Round 2 exconjugants (to be described in RESULTS).

The viability of any cross is customarily expressed as the frequency of *immature* cultures. This practice will be continued in this and subsequent papers, since it is a useful measure of normal conjugation in a cross. This means that *inviable* cultures include both the dead *and* mature categories.

Samples of immature cultures (and mature cultures in some crosses) were tubed up in Cerophyl-Aerobacter medium, and serotype tests were conducted. Tests for esterases and phosphatases were made on samples of these cultures grown in 1% proteose-peptone. Mating type tests were carried out on clones brought to maturity by serial transfers of single cells at 13-fission intervals. Details of the identification of the *H* serotypes, the *E-1* and *E-2* esterases, the *P-1* phosphatases and the mating types may be found elsewhere (ALLEN 1964a, b; 1965; ALLEN, MISCH and MORRISON 1963a, b; NANNEY and CAUGHEY 1955; NANNEY and DUBERT 1960).

*Cytological methods:* A sample containing several thousand paired and/or single Tetrahymena in Dryl's solution was concentrated by centrifugation into a soft pellet, and approximately 0.01 ml amounts of this pellet were diluted and spread on clean glass microscope slides with NISSENBAUM'S fixative (NISSENBAUM 1953). The slides were stored in 70% ethanol before further processing, usually within 2 weeks. They were then further fixed in 3 parts of 95% ethanol to 1 part of glacial acetic acid for 15–20 minutes; hydrated; hydrolyzed in hot 1N HCl (60°C, 15 min); and stained in hot Gomori's haematoxylin (60°C, 10 min; MELANDER and WINGSTRAND 1953). Cytoplasmic staining was removed by 45% glacial acetic acid (23°C, 15 min). The preparation was covered, gently blotted, flattened over steam, and ringed with Beeswax Sealing Compound (1 part of paraffin to 1 part of gum mastic to 1/5 part of beeswax). The slides were stored, flat, at 4°C to prevent evaporation and to retard decomposition of the stain. These methods were based on those described earlier by RAY (1956) and by WELLS (1961; personal communication).

## RESULTS

*Comparison of stopped and unstopped matings:* Mating in Dryl's solution began as early as 45 minutes and as late as 3 hours after mixing the parental cultures, depending upon the total concentration of cells and the proportions in which the two parental cultures were added. Early mating occurred whenever the concentration of cells was high and the proportion was close to 1:1. In most experiments mating began after about 1½ hours. For convenience 0 time was set at 2 hours. All matings were allowed to proceed undisturbed for 4 hours from time 0. When it was desired to stop matings, an equal volume of 1% proteose-peptone was added to all depressions at 4 hours. This was mixed gently with the contents of the depression. In unstopped matings, run at the same time as their stopped counterparts, Dryl's solution was substituted for peptone.

When matings were stopped, no new pairs (A, in attachment through the crescent stage) were observed after 8 hours in either the normal cross (Table 1) or the AB × C* cross (Table 2). By 12 hours more than 90% of the pairs had reached the postzygotic stages (C, late pairs) in the normal cross, but in the AB × C* cross this frequency was not attained before 14 to 16 hours. By 18 hours 100% of the pairs were undergoing postzygotic divisions in both crosses. At 22 to 24 hours and in subsequent samples no pairs were observed in either cross.

The frequency of new pairs in the unstopped mating of the normal cross fell rapidly over the first 18 hours and approached a constant value in later samples (Table 1, Figure 1). The frequency of late pairs rose and later approached a constant (Table 1).

TABLE 1

*Relative frequency of pairs‡ in various stages of conjugation with increasing time in stopped and unstopped matings of* A × B *(normal cross)*

| Hours from 0 time | Stages: Stopped A† | Stopped B | Stopped C | Unstopped A | Unstopped B | Unstopped C |
|---|---|---|---|---|---|---|
| 4 | 99 | 1 | .. | 100 | .. | .. |
| 6 | 64 | 36 | .. | 75 | 25 | .. |
| 8 | 11 | 85 | 4 | 44 | 53 | 3 |
| 10 | .. | 63 | 37 | 29 | 44 | 27 |
| 12 | .. | 7 | 93 | 22 | 38 | 40 |
| 14 | .. | 1 | 99 | 16 | 20 | 64 |
| 18 | .. | .. | 100 | 8 | 13 | 79 |
| 22 | .. | .. | § | 6 | 14 | 80 |
| 24 | .. | .. | § | 9 | 16 | 75 |
| 36 | .. | .. | § | 5 | 10 | 85 |

† Stages A = Attachment through crescent, B = MI metaphase through fertilization, C = Postzygotic.
‡ Each sample consisted of 100 pairs.
§ No pairs were observed.

A pronounced difference was observed in the AB × C* cross, compared to the normal cross. The frequency of new pairs fell to 2.5% at 16 hours, rose to a second peak of 45% at 24 hours, then fell to a new low of 4.2% at 36 hours (Figure 1). The frequency of late pairs also fluctuated. Up to 12 hours it was similar to the stopped series (Table 2); but after 12 hours it continued to rise, dipped at 24 hours and then rose again. The timing of these fluctuations, particularly that of new pairs, was of some interest, since it occurred after 12 to 16 hours, a time interval sufficient to complete a normal round of conjugation. This observation suggested that a second mating takes place during genomic exclusion. By adding peptone, as was done in the stopped series, this mating could be prevented.

TABLE 2

*Relative frequency of pairs‡ in various stages of conjugation with increasing time in stopped and unstopped matings of* AB × C* *cross*

| Hours from 0 time | Stages: Stopped A† | Stopped B | Stopped C | Unstopped A | Unstopped B | Unstopped C |
|---|---|---|---|---|---|---|
| 4 | 97.0 | 3.0 | ... | 96.0 | 4.0 | ... |
| 8 | 7.7 | 91.3 | 1.0 | 28.0 | 71.3 | 0.7 |
| 10 | ... | 88.7 | 11.3 | 14.3 | 76.1 | 9.7 |
| 12 | ... | 70.0 | 30.0 | 7.3 | 62.7 | 30.0 |
| 18 | ... | ... | 100.0 | 15.7 | 11.0 | 73.3 |
| 24 | ... | ... | § | 45.6 | 16.0 | 38.4 |
| 30 | ... | ... | § | 10.3 | 12.3 | 77.4 |

† Stages A = Attachment through crescent, B = MI metaphase through fertilization, C = Postzygotic.
‡ Each sample consisted of 300 pairs.
§ No pairs were observed.

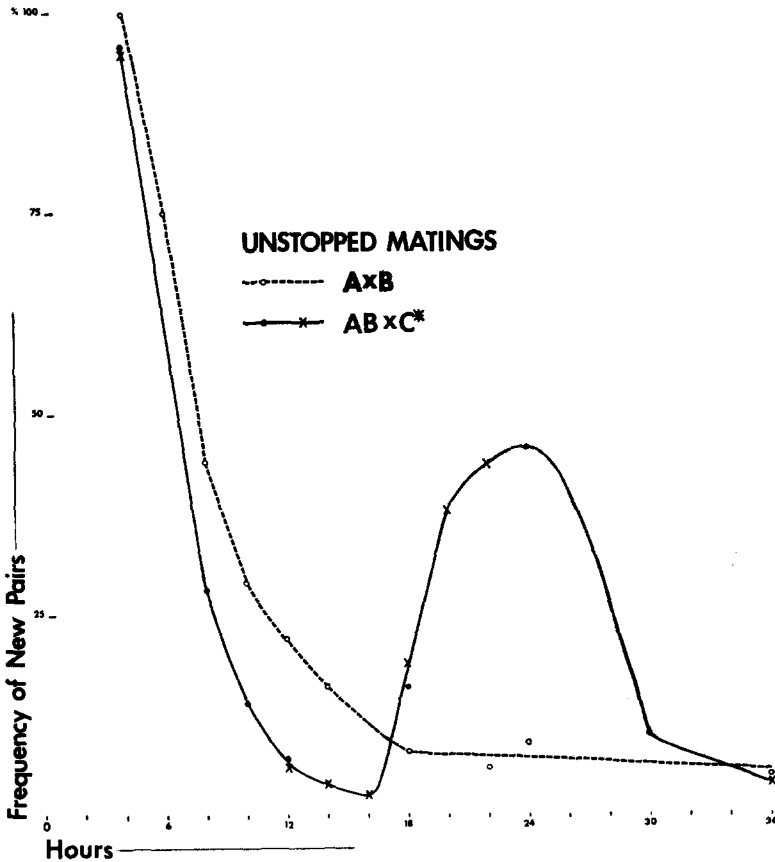


FIGURE 1.—Comparison of the frequency of new pairs over a period of 36 hours in unstopped matings of AB × C* cross and a normal cross (A × B). The percentages of new pairs (stages A) from two experiments (●—●; x—x) are plotted for the AB × C* cross. For the normal cross these percentages (○ - - - ○) were derived from one experiment. Each point is based on observations of 100 pairs (○ - - - ○), 300 pairs (●—●) or 500–560 pairs (x—x).

Attention was, therefore, focussed on the unstopped mating. Its analysis by a combination of cytological and genetic experiments has revealed that genomic exclusion is characterized by specific nuclear behavior that occurs during two rounds of mating. Often the design of a given genetic experiment was predicated by the results of a particular cytological experiment, and *vice versa.* However, each line of evidence will be presented in a separate section.

*Cytological analysis of the unstopped mating:* Samples of cells were taken at different hours after the beginning of mating. Particular attention was paid to the micronuclear condition in unpaired cells and in newly formed pairs (those in stage A) and to the kinds of nuclear complements observed during the anlagen stage (Tables 3 to 5). From the frequencies of certain cell-types, a pattern began to emerge as to the kinds of abnormal nuclear behavior that accompany genomic exclusion. From these samples it was also possible to establish a sequence of

TABLE 3

*Frequencies of micronuclear types in unpaired cells with increasing time in unstopped mating of* AB × C* *cross*†

| Sample | Percent amicronucleate | Percent hypodiploid‡ | Percent diploid | Total No. cells | Percent diploid cells with 2 or more micronuclei |
|---|---|---|---|---|---|
| AB | 0 | 0 | 100 | 1000 | 0.3 |
| C* | 17 | 83§ | 0 | 500 | 0 |
| 4 hr | 12.3 | 36 | 51.7 | 400 | 0.5 |
| 12 hr | 22 | 34 | 44 | 400 | 4.6 |
| 14 hr | 24.5 | 29 | 46.5 | 200 | 42 |
| 16 hr | 21 | 8 | 71 | 200 | 65 |
| 18 hr | 7 | 4 | 89 | 200 | 55 |
| 20 hr | 7.5 | 2.5 | 90 | 200 | 54 |
| 22 hr | 8 | 2.5 | 89.5 | 200 | 50 |
| 36 hr | 12.3 | 2.2 | 85.5¶ | 400 | 13.5 |

† Cells with distinct anlagen not included.
‡ Classified as hypodiploid on the basis of nuclear size. These nuclei were smaller than diploid nuclei, as illustrated by comparison of Figure 2B with 2A.
§ A few C* nuclei (2%) appeared to be hyperdiploid, again on the basis of size.
¶ 77% had one macronucleus; 23% had two macronuclei. Unpaired cells with two macronuclei were not observed in the earlier samples.

stages, which are illustrated by photographs (Figures 2 to 5), and which are summarized in a diagram (Figure 6).

A judgment was often made as to the "ploidy" of the micronucleus, based on the criterion of nuclear size. In normal vegetative cells this is usually the only basis for estimating the degree of ploidy, since individual chromosomes cannot be distinguished during mitosis. They can be distinguished during meiosis, and in cells of the AB clone, ten chromosomes could be counted (note five bivalents in Figures 3A, B). This is the diploid value for *T. pyriformis*. Thus, the size of the micronucleus in cells of the AB clone was considered to be representative of

TABLE 4

*Frequencies of micronuclear types in new pairs with increasing time in unstopped mating of* AB × C* *cross*†

| Hours from 0 time | Percent pairs | | Percent Amicronucleate-diploid pairs | Percent diploid-diploid pairs | | | |
|---|---|---|---|---|---|---|---|
| | Defective-diploid | Diploid-diploid | | With multiple crescents | With relic nuclei | With extra macronuclei | No. pairs observed |
| 4 | 100 | 0 | 4 | 0 | 0 | 0 | 600 |
| 12 | 94 | 6 | 3 | 0 | 17 | 17 | 100 |
| 14 | 91 | 9 | 4 | 33 | 22 | 11 | 100 |
| 16 | 86.5 | 13.5 | 3 | 41 | 20 | 15 | 200 |
| 18 | 31.6 | 68.4 | 4.8 | 27 | 21 | 2 | 250 |
| 20 | 5.5 | 94.5 | 3 | 29 | 22 | 1.6 | 200 |
| 22 | 6 | 94 | 4.5 | 12 | 19 | 0.5 | 200 |
| 36 | 5 | 95 | 5 | 17 | 0 | 1.1 | 100 |

† Data obtained from same experiment as that reported in Table 3.

TABLE 5

*Relative frequencies of pair types in anlagen stage with increasing time in unstopped mating of* AB × C* *cross*‡

| Hours from 0 time | Both mates normal | One empty† | | | One or both mates with abnormal new nuclei | No. of pairs observed |
|---|---|---|---|---|---|---|
| | | One 2+2 | One 4+4 | One abnormal | | |
| 8 | 65% | 6% | 7% | 7% | 15% | 100 |
| 10 | 62 | 6 | 7 | 16.5 | 8.5 | 200 |
| 12 | 62 | 7.3 | 6 | 11.2 | 13.5 | 400 |
| 14 | 61 | 5 | 6.3 | 12.7 | 15 | 300 |
| 16 | 67.5 | 3 | 5 | 9 | 15.5 | 200 |
| 18 | 70.3 | 4.7 | 3.5 | 8.5 | 13 | 600 |
| 20 | 69 | 4.6 | 5.2 | 9 | 12.2 | 500 |
| 21 | 66.5 | 5.5 | 6.0 | 13 | 9 | 400 |
| 22 | 67 | 6.2 | 3.8 | 8.9 | 14.1 | 600 |
| 36 | 95.5 | 0.25 | 0.25 | 0.25 | 3.75 | 400 |

† Translation: One mate empty of new nuclei. The other mate had either two new micronuclei and two new macronuclei (2+2), or four new micronuclei and four new macronuclei (4+4), or abnormal numbers and/or sizes of new nuclei. Abnormalities fell into three categories: macronuclei and micronuclei larger than normal; odd but equal numbers of macronuclei and micronuclei (e.g., 1 + 1; 3 + 3); or, unequal numbers of macronuclei and micronuclei.
‡ Some of data obtained from same experiment reported in Tables 3 and 4.

that of a diploid nucleus (Figure 2A). And so, whenever a statement is made below attesting to the diploid condition of a cell, it is made on the basis of comparison of the size of the nucleus in question relative to that of a known diploid nucleus.

By the criterion of nuclear size, the cells of C* had defective micronuclei, which were usually hypodiploid (Figure 2B) or amicronucleate (Table 3). Confirmation of the hypodiploid condition of C* cells was derived from two sources. First, the chromosomes were clearly thinner, fewer in number, and behaved abnormally during meiosis (e.g. Figures 2E; 3A,B,C). Secondly, the chromosomes could be counted in dividing cells of C* subclones (ALLEN, KOCH and PATRICK, unpublished), and in these cells the chromosome number was always less than 10.

Because C* cells had fewer and/or defective chromosomes (or none at all), when AB and C* cells were mated, the C* conjugant was readily distinguished from the AB conjugant, and it could be followed throughout most of the first round of mating. It is indicated by arrows in Figures 2 to 4.

The micronuclear condition of newly paired cells was examined. Within the first 12 hours all new pairs were made up of one diploid and one defective conjugant (Table 4). Starting at 12 hours, a few new pairs were made up of two diploid conjugants (Figure 5A). The frequency of these diploid-diploid pairs increased slowly between 12 and 16 hours, then rapidly at 18 hours. By 20 hours most pairs were of this type. The qualitative difference in micronuclear composition of new pairs at 4 and 22 hours is compelling evidence for two matings during genomic exclusion.

The frequency of defective cells among those *not* engaged in mating was 48.3% at 4 hours, close to the frequency expected for a 1:1 mixture of AB and C* cells (Table 3). The frequency of hypodiploid cells fell sharply at 16 hours and con-

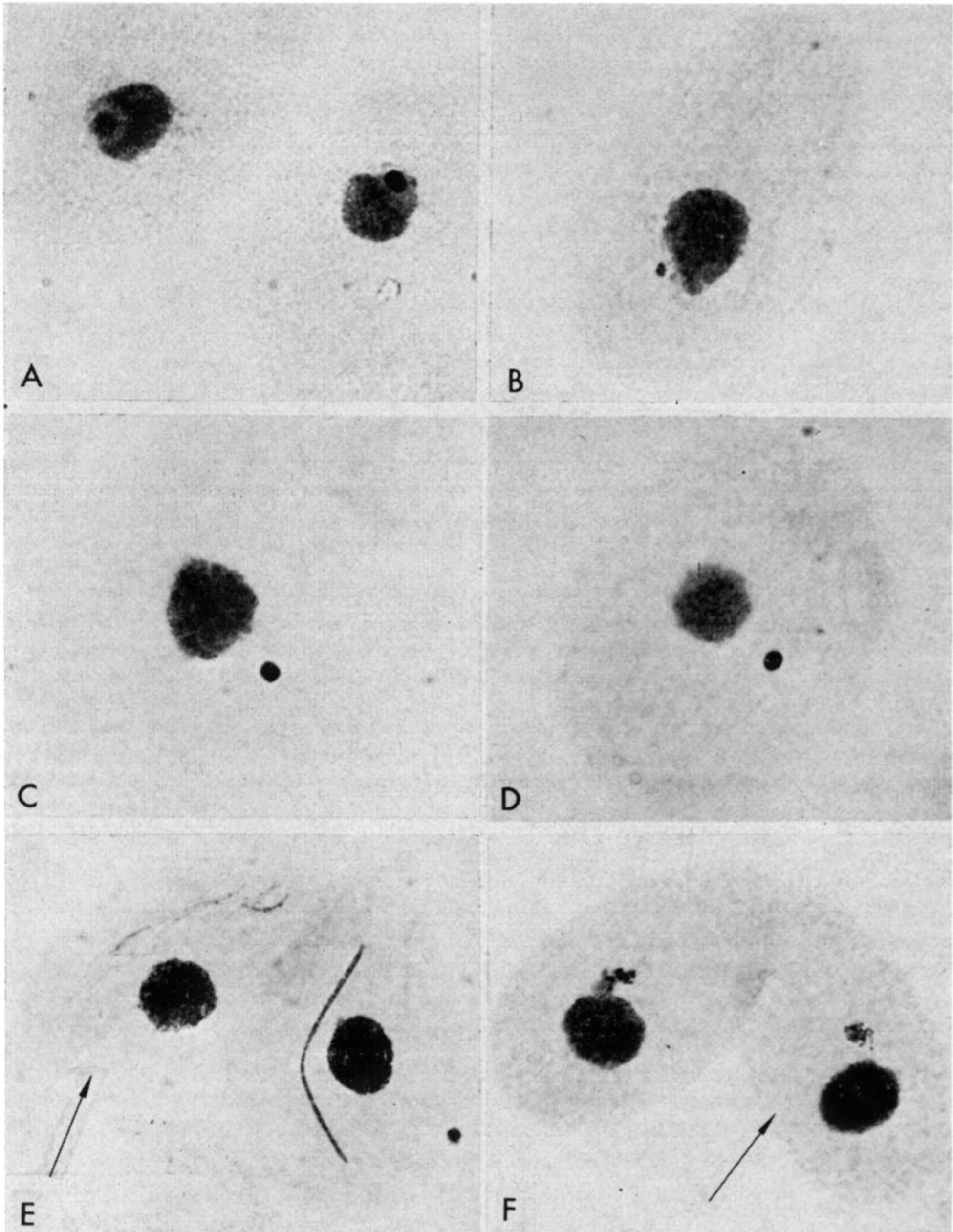


FIGURE 2.—Samples of cells from clones of: (A) AB; (B) C*; (C,D) Round 1 exconjugants with C* phenotype. Stages in genomic exclusion, Round 1: (E) Crescent; (F) Metaphase, MI; × 1550. Where possible, the C* conjugant is identified in these photographs by an arrow. See Figure 6 for a description of the symbols used in describing the stages. Samples E,F were fixed 4 hours after time 0.

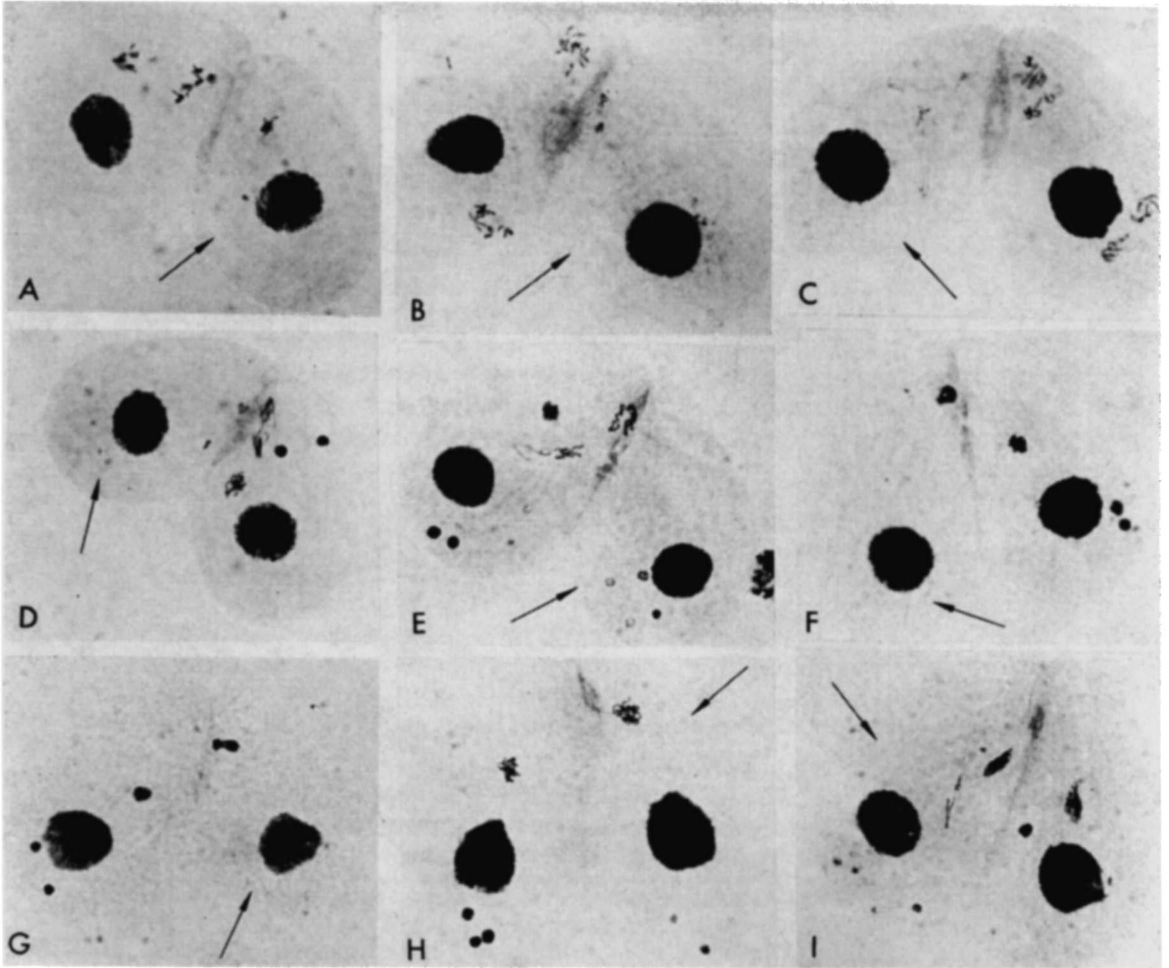


FIGURE 3.—Stages in genomic exclusion, Round 1: (A,B) Anaphase, MI; (C) Anaphase, MII; (D,E) Anaphase, MIII in AB conjugant. Note supplementary division of a second meiotic product. In (E) note four small relics in C* conjugant. (F,G) Migration of pronucleus to C*; (H) Probably metaphase, PZI. However, it might also be an example of endoreduplication, which could not be easily distinguished from metaphase of PZI. (I) Metaphase, PZI; $\times$ 1200. Samples A-C fixed at 6 hours; D-I fixed at 8 to 10 hours after time 0.

tinued its downward trend throughout the remaining samples. The frequency of amicronucleate cells, on the other hand, was somewhat higher than expected for a 1:1 mixture of parental cells and fluctuated up and then down in subsequent samples. The frequency of amicronucleate-diploid pairs was lower than expected and changed little throughout the samples (Table 4). These observations suggest that amicronucleate C* cells do not mate as readily as micronucleate cells, and/or that they do mate, but give rise to amicronucleate exconjugants.

The decreased frequency of unpaired defective cells was compensated by an increased frequency of diploid cells not engaged in mating (Table 3). The in-

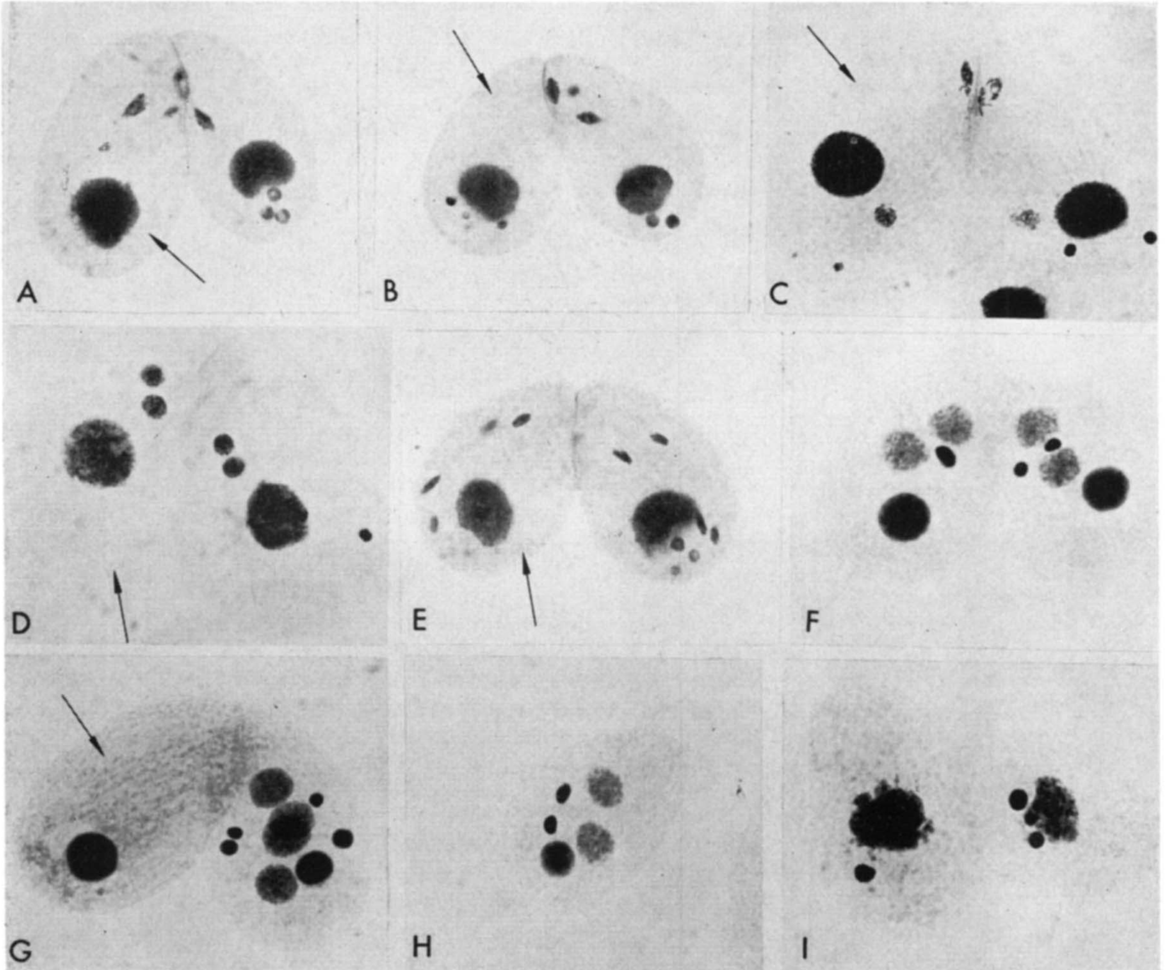


FIGURE 4.—Stages in genomic exclusion, Round 1: (A) Metaphase, PZI in both AB and C* conjugants; (B) Metaphase, 2 PZI in AB; (C) Probably anaphase, PZI. In the anterior configuration in the AB conjugant nine chromosomes could actually be counted; a tenth was probably hidden. A second configuration to the right of the macronucleus is slightly out of focus. These two configurations are probably the products of PZI. Note, however, that the chromosomes in the paroral region are in an unusual configuration, which itself resembles an anaphase figure. (D) Telophase, PZI; or is this MIIIb?; (E) Anaphase, PZII; (F,G) Differentiation of macronuclear anlagen: (F) in both mates, (G) in AB mate only. (H) Exconjugant. Note that the anlagen are still diffuse; compare with Figure 5 H. (I) MR. Cell on left has one micronucleus. Cell on right has three smaller nuclei of different size; × 1200. Samples A-D fixed at 8 to 10 hours; E-I fixed at 12 to 14 hours after time 0.

crease started at 16 hours, and by 18 hours about 90% had diploid micronuclei. Approximately half of these cells (42 to 65%) had more than one micronucleus (Table 3; Figure 4I). This was quite unlike the situation in the diploid AB parental clone in which only 0.3% of the cells were observed to have more than

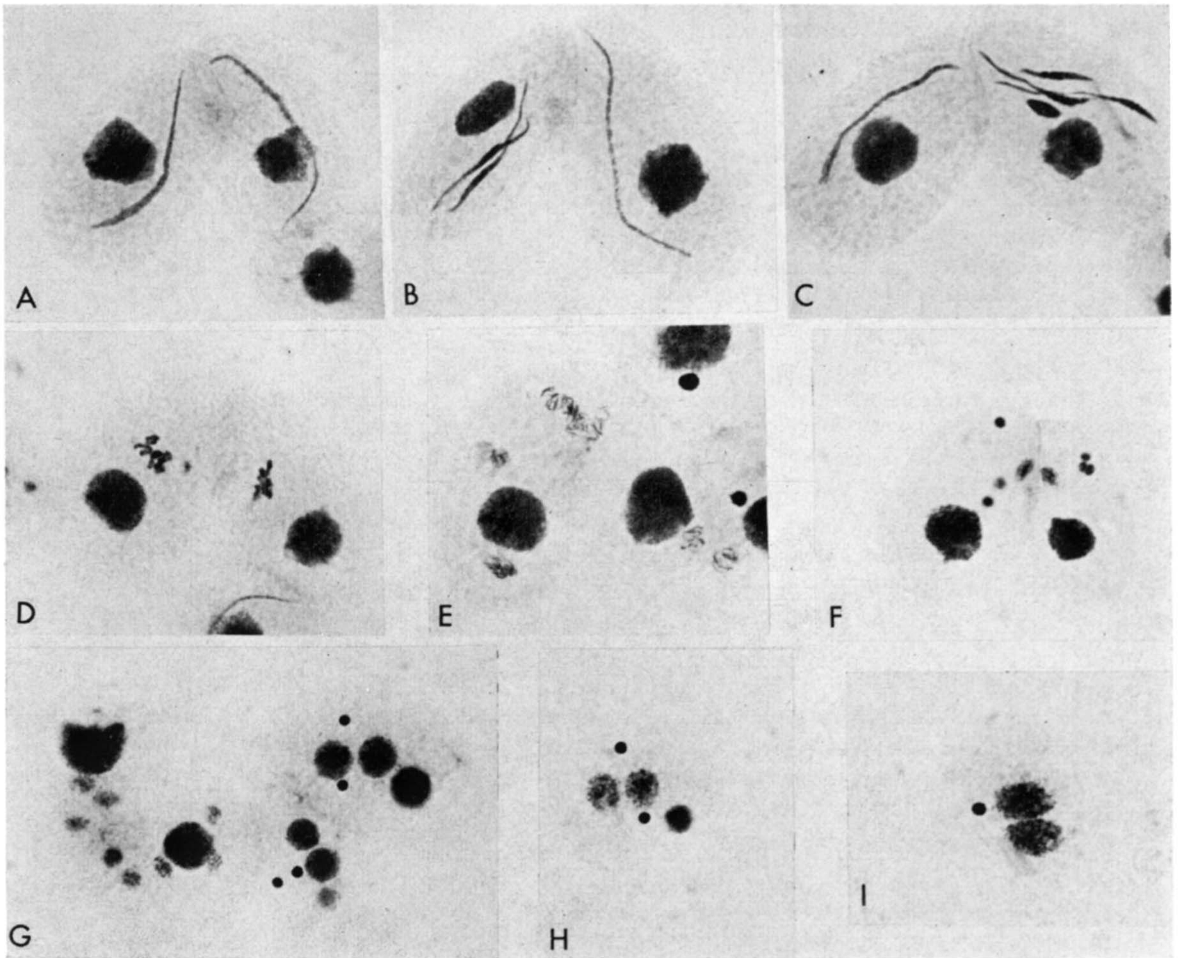


FIGURE 5.—Stages in genomic exclusion, Round 2: (A,B,C) Crescents; (D) Metaphase, MI; (E) Anaphase, MII; (F) MIII. Note the three relics in each conjugant. (G) Pair on left, attachment of pronuclei. Pair on right, differentiation of macronuclear anlagen; note the old macronuclei are degenerating. (H) Exconjugant with degenerating old macronucleus; (I) Exconjugant with two new macronuclei; × 1200. Samples A-F fixed at 24 hours; G-I fixed at 36 hours after time 0.

one micronucleus. This difference was also reflected in the micronuclear composition of the diploid-diploid pairs, and a number of these pairs included one conjugant with extra nuclei. These extra nuclei were observed as multiple crescents (Figure 5B,C), relic (or disintegrating) nuclei, and extra macronuclei (Table 4), which were often in various stages of resorption (judged on the basis of differences in size and/or density). Observation of extra nuclei in paired cells began at about 14 hours and seemed to be closely correlated with the appearance of these unpaired diploid cells. From this observation it is inferred that the diploid-diploid

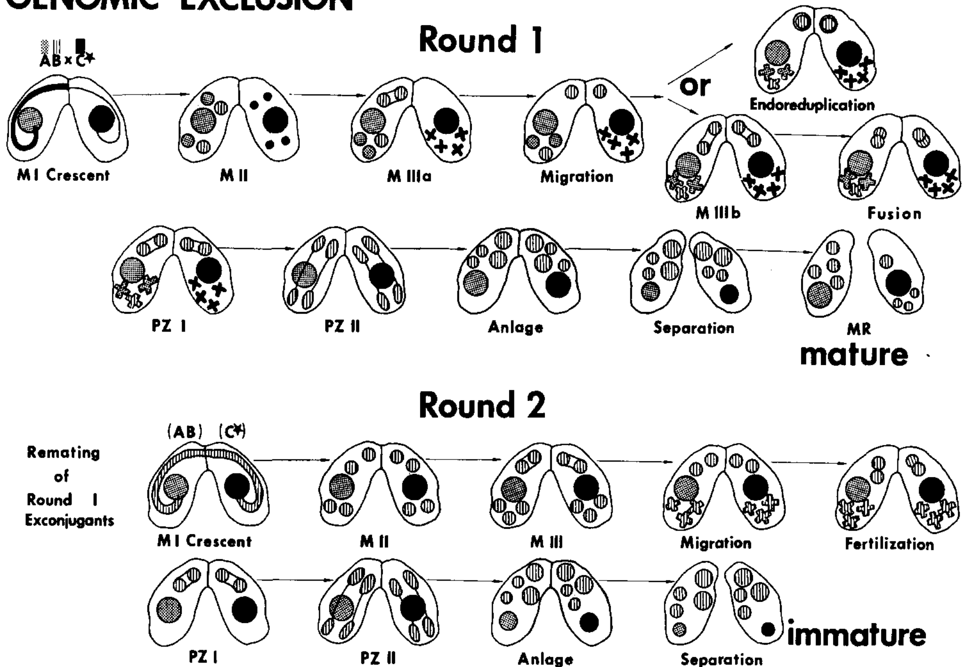


FIGURE 6.—Diagram of the sequence of stages in Round 1 and Round 2 of genomic exclusion. Note that if remating of the exconjugants from the same Round 1 pair occurs (as diagrammed above), then the exconjugants from Round 2 are completely homozygous for all their genes. Key to symbols: MI, first meiotic division; MII, second meiotic division; MIII, MIIIa, third prezygotic division (mitosis); MIIIb, fourth prezygotic division; PZI, first postzygotic division; PZII, second postzygotic division; MR, old macronucleus is retained.

pairs with extra nuclei arise from matings of the anomalous unpaired diploid cells.

At 36 hours 23% of the unpaired diploid cells had one micronucleus and two condensed macronuclei (Figure 5I). This nuclear condition is typical of an exconjugant immediately before the first fission at which time the micronucleus divides and the two new macronuclei do not divide but each goes to one of the daughter cells. Such a cell type was not observed in earlier samples. Present in the exconjugants were the macronuclear anlagen, which are diffuse and take up little of the stain, and the old macronucleus, which is dense and stains deeply, and is located in the cell's posterior. During normal conjugation the old macronucleus can be seen in various stages of resorption (judged on the basis of size and density) and resorption begins as the cells are paired (Figure 5G). By contrast, in these early samples, the old macronucleus was always present in the paired cells, and, upon separation of the exconjugants, it was never observed as being smaller than the anlagen. Moreover, a variety of nuclear conditions was observed in the population of unpaired cells in these samples, and aberrations in the number and size of the extra nuclei were frequent (Figure 4I). These observations, taken in con-

junction with the much stronger genetic evidence reported below, support the proposition that the old macronucleus regains function and that the new macronuclei, once formed, are not capable of further differentiation but are resorbed. The extra nuclei, of various sizes and densities, observed in unpaired cells at this time, would be interpreted as new nuclei, which are in various stages of resorption. Ideally, this process should be followed in a single pair under phase contrast optics; however, *in lieu* of this, inferences are drawn from the population of paired and unpaired cells in various stages. Such observations are in accord with the notion that macronuclear retention regularly occurs at the end of the first round of mating.

Supporting evidence for two matings came from a comparison of the nuclear constitution of pairs during the anlagen stage in early samples (8 to 22 hours) and at 36 hours (Table 5). In the 36-hour sample 95.5% of the paired cells had a normal nuclear complement, whereas an average of 66.4% were normal in the 8 to 22 hour samples. Moreover, particular nuclear abnormalities were observed in the early samples, and these occurred in high frequency relative to the 36-hour sample. In an average of 13.1% of these early pairs, both mates had new nuclei, but one or both had an abnormal nuclear complement. In an average of 20.5% of these early pairs, one of the mates had no new nuclei, only the old macronucleus. The other mate might have a normal nuclear complement (two new micronuclei and two new macronuclei), some multiple of this complement, or abnormal numbers and sizes of micronuclei and macronuclei. In contrast, less than 1% of the pairs had one mate missing new nuclei at 36 hours. The lack of new nuclei in one of the conjugants in the early samples suggests that nuclear transfer from the AB mate to C* occasionally does not take place in the first round of mating. Failure of nuclear transfer may then lead to abnormal numbers of new nuclei in the AB conjugant (see Figure 4G). By the same reasoning, nuclear transfer usually takes place normally in the second round of mating (at 36 hours). The two rounds of mating thus appear to be set apart on the basis of this criterion.

By observing populations of paired and unpaired cells in timed samples, a sequence of nuclear stages could be established for the two rounds of mating that take place during genomic exclusion. Examination of a population of cells obviously suffers from the fact that several stages occur in the sample, particularly where mating is not stopped, or where samples cover a 2-hour period, as they did in the stopped series. Most stages are clearly distinct from others, but others are not; for example, the third prezygotic division is not always recognizably different from the first postzygotic division. Thus, such a study is subject to personal bias and only the best and clearest examples are selected for illustration. It would be difficult, but desirable, to follow a single pair under phase contrast microscopy throughout as much of this process as possible, but this has not been done.

During genomic exclusion abnormal nuclear behavior was observed during the first round of mating (Figures 2E,F; 3A-H; 4A-I). Meiosis was defective in the C* conjugant (Figures 2E,F, 3A-C). Pronuclei were generated by the AB conjugant only (Figure 3D,E), and one pronucleus was transferred to C* (Figure

3F,G). Diploidy was reestablished in both mates, since exconjugants from the first mating were definitely diploid on the basis of nuclear size (Figure 2C,D). Exactly how diploidy is reestablished is not known, but as will be discussed later, it is suspected that it occurs by endoreduplication (Figure 3H may be an example of this, although it is more likely an example of the first postzygotic division.) Usually the postzygotic divisions were normal (Figure 3H,I; Figure 4A-E), and nuclear differentiation occurred in both conjugants (Figure 4F). Occasionally, extra nuclear divisions (Figure 4B) and extra anlagen (Figure 4G) were observed in the AB conjugant, the C* conjugant being empty of new nuclei. Such pairs were interpreted as resulting from failure of pronuclear transfer from the AB to the C* conjugant. At the termination of the Round 1 mating the exconjugants separated (Figure 4H), and MR, or retention of the old macronucleus, occurred (Figure 4I).

During the second round of mating, nuclear behavior, normal for conjugation, was observed (Figure 5A-I). Meiosis occurred normally in both conjugants (Figure 5A-E), pronuclei attached and divided mitotically (Figure 5F,G), they were transferred between mates, and fertilization reestablished diploidy. Postzygotic divisions were normal (Figure 5G) and the old macronucleus was discarded (Figure 5H,I).

*Genetic analysis of the unstopped mating:* Cytological observations offered strong support for two consecutive matings occurring with genomic exclusion.

TABLE 6

*Viability of crosses and distribution of serotypes in progeny of pairs isolated after selected time intervals after mating*

| | Hours isol. | No. of progeny | | | Percent viable | Serotypes of immature progeny | | | Total No. progeny tested |
|---|---|---|---|---|---|---|---|---|---|
| | | Mature | Dead | Immature | | Had | Ha | Hd | |
| A × B ($H^A/H^A$ × $H^D/H^D$) | 4 | 0 | 0 | 30 | 100 | 10 | 0 | 0 | 10 |
| | 8 | 0 | 0 | 30 | 100 | 10 | 0 | 0 | 10 |
| | 12 | 0 | 0 | 30 | 100 | 10 | 0 | 0 | 10 |
| | 16 | 0 | 0 | 30 | 100 | 10 | 0 | 0 | 10 |
| | 20 | 0 | 0 | 26 | 100 | 10 | 0 | 0 | 10 |
| | 24 | 0 | 0 | 28 | 100 | 10 | 0 | 0 | 10 |
| | 36 | 1 | 0 | 28 | 97 | 10 | 0 | 0 | 10 |
| | 48 | 1 | 1 | 27 | 93 | 10 | 0 | 0 | 10 |
| | | | | | | 80 | 0 | 0 | 80 |
| AB × C* ($H^A/H^D$ × $H^E/H^E$) | 4 | 26 | 4 | 0 | 0 | .. | .. | .. | .. |
| | 8 | 24 | 6 | 0 | 0 | .. | .. | .. | .. |
| | 12 | 16 | 13 | 1 | 3 | 0 | 1† | 0 | 1 |
| | 16 | 18 | 8 | 4 | 13 | 1† | 2 | 1 | 4 |
| | 20 | 9 | 11 | 9 | 31 | 4† | 3 | 2 | 9 |
| | 24 | 6 | 6 | 16 | 57 | 7† | 4 | 5 | 16 |
| | 36 | 2 | 8 | 20 | 67 | 6 | 6 | 8 | 20 |
| | 48 | 2 | 11 | 17 | 57 | 5 | 5 | 7 | 17 |
| | | | | | | 23 | 21 | 23 | 67 |

† He was also observed in one or two progeny.

TABLE 7

*Phenotypes of the two clones of the progeny derived from samples of pairs isolated at 12 and 36 hours after mating*

| | Cross and parental phenotypes | | | |
|---|---|---|---|---|
| | A×B | | AB×C* | |
| | (Ha, VI) × (Hd, IV) | | (Ha, VII) × (He, III) | |
| Hours isolated | 12 | 36 | 12 | 36 |
| No. mature progeny/Total No. pairs isolated | 0/53 | 1/60 | 38/60 | 6/60 |
| No. progeny with both clones alive†/ Total immature progeny | 51/53 | 55/59 | 0/22 | 37/48 |
| No. progeny tested with both clones: | | | | |
| (a) Mature and Ha, VI-Hd, IV | 0 | 1 | .. | .. |
| He, III-Ha, VII | .. | .. | 38‡ | 6‡ |
| (b) Immature and Had-Had | 20 | 20 | 0 | 13 |
| Ha-Ha | 0 | 0 | 0 | 8 |
| Hd-Hd | 0 | 0 | 0 | 16 |

† At least one viable exconjugant was recovered from all A×B immature progeny. Some single exconjugant clones were recovered from AB×C* progeny; at 12 hours, 4/22 (1 Hae, 1 Hde, 2 Hd); at 36 hours, 11/48 (4 Had, 4 Ha, 3 Hd).
‡ From the 44 sets of clones, 20 sets were examined cytologically. The cells of all 40 clones had micronuclei whose size was characteristic of normal diploid cells.

They suggested, but did not prove, that a second mating was possible because macronuclear retention took place at the end of the first mating. Cells containing old macronuclei might be expected to be sexually mature and to have parental phenotypes. The genetic experiments were, therefore, concerned with the establishment of the two rounds of mating on genetic grounds (Table 6) and with the distinction between the products of the first and second mating (Table 7). The specific selection of these products then made possible the design of experiments which were concerned with the origin of the syncaryon in these matings and which tested predictions arising from strictly cytological observations (Tables 8, 9).

Genetic evidence for two matings came from experiments in which a cross was made between the AB and C* clones and pairs were isolated at specific time inter-

TABLE 8

*Classification of Round 2 progeny from remated Round 1 exconjugants*

| Locus | No. sets of Round 1 exconjugants remated† | Phenotypes |
|---|---|---|
| *H* | 31 | 13 Ha; 18 Hd |
| *E–1* | 16 | 16 E–1b |
| *E–2* | 16 | 16 E–2b |
| *P–1* | 16 | 9 P–1a; 7 P–1b |
| *mt* | 6 | 3 mt-a; 3 mt-b |

† 20–50 progeny from each of 31 crosses tested for *H*.
Five progeny from each of 16 crosses tested for *P–1*.
Two progeny (4-exconjugant cultures) from each of 16 crosses tested for *E–1* and *E–2*.
Five progeny (10-exconjugant cultures) from each of six crosses brought to maturity and tested for mating type. I was present in three crosses, IV in three crosses. None of crossses had both I and IV

TABLE 9

*Testcrosses of genotypes of Round 2 exconjugants*

| Round 2 pair | Predicted genotypes | Inbred strains | Known genotypes |
|---|---|---|---|
| 1 | $H^D/H^D$ $P\text{-}1^B/P\text{-}1^B$ | A | $H^A/H^A$ $P\text{-}1^A/P\text{-}1^A$ |
| 2 | $H^D/H^D$ $P\text{-}1^B/P\text{-}1^B$ | A1 | $H^D/H^D$ $P\text{-}1^A/P\text{-}1^A$ |
| 3 | $H^D/H^D$ $P\text{-}1^A/P\text{-}1^A$ | B | $H^D/H^D$ $P\text{-}1^B/P\text{-}1^B$ |
| 4 | $H^A/H^A$ $P\text{-}1^A/P\text{-}1^A$ | C1 | $H^A/H^A$ $P\text{-}1^B/P\text{-}1^B$ |
| 5 | $H^A/H^A$ $P\text{-}1^B/P\text{-}1^B$ | | |
| 6 | $H^A/H^A$ $P\text{-}1^B/P\text{-}1^B$ | | |

| Cross | Total No. pairs isol. | Percent of progeny | | Distribution of H in progeny | | | | Distribution of P-1 in progeny | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Mature | Viable | Had | Ha | Hd | He | P-1ab | P-1a | P-1b |
| R2 1 × 1 | 25 | 0 | 96 | 0 | 0 | 15 | 0 | 0 | 0 | 5 |
| 2 × 2 | 28 | 0 | 100 | 0 | 0 | 15 | 0 | 0 | 0 | 5 |
| 3 × 3 | 27 | 15 | 85 | 0 | 0 | 12 | 0 | 0 | 5 | 0 |
| 4 × 4 | 30 | 0 | 93 | 0 | 15 | 0 | 0 | 0 | 5 | 0 |
| 5 × 5 | 30 | 0 | 100 | 0 | 15 | 0 | 0 | 0 | 0 | 5 |
| 6 × 6 | 30 | 0 | 100 | 0 | 15 | 0 | 0 | 0 | 0 | 5 |
| B × R2 1 | 30 | 0 | 100 | 0 | 0 | 15 | 0 | 0 | 0 | 5 |
| 2 | 30 | 0 | 100 | 0 | 0 | 15 | 0 | 0 | 0 | 5 |
| 3 | 30 | 0 | 100 | 0 | 0 | 15 | 0 | 5 | 0 | 0 |
| A × R2 4 | 30 | 0 | 100 | 0 | 15 | 0 | 0 | 0 | 5 | 0 |
| 5 | 30 | 0 | 100 | 0 | 15 | 0 | 0 | 5 | 0 | 0 |
| 6 | 30 | 3 | 97 | 0 | 15 | 0 | 0 | 5 | 0 | 0 |
| A1 × R2 1 | 30 | 0 | 90 | 0 | 0 | 15 | 0 | 5 | 0 | 0 |
| 2 | 30 | 0 | 100 | 0 | 0 | 15 | 0 | 5 | 0 | 0 |
| 3 | 30 | 37 | 63 | 0 | 0 | 18 | 0 | 0 | 5 | 0 |
| C1 × R2 4 | 30 | 7 | 93 | 0 | 15 | 0 | 0 | 5 | 0 | 0 |
| 5 | 30 | 3 | 97 | 0 | 15 | 0 | 0 | 0 | 0 | 5 |
| 6 | 26 | 4 | 96 | 0 | 15 | 0 | 0 | 0 | 0 | 5 |

vals after the onset of mating. A control cross (A × B) was handled in the same way. The progeny could be classified into three categories by the types of cultures they generated—mature, dead and immature. For reasons discussed earlier, the viability of a cross is measured as the frequency of *immature* progeny, since they are produced by normal conjugation, and inviable progeny are those which are either dead or mature. The serotypes of the immature progeny were screened by tests against antisera to Ha, Hd, and He. These are antigens associated with the genotypes $H^A/H^A$, $H^D/H^D$, and $H^E/H^E$, respectively, as well as with particular heterozygotes (i.e., Had is found in $H^A/H^D$).

The viability of the control cross (A × B) was close to 100% in all samples, regardless of when the isolations were made (Table 6). Moreover the expected serotype, Had, was observed in all progeny sampled. This serotype is determined by the heterozygous genotype $H^A/H^D$ and would be expected in the progeny of a cross between clones which were $H^A/H^A$ and $H^D/H^D$.

Results which differed from the expected were observed in the AB × C* cross. Most of the progeny were mature or dead if the isolations had been made 12 hours

or earlier (Table 6). The frequency of mature progeny was particularly high in these samples. Between 16 and 24 hours a gradual increase in the frequency of immature progeny was observed, and between 24 and 48 hours this frequency (or the viability) leveled off at 57 to 67%. If normal conjugation had occurred, two genotypes would be expected in these immature progeny: $H^A/H^E$ and $H^D/H^E$. In the 12 to 20 hour samples antigen He was found in a few of these progeny, suggesting some transmission of genes from the C* parent. (In much more recent experiments, no trace of He has been observed in any of the progeny of this cross, regardless of when the isolations were made.) Most of the progeny, from 20 hours on, fell into three phenotypic classes, Ha, Had and Hd, and they occurred in an approximate 1:2:1 ratio. This type of observation is, by definition, genomic exclusion.

In a subsequent experiment pairs were isolated at 12 and at 36 hours from the control and from the AB × C* cross. Upon separation, the presumptive exconjugants were isolated into separate depressions. Again, little difference in the frequency of immature progeny (viability) was observed in the 12 hour and 36 hour isolates in the A × B cross (Table 7). Both exconjugants from the immature progeny were Had in phenotype. In the one mature progeny, one clone was Ha and mating type VI, similar in phenotype to the A strain parent, and the other clone was Hd and mating type IV, similar in phenotype to the B strain parent.

A pronounced difference in viability was observed between the progeny isolated at 12 hours and 36 hours in the AB × C* cross (Table 7). Among the 12 hour isolates, the progeny were either mature (38/60) or dead (22/60). Among the 36 hour isolates, a few progeny were mature (6/60), some died (17/60), but the majority (37/60) were immature. The phenotypes of both exconjugants from the same immature progeny were alike, but the progeny fell into three different classes: Had, Ha and Hd. In contrast, the phenotypes of the two clones from the mature progeny were unlike. One clone of each of these was Ha and mating type VII (like the AB parent), and the other was He and mating type III (like C*). Tests of other markers (*P-1*, *E-1* and *E-2*) showed that one clone from each of these mature progeny was $P_3$, E-1b and E-2b (like AB), and the other clone was $P_1$, E-1c and E-2c (like C*).

Both mature clones from 20 progeny were examined cytologically, and all 40 clones were diploid on the basis of nuclear size. This included the mature clones with the C* phenotype (see Figure 2 C,D). C* has a defective micronucleus, and there are no diploid cells in the C* population at present (ALLEN, KOCH and PATRICK, unpublished); therefore, the mature cultures that result from this mating do not arise because of "nonconjugation"; that is, because parental cells fail to complete conjugation, come apart, and give rise to clones which are sexually mature. Instead, these cells engage in a form of conjugation that morphologically affects the *micronucleus*, but *not* the macronucleus. The fact that one clone was phenotypically like strain AB for five of the known markers tested, and the other like C*, is very strong evidence for the retention of the old macronucleus in these cells. These cells would be heterocaryons—with a micronucleus derived from a source different from that of the macronucleus. Here, the difference would be

genotypic. Thus, these cells fulfill the requirements expected of the "progeny" of the first round of mating during genomic exclusion. They will be designated Round 1 exconjugants. Products of the second mating will be referred to hereafter as Round 2 exconjugants.

Cytological observations showed that meiosis is abortive in C*, and suggested that the AB conjugant contributes one of its two pronuclei to C*, and that each pronucleus becomes diploid. If this sequence of events is, in fact, as stated, then the two Round 1 exconjugants derived from the same pair should have micronuclei that are genetically identical; but, more important, these micronuclei should be completely homozygous for all their genes. Thus, remated Round 1 exconjugants from the same pair should give rise to Round 2 progeny that are phenotypically identical and homozygous in genotype. For those loci heterozygous in strain AB, half the remated Round 1 exconjugants should give rise to one class of homozygote, the other half to a second class of homozygote. If two meiotic products were contributed by the AB conjugant, but genotypically identical syncarya were formed in the two Round 1 exconjugants, the results would be expected to be more complex for heterozygous loci. Half of these loci would be present in heterozygous condition in the Round 1 exconjugants. Such exconjugants, when remated, would give rise to three different Round 2 genotypes; hence, three classes of Round 2 progeny would be expected within a *single* cross.

With the identification of the Round 1 exconjugants, a direct test of the derivation of the micronucleus in these cells was possible. Forty sets of Round 1 exconjugants were remated. Sixty Round 2 pairs were isolated from each of 20 of the 40 crosses, and 30 from each of the remaining 20 crosses. Of the 40 crosses, 9 were inviable and 31 were highly viable (average viability of Round 2 progeny, 96.6%; range in viability, 83 to 100%). Since 31/40 crosses were viable, the viability of the remated Round 1 exconjugants is 77.5%. And since this figure is similar to the frequency (66.4%) of *normal* pairs in the anlagen stage of the Round 1 mating (Table 5), it argues for a correlation between a normal nuclear complement and the breeding performance of these pairs.

Each cross gave rise to only one class of Round 2 progeny. This conclusion is particularly well supported by the observations on serotypes. More than 50 progeny from each of 12 crosses were tested for their serotypes, while 20 progeny from each of 19 crosses were serotyped. All Round 2 progeny from a single cross were phenotypically identical, although the crosses fell into two categories (Table 8). Thirteen crosses gave rise to progeny that were Ha and 18 to progeny that were Hd. Since strain AB is $H^A/H^D$, this is the result expected if half of the remated Round 1 exconjugants have micronuclei that are $H^A/H^A$, and half have micronuclei that are $H^D/H^D$. Tests were extended to four other loci (*E-1*, *E-2*, *P-1*, and *mt*), and the results obtained were those expected on the basis of the genotype of strain AB ($E\text{-}1^B/E\text{-}1^B$; $E\text{-}2^B/E\text{-}2^B$; $P\text{-}1^A/P\text{-}1^B$; $mt^A/mt^B$). The progeny of 16 crosses were all E-1b, E-2b. Nine of them were P-1a and seven of them were P-1b. With regard to the two heterozygous loci, $H^A/H^D$ and $P\text{-}1^A/P\text{-}1^B$, four classes of progeny were observed among these 16 crosses. They occurred in an approximate 1:1:1:1 ratio—4 Ha, P-1a; 4 Ha, P-1b; 5Hd, P-1a; and 3 Hd, P-1b. Of six crosses tested, two classes were observed for *mt*.

Several Round 2 exconjugants from 6 crosses were brought to maturity, their mating types were ascertained, and testcrosses to determine their genotypes were performed (Table 9). Each of the six Round 2 exconjugants were selfed (Round 2 crosses 1 to 6), and each was outcrossed to two different inbred strains of known genotype. The results of these tests confirmed the genotypes of these Round 2 pairs predicted on the basis of phenotype. These Round 2 exconjugants derived from remated Round 1 exconjugants were therefore, homozygous in genotype.

The results obtained above suggest that the micronucleus in Round 1 exconjugants is usually derived from *one* product of meiosis from AB. Particularly critical were the observations on heterozygous loci. All Round 2 progeny from a single cross were alike in phenotype and only phenotypes associated with homozygotes were observed in different crosses. The crosses fell into two approximately equal classes, and a 1:1:1:1 ratio was observed for the two heterozygous loci. When testcrossed, Round 2 exconjugants could also be shown to be homozygous in genotype. These results do not, of course, eliminate the possibility that two products of meiosis from AB might infrequently give rise to the syncaryon of Round 1 exconjugants; however, they suggest that, as a rule, this does not happen, and that only one product of meiosis is involved.

In contrast, the micronucleus of Round 2 exconjugants is always derived from *two* products of meiosis, one contributed from each conjugant. The best genetic evidence came from crosses of AB × C* in which mating was not stopped and where pairs were isolated after about 24 or more hours (Tables 6,7; ALLEN 1963). Here, the Round 1 exconjugants presumably had separated and the second mating had taken place at random. For heterozygous loci, three classes were observed in the progeny, including the heterozygous class. The appearance of this heterozygous class is compelling evidence for the derivation of the syncarya of Round 2 exconjugants from two meiotic products, contributed by two conjugants, each derived from a different Round 1 pair.

## DISCUSSION

The sequence of stages that occur during genomic exclusion is diagrammed (Figure 6). The only stage that is still uncertain is the method by which Round 1 pairs acquire a diploid micronucleus. Two alternative routes for reestablishing diploidy are shown in the diagram. Either the haploid pronuclei undergo a mitotic division (MIIIb) and the products fuse, or they undergo endoreduplication. There is precedence for both types of chromosome behavior in the Ciliates. The first type is, in essence, what happens during autogamy or cytogamy (SONNEBORN 1947). The second type, or endoreduplication, is a form of endomitosis in which the centromere divides. The form of endomitosis in which the centromere does not divide has been invoked for the cases of polyteny observed in the micronucleus of *T. pyriformis* (ALFERT and BALAMUTH 1957) and in the macronucleus of other Ciliates (GRELL 1964).

A search for pairs undergoing MIIIb or endoreduplication was made. Pairs with the appearance of MIIIb could very easily be mistaken for PZI (Figure 4D). A distinction would depend upon determining a total of five rather than ten

chromosomes. So far, the pairs in which chromosomes could be counted have had ten chromosomes and appear to be in PZI (But, see Figure 4C). This leaves endoreduplication as the more likely mechanism of diploidization. If this is so, it could occur before, during or after migration of the pronucleus to C*. It definitely occurs after MIIIa, since five chromosomes are visible at each pole in AB during this division (Figure 3E). Some pairs were seen that might qualify (Figure 3H); however, these pairs could be confused with pairs in metaphase of PZI. A satisfactory resolution of this problem may only be achieved by following a single pair through these critical stages under phase contrast optics.

Emphasis has been placed in this paper on defining the sequence of stages that gives rise to Round 1 exconjugants and so makes genomic exclusion possible. Approximately two thirds of the pairs were normal when they reached the stage where anlagen were developed (Table 5). In a cytogenetic study on outcrosses of the defective B1 strain, where genomic exclusion had previously been observed, NANNEY and NAGEL (1964) found highly aberrant nuclear behavior. Conjugation in many crosses did not proceed beyond the stage at which a pronucleus was transferred to the defective mate. Presumably B1 had become so defective that few, if any, viable progeny could be produced, and not even genomic exclusion could occur. They made special note of extra divisions of the meiotic products of the normal mate. These supplementary divisions were emphasized since they were searching for nuclear phenomena that would permit the genetic consequences of genomic exclusion with one mating. At that time it was not known that two consecutive conjugations characterize genomic exclusion.

Extra divisions of the meiotic products were also observed in the AB conjugant in some, but not all, of the pairs (Figure 3 D,E). Counts were made of the number of relic nuclei in AB at the stage when one of the pronuclei was being transferred to C* (e.g. Figure 3 F-H). One hundred pairs were counted. The AB conjugant had 3 relic nuclei in 57% of the pairs, 4 relic nuclei in 34% of the pairs, and 6 to 8 relic nuclei in 9% of the pairs.

The significance of these extra divisions for genomic exclusion is not clear. Genetic evidence rules out genic contribution from more than one meiotic product as usually taking place. It does not rule out the possibility that a second meiotic product may occasionally contribute genes. However, it is also possible that these supplementary divisions are irrelevant to genomic exclusion, or they may give rise to those pairs that have an abnormal nuclear complement (see Figure 4 B,G) and which have been presumed to be inviable. It is clear that a number of aberrant nuclear phenomena are associated with genomic exclusion; thus, 33% of the pairs end up without anlagen in one of the mates, or with abnormal numbers and sizes of anlagen in one or both mates. The fact that the frequency of nuclear abnormalities is high in the AB × C* cross makes it difficult to assess how much latitude there is in nuclear sequences leading to Round 1 exconjugants which are viable when bred. Abnormalities that are reminiscent of those seen here have also been described in other ciliates; for example, in *Paramecium trichium* (DILLER 1948), in strain d59 of *P. aurelia* (SONNEBORN 1954) and in diploid-haploid crosses of *P. aurelia* (KIMBALL and GAITHER 1955). In these latter crosses extra nuclear divisions and extra pronuclei were also seen.

One of the most important features to come out of this study on genomic exclusion is the finding that macronuclear retention regularly occurs at the termination of Round 1. Why it happens with regularity is not known. Its occurrence cannot be a direct consequence of micronuclear homozygosity since homozygosity can be brought about by repeated inbreeding in syngen 1 of *T. pyriformis*. When these inbred strains undergo normal conjugation, the old macronucleus is regularly resorbed. Possibly the step that makes Round 1 of genomic exclusion analogous to autogamy (MIIIb and fusion; or endoreduplication) induces the old macronucleus to regain function.

Macronuclear retention in *T. pyriformis* is similar to macronuclear regeneration in *P. aurelia*, described some time ago by SONNEBORN (1942), except that the changes that occur in the old macronucleus are much more dramatic in the latter organism. In Paramecium the old macronucleus forms a skein early in conjugation and breaks up into 30 to 40 pieces. Regeneration occurs from one of the pieces. In contrast, the macronucleus of Tetrahymena does not break up into pieces nor does resorption begin before the anlagen stage. In either organism when "MR" occurs, the net result is the same: a heterocaryon may be produced.

When a heterocaryon is formed in Paramecium, the phenotype of the cell is determined by the macronucleus, not the micronucleus (SONNEBORN 1942). This also appears to be the case in Tetrahymena. As shown in this paper, for five different loci, the *macronuclear genes were expressed, not the micronuclear genes.* Thus, the micronucleus of *T. pyriformis*, like that of *P. aurelia*, appears to be silent in its gene expression when the macronucleus is active.

Genomic exclusion is unlike any of the standard forms of sexual reorganization in the Ciliates. Autogamy, cytogamy, conjugation, and Round 1 of genomic exclusion are compared in Figure 7. Since one meiotic product from only one mate is involved, and homozygosity of both exconjugants is the result, genomic exclusion resembles autogamy more closely than the other forms in its genetic consequences. Although autogamy occurs in other Ciliates, such as *P. aurelia* (DILLER 1936) or *Tetrahymena rostrata* (CORLISS 1952), there is no definitive proof that it ever occurs in *T. pyriformis*. Nor is there evidence that cytogamy occurs in *T. pyriformis* (ORIAS 1960). Conjugation is the normal sexual process. It is, therefore, of extreme interest to find a variant form of conjugation that has the same genetic consequences as autogamy in this organism.

In subsequent papers the generality of genomic exclusion in *T. pyriformis* and its association with cells bearing a defective micronucleus will be discussed (ALLEN, FILE and KOCH, 1967; ALLEN, KOCH and PATRICK, unpublished). Certain evidence suggests that the phenomenon may not only occur in syngen 1 but that it may be more widespread. The high frequency of amicronucleate clones and of clones with defective micronuclei in field collections of all 12 syngens suggests that loss of the micronucleus is of common occurrence in this organism. Some of these amicronucleate clones are highly viable in the vegetative state, but they are sexually dead. Genomic exclusion results in the re-establishment of a micronucleus. Therefore, it may be of importance to the survival of the species, since it restores sexuality to a cell.

Although several cases of parthenogenesis of the haploid pronuclei have been

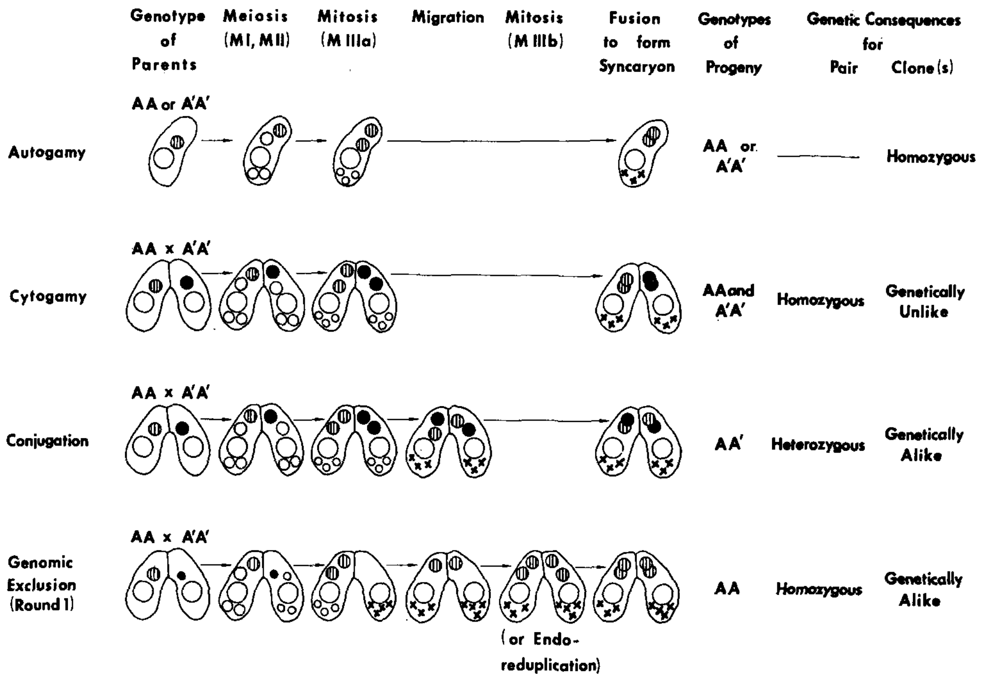


FIGURE 7.—Comparison of the significant stages in autogamy, cytogamy, conjugation, and Round 1 of genomic exclusion. For each process, the genotypes of the progeny are shown, as well as the genetic consequences for the exautogamous clone, and for the pair and "exconjugant" clones.

described in other Ciliates, genomic exclusion has not (KUDO 1966). In "parthenogenetic conjugation" (DILLER 1936), one of the conjugants fails to form functional pronuclei but it receives a migratory pronucleus from its normal mate. The haploid pronucleus in each conjugant then undergoes postzygotic divisions and new micronuclei and macronuclei are developed. The new micronuclei are haploid, however. Examples of parthenogenesis may be found in *P. aurelia* (SIEGEL 1954; SONNEBORN 1954), in *P. trichium* (DILLER 1948), and in double animal-single animal matings of *Euplotes patella* (POWERS 1943). DILLER (1948) does mention the possibility that endomitosis may have occurred in a few cases in *P. trichium*. Endomitosis may have also been responsible for the diploidization of some haploid lines of *P. aurelia* that occurred during clonal growth (SONNEBORN *et al.* 1953; KIMBALL and GAITHER 1955). Parthenogenetic conjugation also occurs in *T. pyriformis* (ELLIOTT and CLARK 1956, 1957; WELLS 1961).

Genomic exclusion is characterized by two consecutive conjugations. These conjugations are distinguishable by time (Figure 1), by the final micronuclear constitution of the mates (Table 4), and perhaps by the ripeness of the cells for mating. Moreover, the second conjugation is possible because the old macronucleus is regularly retained at the end of the first conjugation. Genomic exclusion is thus of interest for the biological questions it raises concerning the process

of mating. In a subsequent paper factors which influence this mating will be examined (ALLEN, KOCH and PATRICK, unpublished).

Genomic exclusion was originally observed in unstopped matings in Cerophyl-Aerobacter medium where pairs were isolated some 36 hours after they were first observed (ALLEN 1960; 1963). When the normal parent was a heterozygote, an approximate 1:2:1 ratio was observed in the progeny. This observation would be expected on the basis of present findings provided the remating of Round 1 exconjugants occurs completely at random and the two homozygous genotypes were equal in viability.

Departures from the 1:2:1 ratio were found for the *H* locus (Tables 6, 7; ALLEN 1963; and unpublished observations), and an increase in the homozygous classes was observed. This increase was usually of the order of 20%. Departures from the 1:2:1 ratio were also observed for the *P-1* locus when *H* homozygotes were selected (ALLEN 1963), but an unselected group of progeny was not scored for *P-1* so it is not known how general these departures are.

An increased frequency of homozygotes might arise if the second conjugation took place without separation of the Round 1 exconjugants, or if Round 1 exconjugants upon separation tended to remate preferentially. The first hypothesis might be ruled out by observations on the *stopped* mating, if immature progeny *never* were found, or if diploid-diploid newly paired cells were never observed cytologically. Isolations from this cross have been made as early as 4 hours after the commencement of mating, and immature progeny were not found; however, this experiment has not been done on a large enough scale to rule out their appearance in low frequency. Samples of 300 pairs from a stopped mating were examined cytologically at various intervals after time 0 (Time 2). None of the paired cells were in stage A after 8 hours. It would appear that diploid-diploid pairs in stage A, if they occur at all, must occur very rarely. Of course, it is possible that stopping the mating by the addition of peptone prevents Round 2 from occurring altogether, and such pairs may not be observed. Perhaps they only occur under the conditions of the unstopped mating. Isolations have been made as early as 8 to 10½ hours from an upstopped mating. A few immature progeny, usually of the order of 2 to 3% were found, and all were homozygous for the H-serotypes (ALLEN, KOCH and PATRICK, unpublished). This observation could be used as evidence supporting the first hypothesis, i.e., Round 2 takes place in some pairs without separation of Round 1 exconjugants. However, the time that exconjugants separate may be as early as 8 to 10 hours, so the second hypothesis—separation and preferential remating—is not, as yet, ruled out.

Knowledge of the cytogenetic basis of genomic exclusion has helped to restore order to the breeding program of *T. pyriformis*. If the micronuclear condition of the parents is determined, and timed matings are followed, genomic exclusion can be prevented—and only progeny produced by normal conjugation selected.

Genomic exclusion can also be promoted and utilized in genetic studies. Since it has the same genetic consequences as autogamy, genetic analysis will be greatly facilitated in future work with this organism. A clone such as C* can be used in a variety of experimental situations to induce the production of homozygotes from

a normal micronucleate heterozygote: (a) for the testing of the inheritance of a new marker under nuclear control; (b) in searching for new markers after treatment of a normal clone with mutagenic agents; and (c) for obtaining new gene combinations by creating new homozygous diploid lines almost instantly, thereby eliminating much of the current tedium in deriving new inbred strains. The method of production of these homozygotes is relatively simple and has been summarized elsewhere (ALLEN 1967).

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## SUMMARY

A cytological and genetic analysis was made on timed matings of a cross of a clone belonging to the heterozygous AB strain and C*, a clone belonging to the inbred C strain in syngen 1 of *Tetrahymena pyriformis*. Cells of the AB clone have a normal diploid micronucleus, and C* cells are hypodiploid or amicronucleate. The progeny of this mating receive only the genes derived from the AB parent, and, in mass matings, genetic ratios approaching 1:2:1 are observed for genes present in heterozygous condition in the AB strain. Since the genes from C* are excluded, the phenomenon has been termed genomic exclusion.—Genomic exclusion includes two consecutive rounds of mating. The first round of conjugation is abnormal. The diploid syncarya of both exconjugants are derived from one meiotic product of AB. However, since the old macronucleus is regularly retained, the products that arise are heterocaryons, express the macronuclear genes, and are sexually mature. The second round of conjugation is normal, and the syncarya are derived from two meiotic products, one from each conjugant. If exconjugants from different Round 1 pairs are allowed to remate at random, the progeny fall into three phenotypic classes. If, however, the two exconjugants from the *same* Round 1 pair are remated, the progeny can be shown to be genetically homozygous at all known loci. The establishment of the cytogenetic basis for genomic exclusion accounts not only for all earlier genetic observations, but it also has useful and important implications for future genetic work with this organism. For, by using a clone, such as C* in crosses to heterozygotes, homozygous diploid lines can be synthesized almost instantly.

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