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Defective Micronuclei and Genomic Exclusion in Selected C* Subclones of *Tetrahymena*[1]

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SYNOPSIS. C* is a structurally heterogeneous clone containing normal and crinkled cells which are amicronucleate. Selection for structurally normal subclones did not result in the genesis of a diploid cell line. The stem cell of C* appears to be aneuploid with about 4 chromosomes instead of the usual 10, and from this line cells with fewer chromosomes or without micronuclei are generated. Only cells containing a portion of the micronucleus are viable. Amicronucleate cells die. They also mate very poorly. Consequently, little difference is observed between subclones having different proportions of amicronucleate cells in their ability to generate viable offspring by genomic exclusion. Our observations are discussed in terms of the separate functions of micronucleus and macronucleus and ways in which the nuclei seem to interact.

THE micronucleus of *Tetrahymena pyriformis*, essential for sexual reproduction, is frequently lost from the cell. Amicronucleate cells are common in collections made from different parts of the world. Some of these natural lines reproduce asexually and can be maintained for years in the laboratory. However, newly produced amicronucleate cells are rarely ever capable of dividing in the laboratory and they die.

In the laboratory clones often develop containing cells with defective micronuclei as well as cells which are amicronucleate. Cells with defective micronuclei cannot conjugate normally but instead can induce genomic exclusion if the other parent in the cross has a normal diploid micronucleus. Progeny are produced which are diploid and contain only genes contributed by the normal parent. There is direct genetic and cytologic proof for the occurrence of genomic exclusion in syngen 1 and indirect evidence for its occurrence in other syngens which make up the species complex (2, 3, 5).

The genetic and cytologic evidence for the details of the process came out of an analysis of crosses of an aberrant clone called C* to a normal cell line which was heterozygous for several of the known genes in syngen 1 (2, 3). These studies showed that 2 consecutive rounds of mating are characteristic of genomic exclusion. The first mating leads to exconjugants which are heterocaryons containing in each exconjugant the old macronucleus and a new diploid micronucleus derived from a single meiotic product from the normal parent. These exconjugants are also sexually mature and can remate immediately. When the exconjugants from the same Round 1 pair remate, then the progeny are homozygous for all genes. If, however, they remate at random with exconjugants from other Round 1 pairs, then the Round 2 progeny can be heterozygous for some of the genes. For any one gene locus 3 classes of progeny can be found for genes known to be heterozygous in the normal parent. The aberrant genetic ratios characteristic of genomic exclusion are then attributable to unusual nuclear behavior.

Aberrant genetic ratios are common in crosses of certain strains of syngen 1 (1, 10, 11) and are associated with crosses in which one of the clones has a defective micronucleus, and many of the progeny which appear are sexually mature. This syndrome—immediate maturity, a parent with a defective micronucleus, coupled with low viability in crosses—is also found in other syngens implying that genomic exclusion probably is widespread within the species complex (5).

Altho there are several clones which can induce genomic exclusion, usually this is only partial and some of the genes of the defective parent are also transmitted. C* is exceptional in that it is now 14 years old and all the progeny which result are produced by genomic exclusion.

When C* reproduces asexually a structurally diverse population of cells is generated. Some of the cells appear normal, but others are extremely abnormal and "crinkled." The crinkled cells are amicronucleate and do not divide. We were interested in examining this heterogeneous population cytologically and genetically. Three questions were asked: (1) Can diploid cell lines be extracted from C*

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by rigorous selection for normal cells? (2) What is the chromosomal complement of the stem cell that perpetuates C*? (3) Is there a relationship between the severity of the micronuclear defect, viability in crosses and the ability to induce genomic exclusion? To answer these questions subclones of C* were produced which differed in their complement of normal appearing cells. The results of their analysis are the subject of this report.

MATERIALS AND METHODS

Strains: C* is a semi-amicronucleate clone and was derived by inbreeding of the C strain (C-5573). It is H^E/H^E in genotype.

AB-7a has a normal diploid micronucleus and was derived from a cross of inbred strains A and B (A-11613 c B-12614d). It is heterozygous at the *H* locus (H^A/H^D).

Terms: *Semi-amicronucleate clone*: Contains a mixture of cell types, some of which have a micronucleus and others which are amicronucleate.

Crinkled cells: These are grossly abnormal and misshapen cells usually found at the bottom of the well of a depression slide. They lack a micronucleus.

Normal cells: These cells are structurally normal and can contain either a diploid micronucleus or a defective micronucleus.

Maturity: Sexual reactivity can be engendered under appropriate culture conditions.

Immaturity: Lack of sexual response under optimal conditions for mating.

Normal conjugation: Where each mate contributes a replica of a single meiotic product to the diploid fertilization nucleus of the other conjugant. In syngen 1 exconjugant cells are sexually unreactive, or *immature*, following normal conjugation. Maturity occurs only after the exconjugant goes thru 60-80 fissions.

Genomic exclusion: Where only one of the mates contributes a replica of a single meiotic product to the diploid fertilization nucleus of each conjugant. There are 2 rounds of mating. Round 1 exconjugants are *mature* and remate to give rise to the Round 2 exconjugants which are *immature*.

Viability: Percentage of progeny which are *immature*.

Culture technics: Cultures of C* and AB were grown axenically in 1% proteose-peptone in tubes at 23 C and were maintained by subculturing at 2 week intervals. All experiments were carried out at 23 C.

Cloning was performed on samples of washed peptone cultures grown in Cerophyl-*Aerobacter* medium (0.15% Cerophyl rye grass infusion inoculated with *Aerobacter aerogenes*). Single cells were isolated from the population into individual wells of depression slides. After 3 days (10-12 fissions) a single cell from each subclone was transferred to fresh medium. This process was repeated for a number of transfers.

For the crosses parental cultures were washed in Dryl's salt solution (7) and mixed. Mating occurs after 1-2 hr. Single pairs were separately isolated after 36 hr into different wells of depression slides containing Cerophyl-*Aerobacter* medium. Exconjugants were not separated in these experiments but were permitted to grow up together in the same well.

After 3-4 days all cultures were examined and were classified into 3 types: Dead, mature and immature. Dead cultures included those which were dead as well as those unable to undergo more than 2 or 3 fissions. Mature cultures contained pairs and reacted sexually to samples of a tester culture of non-parental mating type in a "maturity test." Such cultures contain either Round 1 exconjugants or non-conjugants. Immature cultures did not contain pairs nor were they sexually reactive in the maturity test. The viability of a cross was expressed as the percentage of *immature* cultures. H-serotype tests were conducted on samples of immature cultures according to the method of Nanney and Dubert (12).

TABLE 1. *Fate of AB and C* subclones*†.

	Transfers of AB				Transfers of C*			
	0	*1*	*2*	*3*	*0*	*1*	*2*	*3*
No. Subclones:								
Dead	0	1	0	0	6	29	12	9
Slow	0	0	1	2	3	4	5	8
"Normal"	82	81	80	79	0	3	7	11
With Crinkled Cells#	0	0	0	0	110	77	60	44
TOTAL	82	82	81	81	119	113	84	72

† Design of experiment: A number of structurally normal single cells was isolated from populations of AB or C*. At 3 day intervals (and after 10-12 fissions) a single cell, also structurally normal, was transferred from each subclone to fresh medium.

Presence of crinkled cells was determined by examination of cultures after 3 days. These abnormal cells are usually found resting on the bottom of the well of the depression slide. Cultures were designated as "normal" if no crinkled cells were observed. However, a very few such cells could easily be missed in populations of 1-4,000 cells.

Cytologic methods: All cultures used for cytologic work were grown axenically in 1% proteose-peptone at 23 C. Mass samples of subclones in Cerophyl-*Aerobacter* were transferred thru antibiotics (penicillin-streptomycin) to peptone. To obtain cells in division, tubes containing 5 ml of peptone, inoculated with 0.1 ml of a plateau culture, were used after 1-3 days, depending upon the growth rate of the cell line. The cells were concentrated by centrifugation, washed in Dryl's salt solution and processed as described previously (3) using Gomori's hematoxylin as a nuclear stain (9).

RESULTS

Cloning C and AB.* Subclones of C* were initiated by selection of structurally normal cells and were propagated by picking normal-appearing cells during the serial transfers. As a control subclones were also initiated from AB and handled in the same way. At each transfer the cultures were classified into 4 categories: Dead, slow, those containing crinkled cells, and those not containing crinkled cells (or normal).

The results are shown in Table 1. In the AB series 1/82 of the subclones died, 2 were slow and the rest were completely normal as to growth rate and appearance. In the C* series almost half of the subclones died during the course of the transfers. Most contained crinkled cells, altho with selection a few appeared which seemed to be normal. By the 3rd transfer 11/72 fell into this category.

For cytologic and genetic studies we selected 7 of the AB subclones, 5 of the C* subclones which appeared normal and 5 which contained a high proportion of crinkled cells. We also cloned the 5 normal C* subclones further, again selecting for normal cells at each transfer. Two of the most normal sets of lineages were examined cytologically and in crosses.

Micronuclear chromosomes of C and AB subclones.* We prepared slides of samples of each of the subclones and examined a number of cells as to the size and number of micronuclei per cell. In cells in division we attempted to determine the number and condition of the chromosomes.

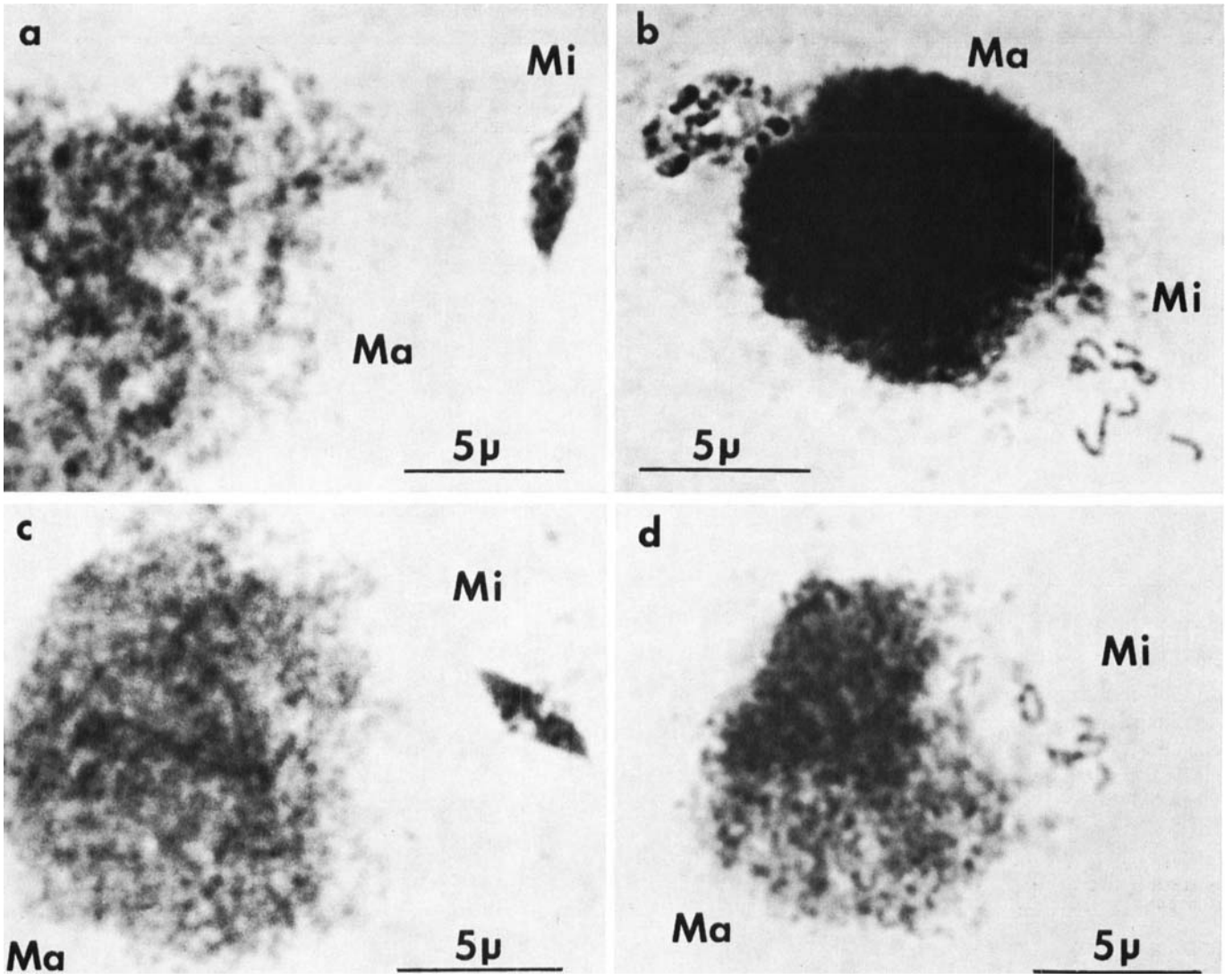

Fig.1. (a,c) Micronucleus of AB cells during mitosis: AB-1 (a, metaphase?), AB-5 (b, anaphase). Note spindle-shaped aggregate. Individual chromosomes are not resolved, as is normal for the micronucleus of *Tetrahymena*. During meiosis AB cells have the normal number of chromosomes for *T. pyriformis* (2n = 10). (b,d) Mitotic chromosomes of C* cells at anaphase: C*27 (b), C*3 (d). The aggregate is not observed, and individual chromosomes are clearly resolved. These chromosomes are about ¼ the size of normal meiotic chromosomes and some are fragmented. Ma, macronucleus; Mi, micronucleus.

The results are shown in Table 2 and Fig. 1. We found that all the AB cells had micronuclei and that all their micronuclei were diploid. Some of the subclones had more than one micronucleus, altho in only a few percent of the cells. The maximum number of micronuclei was 2 per cell.

A very different picture emerged when we examined the C* subclones. Most of the subclones contained some cells with more than one micronucleus, up to 10 micronuclei being observed in a single cell. The distributions of the numbers of nuclei varied between subclones, some containing predominantly one nucleus and others predominantly 2 nuclei. All of these nuclei were smaller than the normal diploid nucleus, but there were variations in nuclear size not only between cells but within the same cell. The nuclei of cells of some subclones were more uniform in size than others.

All subclones had some amicronucleate cells, but the 2 groups of C* subclones differed in the frequency with which amicronucleate cells were present in the cultures. The first group which appeared free of crinkled cells had an average of 5% amicronucleate cells in the population (range 0.8-7.8%). The 2nd group which had a high frequency of crinkled cells had an average of 27% amicronucleate cells in the population (range 19.8-31.0%). On the slides many of these amicronucleate cells appeared round and lacked any vestige of an oral apparatus.

No diploid micronuclei were observed in any of the C* cells. Upon further cloning of 2 of the "normal" subclones (27 and 45) 20 lineages which appeared free of crinkled cells were established. None of the cells in these lineages had a diploid micronucleus. We conclude that despite selection for normal cells we cannot reverse the process which has taken place; all nuclei are defective.

TABLE 2. *Distribution of micronuclear types in selected subclones of AB and C*.*

Sample #	Diploid No. of nuclei		Aneuploid No. of nuclei			Amic	No. Chromosomes	Remarks
	1	*2*	*1*	*2*	*3+*			
AB-1	100.0	0	0	0	0	0	(10)	Difficult to observe
2	99.6	0.4	0	0	0	0	(10)	chromosomes.
3	100.0	0	0	0	0	0	(10)	Compacted into
4	99.8	0.2	0	0	0	0	(10)	spindle-shaped
5	100.0	0	0	0	0	0	(10)	aggregate even at
6	98.8	1.2	0	0	0	0	(10)	metaphase-anaphase.
7	98.6	1.4	0	0	0	0	(10)	
								Nuclei:
C*-27	0	0	98.6	0.4	0.2	0.8	3-4	Uniform in size
23	0	0	33.0	53.2	8.2	5.6	2-5	Variable in size
25	0	0	22.6	50.4	22.2	4.8	2-3	Variable
45	0	0	89.2	3.6	0.4	6.8	3-5	Uniform
68	0	0	37.0	51.0	4.2	7.8	2-5	Variable
C*- 3	0	0	63.4	5.2	2.0	29.4	2-5	Variable
7	0	0	53.8	10.2	5.0	31.0	2-5	Highly variable
10	0	0	54.0	11.6	4.0	30.4	2-5	Highly variable
24	0	0	71.4	4.2	1.8	22.6	3-5	Variable
28	0	0	52.2	23.8	4.2	19.8	2-5	Highly variable

500 cells per sample observed.

The stem cell of C* appears to be aneuploid. The diploid number of chromosomes is 10 (13). The predominant type in C* seems to be characterized by 4 (Fig. 1b) or 3 (Fig. 1d) chromosomes. Some subclones like C*27 showed little variation in numbers (3-4) while others like C*3 showed more extensive variation (2-5). These chromosomes seem to be smaller and thinner than meiotic chromosomes and often appear fragmented. The fact that chromosomes of the micronuclei could be seen at all during mitosis, and in some cells actually counted, was in itself an unusual finding. Normally the chromosomes are held tightly together in a skein-like formation (Fig. 1a) and all that is seen is a pinching in 2 of the chromatin during division (Fig. 1c). Apparently the forces which normally cause the formation of this aggregate of chromosomes have been relaxed in C* cells. A similar loosening of the chromatin in a clone losing its micronucleus was reported by Wells (14).

Thus, the micronucleus of C* is truly defective and all cells contain fewer than the diploid number of chromosomes. The nuclear divisions appear to be highly abnormal and as a result not only amicronucleate cells are produced but also cells with several small nuclei. Since amicronucleate cells are incapable of dividing (5) and no diploid cells are present in the population, we conclude then that the cell line is maintained by replication of cells having a defective micronucleus. These would appear to be the cells which are structurally normal.

Behavior of C subclones in crosses.* We next examined the breeding behavior of the C* subclones in crosses to AB. We were interested in determining if lines which are free of crinkled cells produce more viable progeny than those which are not, and if they ever transmit their own genes. We scored the progeny for their H serotypes. Since AB is H^A/H^D and C* is H^E/H^E then the presence of the He antigen in any of the progeny would signal transmission of C* genes.

The results are shown in Table 3 for the 2 groups of subclones. We found no difference between the groups in their average breeding performance (74% and 75% viability, respectively). When tested for their H serotypes, none of the progeny was He, and all must have been produced by genomic exclusion. Cytologic examination of an early stage of conjugation (crescent stage) of crosses of 4 subclones which differed in the frequency with which amicronucleate animals were present (0.8, 6.8, 29.4, and 31.0%) showed a similar but low frequency of mating amicronucleate animals (0.5, 3.0, 3.5, and 3.0%). We conclude that amicronucleate animals do not readily mate and are usually excluded during conjugation. Micronucleate animals, regardless of how much chromatin is present, do participate in the mating and seem to be potentially equivalent in their breeding performance and in their ability to induce genomic exclusion. There are differences in viability between crosses but they do not seem to be correlated with the degree to which amicronucleate animals are present.

The results of the 2nd series of clones are shown in Table 4. All of these appeared to be free of crinkled cells but all contained a low percentage of amicronucleate animals. Altho there is variation in the breeding performance of the 20 lines (33-97% viability) the overall average (77%) is not different from the averages shown in Table 3. Thus, further selection for "normalcy" was not effective in raising the average breeding performance of these lines. Nor was it effective in producing progeny which showed transmission of C* genes. All the progeny in these crosses had either the Ha, Had, or Hd pheno-

TABLE 3. *Viability and serotype distribution of progeny and percentage of mated amicronucleates in crosses of AB to selected C* subclones.*

C*	Distribution of Progeny			Viability	Distribution of Serotypes			% Amicronucleate Cells in	
Subclone	Dead	Mature	Immature	(% Immature)	Ha	Had	Hd	Subclones	Crescents[1]
27	4	0	26	87	7	12	7	0.8	0.5
23	2	6	20	72	3	11	6	5.6	
25	2	9	18	62	5	8	5	4.8	
45	2	8	16	62	4	7	5	6.8	3.0
68	4	0	23	85	7	9	7	7.8	
Total	14	23	103	74	26	47	30	5.2	1.8
3	1	7	22	73	7	10	5	29.4	3.5
7	6	8	16	53	5	8	3	31.0	3.0
10	0	5	22	81	8	7	7	30.4	
24	1	2	24	89	6	11	7	22.6	
28	2	4	24	80	5	11	8	19.8	
Total	10	26	108	75	31	47	30	26.6	3.3

[1] 200 crescents examined per sample.

types which are specified by genes contributed from AB. This means then that all the progeny were produced by genomic exclusion.

These observations reinforce our conclusions that none of the C* cells contains a normal micronucleus. All must have defective nuclei, and there does not seem to be a strong correlation between the degree of the defect, breeding performance and the ability to induce genomic exclusion. This situation apparently arises due to the exclusion of amicronucleate animals from the act of conjugation.

DISCUSSION

C* is a clone which seems to lack any cells which have a diploid micronucleus since rigorous selection against crinkled cells failed to establish a stable normal cell line. The stem cell appears to be aneuploid, with about 4

TABLE 4. *Effects of further cloning and selection for "normalcy" on percentage of amicronucleates, viability and serotype distribution in crosses to AB.*

C*	Distribution of Progeny			Viability	Distribution of Serotypes			% Amicronucleate Cells in	
Subclone	Dead	Mature	Immature	(% Immature)	Ha	Had	Hd	Subclones[1]	Crescents[2]
27-1	3	4	23	77	6	10	7	1.5	1.0
2	2	3	25	83	9	10	6	2.5	2.0
3	1	4	25	83	6	11	8	2.0	1.0
4	9	3	18	60	4	8	6	5.0	2.5
5	1	1	28	93	8	12	8	0.5	0.5
6	5	7	18	60	6	7	5	3.0	2.0
7	1	1	28	93	8	11	9	1.5	1.0
8	4	5	21	70	7	9	5	4.5	2.0
9	7	7	16	53	4	6	6	4.0	2.5
10	0	1	29	97	6	15	8	1.0	1.0
Total	33	36	231	77	64	99	68	2.6	1.6
45-1	0	3	27	90	8	12	7	5.0	3.0
2	0	1	29	97	6	14	9	1.0	1.0
3	3	1	26	87	8	10	8	4.0	2.0
4	12	8	10	33	3	5	2	7.5	3.5
5	1	2	27	90	10	10	7	0.5	0
6	8	4	18	60	6	8	4	4.5	3.0
7	2	2	26	87	6	12	8	2.0	1.0
8	4	2	24	80	7	9	8	3.0	1.0
9	0	9	21	70	8	8	5	2.5	1.5
10	1	5	24	80	9	8	7	4.0	2.0
Total	31	37	232	77	71	96	65	3.4	1.8

[1] 200 cells observed per sample.
[2] 200 crescents examined per sample.

chromosomes instead of the usual 10, and from this line cells with fewer chromosomes or without micronuclei are generated. Only cells which have some micronuclear chromatin survive. The fate of amicronucleate cells is death, and they seem to mate very poorly. Since they do not mate well, they are selected against during conjugation. As a result, there is little difference between populations of cells having high or low numbers of amicronucleate animals in their ability to generate viable offspring. All progeny which are produced result from genomic exclusion and none receive genes from C*.

We have never observed a viable amicronucleate cell line in syngen 1. Amicronucleate cell lines have been collected in nature but only rarely has a newly developed line been observed to divide and multiply in the laboratory. For example, Wells (14) reported the genesis of a viable amicronucleate line in syngen 6. The fact that newly formed amicronucleate lines usually die argues for an essential function carried out by the micronucleus. In those lines which do make it Wells (14) has suggested that this function has been taken over by the macronucleus.

In *Stylonychia mytilus* Ammermann (6) was able to produce amicronucleate cells by uv-microbeams or x-irradiation. Altho most of the cells died and many structurally abnormal cells were observed, in some cases clones developed which appeared structurally normal and which resumed the normal rate of division. By appropriate crosses Ammermann was able to show that the micronuclear function was indeed taken over by the macronucleus. He also was able to show that in some of the recovered lines chromatin was extruded from the macronucleus and that it was then perpetuated in the form of "pseudomicronuclei." These bodies are very much smaller than normal micronuclei, contain less DNA, and often several are present in the same cell. By autoradiographic experiments he was able to show a low level incorporation of radioactive uridine, implying that the pseudomicronuclei are functional and synthesize RNA in small amounts. A low level of RNA synthesis by normal micronuclei was also observed in *Stylonychia*.

In *Tetrahymena* RNA synthesis by normal micronuclei could not be detected (8). The macronucleus does synthesize large amounts of RNA. Moreover, in heterocaryons, where the micronucleus and macronucleus differ in genotype, only the gene which is present in the macronucleus is expressed (2). On the basis of these observations we would then not expect that the loss of the micronucleus would have any dire effect on a cell. Yet it does. It is possible that only a few essential functions are carried out by the micronucleus and that they are not detected in either the autoradiographic or genetic experiments.

Altho some amicronucleate *Tetrahymena* seem to recover, most do not. Reversion to complete normalcy was never observed for C*. These cells persist in spite of the fact that they have only a partial micronuclear genome. This partial genome seems to be essential for survival.

In C* the macronucleus has an effect on the behavior of the micronucleus during its division. We showed this earlier (5) by following the fate of heterocaryons in which a new diploid micronucleus replaced the defective micronucleus. In the presence of the C* macronucleus the new micronucleus became defective and was lost from some of the cells in the population. Only by also replacing the macronucleus were cell lines generated in which the micronucleus remained diploid and behaved normally during division.

The aneuploid condition of the C* micronucleus is accompanied by a reduced amount of DNA. Microspectrophotometric measurements of the micronuclei of C*, strain 7 (a second semi-amicronucleate cell line which behaves in crosses like C*) and 3 normal strains in syngen 1 have been made (4). If we express our results in terms of the amount of DNA in the *haploid* genome, we find an average value of 1.31×10^{11} daltons for the normal strains and values of 0.28×10^{11} and 0.43×10^{11} for C* and strain 7.

The reduced genome size of the micronucleus in C* and strain 7 is also expressed in the macronucleus. The evidence for this comes from renaturation experiments on denatured sheared DNA which can give an estimate of the size of the haploid genome (4). When carried out on whole cell DNA we found a haploid genome size of 0.88×10^{11} daltons for C* and 0.68×10^{11} daltons for strain 7. The haploid genome size of the 3 normal strains ranged between 1.45 and 1.72×10^{11} daltons. Since a minimum of 89% of whole cell DNA originates from the macronucleus, this means then that the genome size is reduced in the macronucleus of C* and strain 7. Genome reduction does *not* occur in the macronucleus of normal strains. This suggests that in defective strains like C* or strain 7 there are mechanisms which affect *both* the micronucleus and macronucleus to bring about loss of DNA molecules.

The micronucleus may have specialized functions which can be carried out by the macronucleus when the micronucleus is lost. The macronucleus can also interact with the micronucleus to affect its mode of replication. Both nuclei may be subject to a common stimulus which results in reducing their genomes.

Cell lines like C* are grossly abnormal yet they survive. C* is now 14 years old. By cloning it before use, we can always generate a line which will induce nearly 100% viable progeny by genomic exclusion.

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Is There Selective Mating in *Tetrahymena* During Genomic Exclusion?[1]

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SYNOPSIS. Genomic exclusion is characterized by 2 rounds of mating. If exconjugants from different pairs remated at random after the first mating, we would expect a 1:2:1 ratio for genes present in heterozygous condition in the normal parent. An excess of homozygotes is observed which is similar for 2 different genes, suggesting that 10% of the rematings occur between exconjugants from the same Round 1 pair. Some but not all of these homozygotes can be attributed to a lack of separation of mates after the first round of mating. The rest may result because of differential mortality, induced autogamy or preferential remating.

UNIPARENTAL transmission of genes occurs in *Tetrahymena pyriformis* as a consequence of an abnormal form of conjugation called genomic exclusion. This unusual gene behavior is observed in the mating of a cell with a defective micronucleus and one with a normal micronucleus. Only the genes from the cell with the normal micronucleus appear in the progeny. If the normal cell is heterozygous, then more than one phenotypic class is observed.

The basis for uniparental transmission of genes resides in a series of unusual nuclear events which take place during conjugation (2). The normal mate contributes replicas of a single meiotic product to the diploid fertilization nucleus of each exconjugant, and when the exconjugants come apart the old macronucleus becomes the functional nucleus. The resulting exconjugants are heterocaryons, sexually mature and can remate immediately. When the exconjugants from the same pair remate, the progeny that appear are homozygous for all genes. If instead they remate with an exconjugant from a different pair, then the progeny can be heterozygous for some of the genes. For genes which are known to be heterozygous in the normal parent the 2 homozygous classes appear in a 1:1 ratio when all rematings involve exconjugants from the same pair. When remating of exconjugants from different pairs occurs, then the heterozygous class also appears. We would expect a 1:2:1 ratio of the 3 phenotypic classes if remating occurred at random.

Very early we observed departures from the expected 1:2:1 ratio for the H serotypes in such rematings and a deficiency of the heterozygous class (1). We also observed a deficiency of heterozygotes for the phosphatases when we examined *H* homozygotes. In this selected group of progeny the phosphatases seemed to be segregating in a 1:1:1 ratio. We did not, however, look at the distribution of phosphatases among *H* heterozygotes.

Does the distortion in the genetic ratio affect other genes or is it special to the *H* locus? The *H* character is a surface character and, since mating also involves a surface reaction, perhaps there is something peculiar about surface reactions which are manifested in the distorted ratio.

In this report we shall show that the same type of distortion occurs in the genetic ratios for the phosphatases, with an excess of homozygotes. How do these homozygotes arise during genomic exclusion? Is remating selective?

MATERIALS AND METHODS

Strains: C* is a semi-amicronucleate clone containing a mixture of cell types, some of which have a defective micronucleus and others which are amicronucleate (3). It was derived by inbreeding of the C strain (C-5573). In genotype it is H^{E}/H^{E} $P\text{-}1^{B}/P\text{-}1^{B}$.

AB clones have a normal diploid micronucleus and are derived from crosses of inbred strains A and B. In this paper we used 2 AB clones (AB-6 and AB-7a) which came from a cross of A-11613 and B-12614d. AB clones are heterozygous at both the *H and P-1* loci (H^{A}/H^{D} $P\text{-}1^{A}/P\text{-}1^{B}$).

Culture technics: Cultures of C* and AB were maintained at 23 C in bacterized medium (0.15% Cerophyl rye grass infusion inoculated with *Aerobacter aerogenes*). Previous to using them in crosses C* and AB-6 and 7a were cloned. Crosses (AB-6 × C* and AB-6 × AB-7a) were then set up with the subclones on a small scale and crosses yielding maximum viable progeny were then repeated on a large scale.

In preparing for a cross parental cultures were washed in Dryl's salt solution (6) and mixed. Mating begins after about 2 hr. Pairs were isolated at 4, 10, or 36 hr after the beginning of mating in different experiments. Single pairs were pipetted into different wells of depression slides containing bacterized medium and the exconjugants from the same pair were permitted to grow up together in the same well.

After 3-4 days the cultures were examined and classified into 3 types: Dead, mature and immature. *Dead* cultures included those which had died as well as those unable to undergo more than 2 or 3 fissions. *Mature* cultures contained pairs and reacted sexually to samples of a tester culture of non-parental mating

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