CYTOCHEMICAL STUDIES ON THE PROBLEM OF MACRONUCLEAR SUBNUCLEI IN TETRAHYMENA

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ABSTRACT

DNA amounts in macronuclei and micronuclei of *Tetrahymena pyriformis* were measured by Feulgen microspectrophotometry. Assuming that the unreplicated micronucleus is diploid, the unreplicated macronucleus was found to contain approximately 45 times the haploid DNA amount. The relationship of these findings to the 45 independently assorting genetic subunits characterized by ALLEN and NANNEY and their collaborators is discussed. The pattern of DNA synthesis in macro- and micronuclei during the cell cycle is also described.

CERTAIN ciliates show phenotypic differences within a genetically homogeneous clone. The elegant studies of SONNEBORN first indicated that this intraclonal variability in *Paramecium aurelia* had its basis in macronuclear differentiation. SONNEBORN's studies on macronuclear regeneration in *P. aurelia* also clearly demonstrated the compound nature of the ciliate macronucleus, giving rise to the idea that the macronucleus was composed of independent subnuclei which were capable of random segregation at each macronuclear division (see SONNEBORN 1947, for review). More recently, genetic studies have indicated that the macronucelus of Tetrahymena contains independently assorting genetic subunits (NANNEY, CAUGHEY and TEFANKJIAN 1955; ALLEN and NANNEY 1958; NANNEY and DUBERT 1960; NANNEY 1964; ALLEN 1965; BLEYMAN, SIMON and BROSI 1966; PHILLIPS 1967 a,b; BLEYMAN and SIMON 1968). Based on the rate of assortment of pure types from clones manifesting mixed phenotypes, SCHENSTED (1958) calculated that there were 90 such subunits present in the macronucleus immediately prior to cell division (G_2 macronucleus) or 45 subunits in a recently divided (G_1) macronucleus. To date, the inheritance of at least 6 different codominant genetic loci has been analyzed in Tetrahymena. Each shows the same rate of production of pure types which can be explained by a model in

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which there are 45 subunits in the G_1 macronucleus. It has also generally been assumed that these subunits (subnuclei) are diploid since diploidy best explains the genetic data on assortment patterns (see NANNEY 1964 and DISCUSSION, below). If this model is correct, the G_1 macronucleus should contain 45 times as much DNA as the (presumably diploid) unreplicated (G_1) micronucleus. In this paper, we present microspectrophotometric measurements of the DNA amounts of macro- and micronuclei which indicate that there are, in fact, 45 subunits in the G_1 macronucleus of *Tetrahymena pyriformis*, but that these subunits are haploid.

MATERIALS AND METHODS

A clone of *Tetrahymena pyriformis*, derived from Syngen 1, strain A-17681 (kindly provided by Dr. S. L. ALLEN), were grown axenically in an enriched proteose peptone medium as described previously (GOROVSKY and WOODARD 1969).

Synchronous populations of cells were obtained by selecting dividing animals from a log phase culture over a 5-min interval (STONE and CAMERON 1964). The cells were placed on a microscope slide in a small drop of conditioned medium and were allowed to progress through the division cycle for various lengths of time, after which the slides were placed on an iced metal tray and the excess medium was drawn off with a braking pipet. The small amount of remaining medium dried rapidly when the slides were returned to room temperature. Slides were air dried overnight, and were post-fixed either in 10% formalin, absolute ethanol or a 3:1 mixture of absolute ethanol and glacial acetic acid.

An example of the degree of synchrony obtained by this method is seen in Figure 1. At

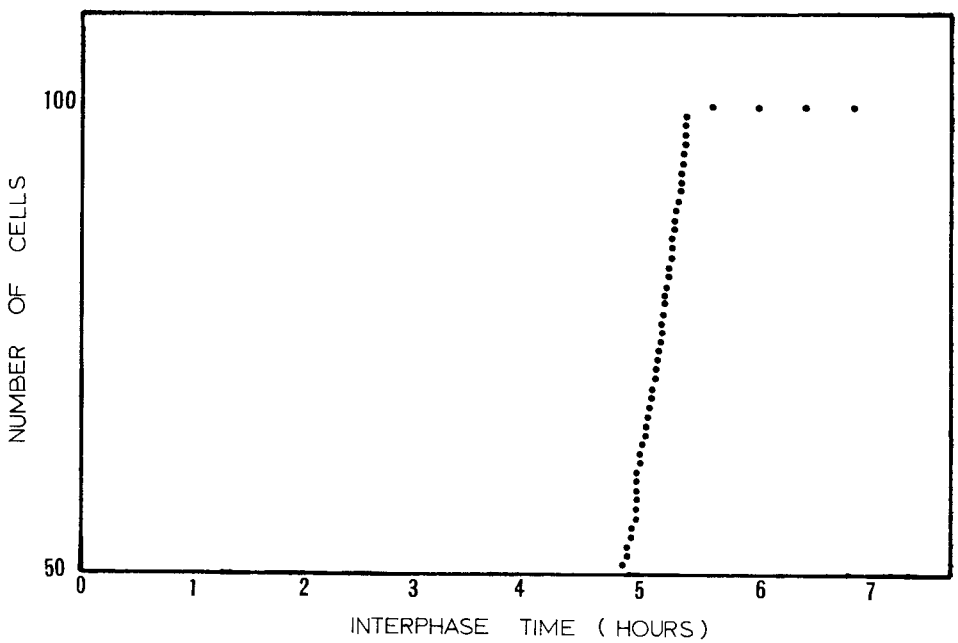

FIGURE 1.—Twenty-five dividing cells were collected from log phase cultures over a 5 min period and were allowed to proceed through the division cycle. Cell counts at the end of interphase show the degree of synchrony.

temperatures of 25°C or 28°C the duration of interphase was between 3½ and 5½ hr, depending on the temperature and the strain used.

Since determination of ploidy must be made on nuclei not engaged in DNA replication, it was necessary to devise methods for selection of macronuclei either preceding (G_1) or following (G_2) DNA synthesis (S). This problem was approached in two ways. In method 1, photometric measurements were made on synchronized cells fixed at 30-min intervals during interphase. When mean macronuclear DNA amounts were plotted against interphase time (Figure 2), interphase could be divided into three periods; G_1, S and G_2. Only G_1 or G_2 nuclei were used to calculate macronuclear : micronuclear DNA ratios. To confirm this time sequence, DNA synthesis was studied in synchronized cells using ^{3}H-thymidine. Animals were fixed at thirty minute intervals after being exposed to ^{3}H-thymidine for 20 min preceding fixation, and the slides were processed for autoradiography. These experiments allowed us to determine the time of micronuclear DNA synthesis and to compare the autoradiographic data (Figure 3) on the micronucleus with the microspectrophometric data.

In Method 2, a sample of log phase cells was grown in the presence of ^{14}C-thymidine for 5 min, air dried, fixed, and stained by the Feulgen reaction for DNA, and autoradiographs were then prepared. Nuclei undergoing DNA synthesis could be identified by the presence of silver grains above them. Non-radioactive cells were considered to be in either G_1 or G_2 stages of the cell cycle. Under these conditions, the non-radioactive cells selected for measurement were of 3 types:

1. Replicated (G_2) micronuclei and non-replicated (G_1) macronuclei.
2. Replicated (G_2) micronuclei and replicated (G_2) macronuclei.
3. Dividing animals with replicated (G_2) but undivided macronuclei and unreplicated (G_1) micronuclei.

DNA amounts were measured by the two-wavelength method (Ornstein 1952; Patau 1952) using wavelengths of 5000 Å and 5400 Å, by photographic plate microdensitometry (Woodard, Gorovsky and Swift 1966; Gorovsky and Woodard 1967) or with a Vickers scanning and integrating microspectrophotometer. In some experiments, the absolute amounts of DNA in the macronucleus and micronucleus were estimated by measuring DNA amounts in the nuclei of a rat liver imprint (kindly provided by Dr. David Grove) fixed and stained along with the Tetrahymena used in this study. This method assumes that hydrolysis maxima and other variables of the Feulgen reaction are similar for rat liver and for Tetrahymena.

RESULTS

Pattern of DNA synthesis in the cell cycle: Figure 2 shows that DNA synthesis, as measured by Feulgen microspectrophotometry began in most macronuclei at about 120 min, and was complete at about 270 min. As might be expected, the DNA amounts per nucleus showed approximately a 1:2 ratio before and after replication (Table 1). An autoradiographic analysis of DNA synthesis in the cell cycle is seen in Figure 3. Although some variability in this type of analysis is to be expected due to self absorption of beta particles by overlying cytoplasm, it is clear from this study that the maximum number of macronuclei undergo DNA synthesis in mid-interphase. Macronuclear labelling at the beginning and end of interphase may be due to small amounts of early (or late) DNA synthesis as for example has been shown to occur in nucleoli in Tetrahymena (Charret 1969). Micronuclei, on the other hand, undergo DNA replication within a relatively short period, starting at late anaphase, and ending approximately 30 min after fission. Only one labelled micronucleus was found at time points later than 30 min.

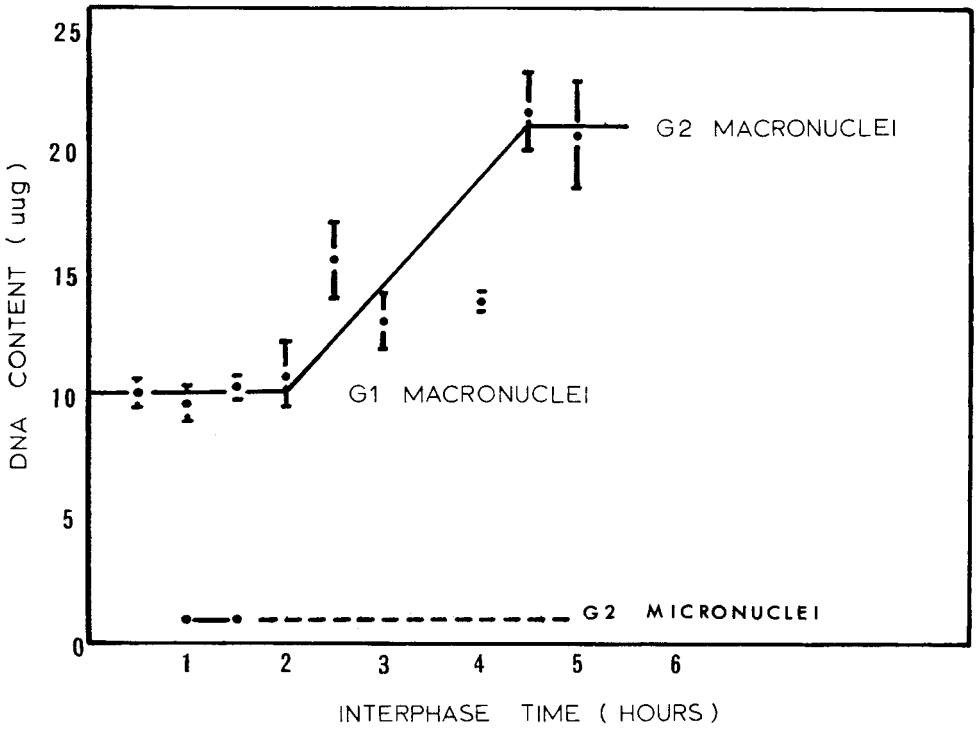

FIGURE 2.—DNA (Feulgen) content of synchronized macronuclei plotted against interphase time.

Comparison of DNA amounts in macro- and micronuclei: Six macronuclei having values close to the mean unreplicated (G_1) DNA value were selected from the experiment in Figure 2, and their DNA amounts were measured by the highly accurate method of photographic plate microdensitometry. Six G_2 micronuclei (any interphase micronucleus in a cell at a time 90 min or later after division) were also photographed and measured. The data are summarized in Table 2. Assuming that the variables associated with the Feulgen reaction are

TABLE 1

DNA amounts (2 wavelength photometry of Feulgen-stained nuclei) in macronuclei and rat liver diploid nuclei

Nucleus	Number of measurements	Feulgen DNA	Actual DNA
Diploid rat liver	5	650 ± 13*	6.55 μμg†
G_1 macronucleus	25	1009 ± 33	10.16 μμg‡
G_2 macronucleus	20	2114 ± 127	21.29 μμg‡

* Mean ± standard error in arbitrary units.

† Average value of chemically determined DNA amounts in non-polyploid, non-growing rat tissues. Calculated from data in MIRSKY and OSAWA (1961).

‡ Calculated using 6.55 μμg DNA/650 absorption units.

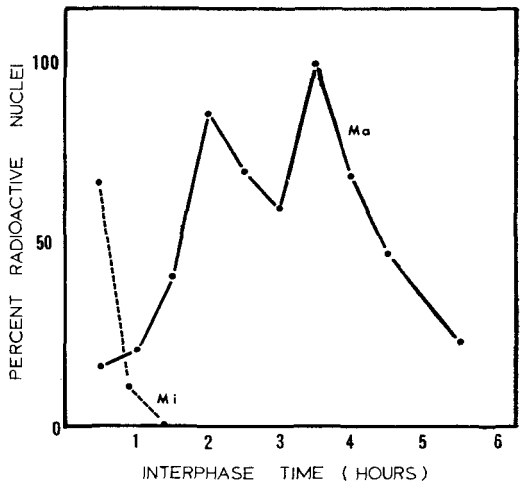

FIGURE 3.—Percentage of radioactive macronuclei and micronuclei plotted against interphase time. 13–40 macronuclei and 9–10 micronuclei were counted at each time point.

the same for both macro- and micronuclei, these results show that the G_1 macronucleus contains the equivalent of 23.6 diploid genomes or 47.2 haploid genomes.

It was possible that we were dealing with a micronuclear tetraploid (ALFERT and BALAMUTH 1957) in our studies, whereas the genetic studies of ALLEN and NANNEY on subnuclear assortment had been performed with a diploid clone. Animals of a second clone (Syngen 1, A-16671d) were obtained from Dr. DAVID NANNEY and a group of non-replicating nuclei were selected (by method 2) for DNA measurements. The data are summarized in Table 3. Macronuclear : micro-

TABLE 2

DNA amounts (plate densitometry of Feulgen-stained nuclei) of macro- and micronuclei of synchronized cells

Nucleus	Number of measurements	DNA amounts
G_2 micronucleus	6	0.86 ± 0.03 μμg* (4C)
G_1 macronucleus	6	10.16 ± 0.42 μμg (47C)

* Mean ± standard error.

TABLE 3

Macronuclear and micronuclear DNA amounts in animals of strain A-16671d

Nucleus	Number of measurements	Feulgen DNA
G_1 macronucleus	7	11.46 ± 1.1*
G_2 macronucleus	6	28.94 ± 2.3
G_1 micronucleus	7	0.59 ± 0.06
G_2 micronucleus	2	1.34, 1.36

* Mean ± standard error in arbitrary absorption units.

TABLE 4

Macronuclear and micronuclear DNA amounts in strains F-5 and C of* Tetrahymena pyriformis

Strain	Nucleus	Number of measurements	Feulgen DNA
F-5	G_2 macronucleus	8	27.15 ± 2.3†
F-5	G_2 micronucleus	6	1.19 ± 0.01
C*	G_2 macronucleus	10	21.02 ± 0.90
C*	G_2 micronucleus	4	0.10, 0.16, 0.05, 0.11

† Mean ± standard error in arbitrary absorption units.

nuclear DNA ratios fell into 3 categories as described in materials and methods:

1. Nonreplicated macronuclei–replicated micronuclei: 9.4
2. Replicated macronuclei–replicated micronuclei: 23.7
3. Replicated macronuclei–unreplicated micronuclei: 47.4

Two additional strains of *Tetrahymena pyriformis* were also investigated using Method 2. Strain F-5 (obtained from Dr. J. O. CORLISS) showed a DNA ratio of G_2 macronuclei to G_2 micronuclei of 22.8, in good agreement with our measurements on the other strains. Strain C* (obtained from Dr. S. L. ALLEN) is considered by ALLEN (1967a,b) to have a hypodiploid micronucleus. That this is in fact the case can been seen in Table 4. Although DNA amounts in the macronuclei of strain C* were similar to those of F-5, the mean micronuclear DNA content was only about 10% that of the "normal" micronuclei of F-5.

DISCUSSION

DNA synthesis in the cell cycle: The value obtained in this study of 10–20 $\mu\mu$g of DNA per cell in growing Tetrahymena agrees reasonably well with biochemical determinations of DNA amounts in other strains of Tetrahymena (LEE and SCHERBAUM 1966; CAMERON and JETER 1970). In addition, a similar pattern of DNA synthesis to that observed here has also been reported (MCDONALD 1962, PRESCOTT, see FLICKINGER 1965). Our findings on the timing of DNA synthesis during the cell cycle are summarized in Figure 4. Although our values for DNA amounts in G_1 and G_2 macronuclei showed an approximate 1:2 relationship, the variations which were observed (Figure 2) about the mean values were greater than that usually found in photometric measurements of plant and animal diploid nuclei which undergo an orderly partition of chromatin at mitosis. Although some of this variability may have been caused by trauma due to handling while selecting dividing cells, and to asynchrony of DNA synthesis, another explanation is found in the recent experiments of CLEFFMANN (1968). He found that DNA-containing bodies in Tetrahymena (HSM) were lost during macronuclear divisions until a lower limit of DNA content per macronucleus was reached, at which time an extra replication of DNA occurred. These two contrasting events —DNA elimination and additional replications without intervening divisions— apparently serve to maintain an "average" amount of DNA in the macronuclei of the population as a whole and, at the same time, to explain the high degree

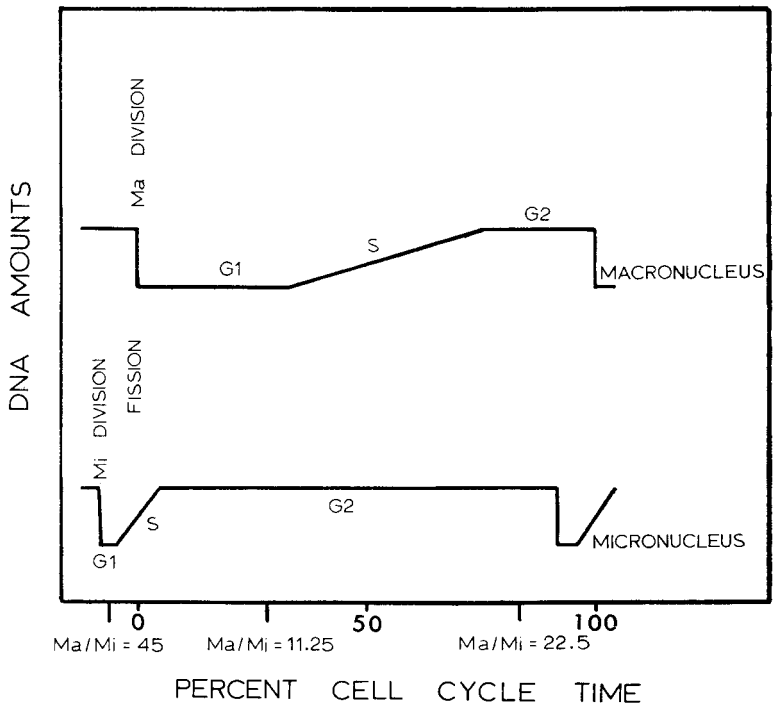

FIGURE 4.—A summary of the pattern of DNA synthesis during the cell cycle and the resultant macronuclear : micronuclear DNA ratios expected if unreplicated macronuclei contain 45 haploid subunits and unreplicated micronuclei are diploid. The vertical axis is logarithmic. Ma= macronucleus, Mi=micronucleus.

of variability between cells. Our results, like CLEFFMANN's, indicated that "on the average" DNA was doubled prior to each division.

Macronuclear subnuclei: Both the genetic data and the photometric data presented here are in good agreement in that the G_1 macronucleus contains approximately 45 subunits. The number 45, inasmuch as it is not part of a geometric progression starting with a diploid nucleus (2,4,8, 16, ...) indicates at least some disparity between replication and division during the formation of the macronucleus from a diploid synkaryon. In this respect, the macronucleus of Tetrahymena appears to differ from that of Stylonichia, which contains approximately the 64C amount of DNA in G_1 (AMMERMAN 1971). It should be particularly rewarding to follow the course of DNA synthesis during differentiation of the macronucleus of Tetrahymena to determine if there is ever a stepwise doubling of DNA and if there is, at which stage does the DNA content differ from a geometric progression.

One must be cautious in the interpretation of data obtained by microspectrophotometry of Feulgen-stained nuclei of widely different staining intensity. GARCIA (1970) has shown that, in some tissues, the amount of Feulgen staining is inversely proportional to the degree of nuclear compaction. If the relatively greater concentration of micronuclear DNA has resulted in an underestimate

yielding macronuclear : micronuclear ratios of 45 rather than 32, the percentage of underestimate would have to be 29%. An error of such magnitude is unlikely for a number of reasons: (1) 3 different fixations and preparative procedures gave similar results, (2) measurements of DNA amounts in isolated macro- and micronuclei gave similar ratios to those obtained here (GOROVSKY and WOODARD 1969) even though isolated macronuclei shrink sufficiently to make their Feulgen staining intensity similar to that of micronuclei; (3) it is unlikely that the agreement between the genetic studies and the photometric data is fortuitous (see below).

Haploid vs. *diploid subnuclei:* The genetic studies of ALLEN and NANNEY and their collaborators led to the suggestion that the macronuclear subunits have the properties of diploid subnuclei (see NANNEY 1964 for review). However, the DNA measurements presented here in three strains of Tetrahymena indicate that there are approximately 45 *haploid* equivalents in the G_1 macronucleus (assuming that the G_2 micronucleus is 4C). It must be kept in mind that the model containing 45 diploid subnuclei is based upon assorting genetic units, while the microspectrophotometric measurements present genomic units which might be considered chemical or structural units. Assorting units may be very different from structural units. As indicated by ALLEN and NANNEY, the number of assorting subunits is dependent upon the mechanics of separation of daughter subnuclei after replication. Deviations from random assortment of genetic subunits could lead to overestimates of the number of subunits if daughter subnuclei tended to segregate from one another, and an underestimate of their number if daughter subnuclei tend to remain together. The discrepancy between the cytochemical data and the genetic data may, therefore, reflect a non-random assortment mechanism at macronuclear division. On the other hand, the validity of the cytochemical studies might be questioned since differences in the physical or chemical state of the macro- and micronucleus might introduce errors in their quantitation. However, we feel that this is unlikely in light of the good agreement between the DNA amounts per cell determined cytochemically in this study and those determined biochemically by others. Moreover, any underestimate of the micronuclear DNA amount introduced by the compaction error (see DISCUSSION, above) would lead to an overestimate of the ploidy of the macronucleus, and the disparity between the genetic and the cytochemical studies would be even greater.

It is clear, however, that despite the differences in the techniques, genetic on the one hand, cytochemical on the other, our results can be thought to differ from those of ALLEN and NANNEY and their collaborators, not in the number of macronuclear subnuclei, but in their ploidy. It seems improbable that such remarkable agreement between two such disparate approaches would occur by coincidence, and we are encouraged to propose that assortment of subnuclei is random, and that the assorting units are haploid rather than diploid.

Recently, two other estimates of the ploidy level of macronuclei have appeared. NILSSON (1970) found morphological evidence for the presence of approximately 80 subnuclei in the replicated (G_2) macronucleus of an amicronucleate strain (GL) of Tetrahymena. Comparison of the number of chromatin bodies in these

subnuclei with the number of chromatin bodies in micrographs published by FLICKINGER (1965) of the micronucleus of strain HSM, led her to suggest that these morphological subnuclei were haploid. FLAVELL and JONES (1970), on the other hand, measured the complexity of the haploid genome of Tetrahymena by a kinetic analysis of the rate of renaturation of isolated DNA. Their results were in approximate agreement with the diploid model of macronuclear subnuclei. In summary, it is likely that the unreplicated (G_1) macronucleus contains approximately 45 subnuclei, but additional evidence will be necessary to reconcile the different estimates of the ploidy of these subnuclei.

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