SIMULTANEOUS SYNTHESIS OF HISTONE AND DNA IN SYNCHRONOUSLY DIVIDING *TETRAHYMENA PYRIFORMIS*

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ABSTRACT

Histone and DNA syntheses have been studied in synchronously dividing *Tetrahymena pyriformis* GL. During the heat treatment necessary to synchronize cultures of this amicronucleate protozoan, the DNA content of the already polyploid macronucleus increases. When the cells begin synchronous division, their DNA content is reduced in a stepwise process which is closely paralleled by reduction of macronuclear histone content. During cell division, the contents of DNA and histone decrease by slightly more than twofold, and in the subsequent S phase, DNA and histone increase simultaneously to 85% of the values expected if all chromosomes were to double. The first step in the process of reduction of DNA and histone contents is their decrease in excess of twofold, and this is accomplished by removal of extrusion bodies from the nuclei of dividing cells. The second step is a mechanism which allows, in effect, only 70% of the chromatin in the average nucleus to duplicate. Such partial duplication suggests that both histone and DNA syntheses in synchronous *Tetrahymena* depend upon a regulatory mechanism, the mediating elements of which are localized in only certain chromosomes.

INTRODUCTION

The organization of histone within the chromosomes is poorly understood. Histochemical and biochemical studies of DNA and histone indicate that these two substances are bound together by ionic bonds (15) and are in constant proportion to each other (1, 2, 26). Bloch and Godman (2) have suggested that this proportionality is maintained by simultaneous proportional synthesis of histone and DNA. Studies of regenerating liver show that this expectation is at least partially correct, for histone synthesis begins before and continues during the period of DNA synthesis (3, 16, 24, 33). Furthermore, proteins, of which some may be presumed to be histones, are synthesized rapidly in the DNA-replicating band of the ciliate protozoan, *Euplotes* (9, 25). Another protozoan, *Tetrahymena*, which may be grown in synchronous culture, provides a very useful system for study of histone and DNA syntheses during the cell cycle, and the experiments we describe in this paper demonstrate that Bloch and Godman's suggestion (2) is correct for *Tetrahymena*. Our results further suggest that control of histone and DNA syntheses is mediated by nondiffusible agents operating at the chromosomal level (12).

MATERIALS AND METHODS

Synchrony

The amicronucleate strain GL of *Tetrahymena pyriformis* was obtained from Dr. George Holz and grown in 2.0% proteose peptone medium with

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added salts (30). Cultures were synchronized by the heat treatment method of Scherbaum and Zeuthen (30). In each experiment, an exponentially growing culture of 25°C was inoculated into 1 liter of medium at 28.5°C contained in an automatic synchrony apparatus.[1] The volume of the inoculum was adjusted to provide 5×10^4 cells per ml after the end of the heat treatment. Usually after 6 hr of growth, the apparatus heated the culture for eight ½-hr periods at 34°C alternating with seven ½-hr periods at 28.5°C. Thereafter, the temperature was maintained at 28.5°C. During the experiments, the culture was stirred by a water-driven "Mag-Jet" stirrer mounted beneath the synchrony flask and was aerated by continuous flow of sterile air into the flask. In some experiments, the cells were diluted with fresh medium at the same temperature to keep the cell concentration below 10^5 cells per ml. When the end of heat treatment occurred, the culture was sampled at appropriate intervals, a small portion of the sample was fixed for cell counts, and the remainder quickly chilled on ice to stop division processes. Cells were counted with a Sedgwick-Rafter chamber and optical grid after fixation with 0.1 volume of a mixture of 3 vol methyl green and 7 vol 40% formalin. The volume of each chilled sample was adjusted to provide 1.6×10^6 cells for preparation of histone and DNA from partially purified macronuclei.

Preparation of Macronuclei

Partially purified macronuclei were prepared in high yield by centrifugation of lysates obtained from washed cells.[2] Cell samples were kept on ice and processed in batches of four or six at about 5°C. Each sample was centrifuged to sediment cells, and the medium was drawn off. The cells were resuspended in ice-cold distilled water and recentrifuged. The supernatant was drawn off and the cells were lysed in 5.0 ml of medium A (0.33 mg/ml digitonin, from Fisher Scientific Co., Pittsburgh, 8.5×10^{-5} M $CaCl_2$, 0.80 mg/ml spermidine trihydrochloride, from Sigma Chemical Co., St. Louis) at room temperature. Complete lysis was insured by gently drawing the cells through the mouth of a pipette. After 5–10 min, 5.0 ml of ice-cold medium B (1.8×10^{-4} M $CaCl_2$, 0.003 mg/ml spermidine trihydrochloride, 0.29 M sucrose, 0.01 M phosphate buffer, pH 6.2) was added to dilute the digitonin, and the suspension was centrifuged at 500 *g* for 5 min. The pellet containing nuclei and some heavy debris was resuspended in a 1/1 mixture (vol/vol) of media A and B and recentrifuged. The resulting pellet contained partially purified macronuclei representing 70–80% of the cells originally in the sample and was used for preparation of histone and estimation of DNA.

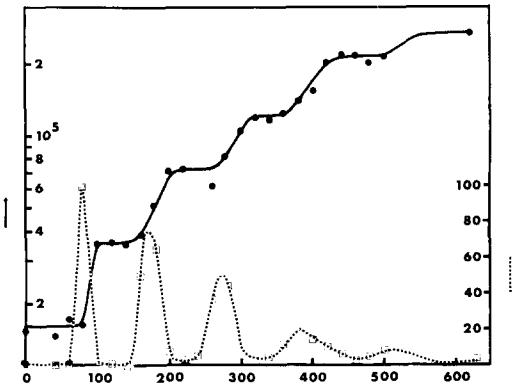

FIGURE 1 Characteristics of synchronous division in *Tetrahymena pyriformis* GL. Ordinate: solid line, cell concentration (cells per ml); dotted line, percent of cells dividing. Abscissa: minutes after the end of heat treatment.

Histone and DNA

The pellet containing partially purified macronuclei was resuspended in 2.0 ml of ice-cold 0.2 N HCl and allowed to stand for 15 min. The suspension was then centrifuged (10 min, top speed, IEC clinical) and the supernatant collected. This extraction was repeated and both supernatants were pooled to provide macronuclear histone which was assayed by the microbiuret method of Zamenhof (34). For the purposes of this paper, histone is defined as that protein of partially purified macronuclei which is soluble in dilute HCl. Certain problems attending this definition are considered in the Discussion. The pellet was incubated at 37°C in 2.0 ml of 1 N NaOH to hydrolyze RNA, and then 0.2 ml each of 10 N HCl and 3 N trichloracetic acid were added to precipitate DNA and protein. The suspension was chilled, centrifuged, and the supernatant dis-

[1] Basic design follows Scherbaum and Zeuthen (30). A low form culture flask (Pyrex) is bathed in a circulating water bath which is continually supplied with cold water. Temperature of the bath is controlled by two thermostats which intermittently actuate a pump to deliver hot water. One thermostat is set for 34°C, the other for 28.5°C, and a time switch alternately activates each one to deliver the heat treatment.

[2] J. L. Rosenbaum. Unpublished.

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carded. The pellet was resuspended in 2.0 ml of ice-cold 0.3 N trichloroacetic acid and centrifuged. The resulting pellet was taken up in 2.0 ml of 0.3 N trichloracetic acid, heated at 90°C for 15 min to hydrolyze DNA, and then centrifuged. This step was repeated, and the two supernatants were pooled and the DNA content assayed by the indole method of Iverson and Giese (17). Histone and DNA values were corrected by a factor of 1.33 to compensate for loss of cells and nuclei during preparation. The yield of nuclei and cells was not influenced by the stage of the cell cycle.

Macronuclear Volume

The volumes of macronuclei were calculated from the diameter of spherical macronuclei in the digitonin lysate. A sample (0.1 ml) of lysate in medium A was removed and fixed in methyl green–formalin as described for whole cells. Essentially all nuclei in these samples are spherical, and in the major proportion (the quantity of which is unrelated to the stage of the cycle) the chromatin is homogeneously distributed. The diameter of such nuclei was measured at 400 magnification with a phase contrast microscope equipped with an optical grid, and the volume was expressed in cubic microns.

Electrophoresis

Heterogeneity of histone was demonstrated by disc electrophoresis (Canalco) at pH 4.3 according to the method of Reisfeld et al. (27).

RESULTS

During the first two synchronous divisions, all cells divide rapidly to provide four times as many cells as at the end of heat treatment. Fig. 1 demonstrates the characteristics of a typical synchronous culture over a long time period. The percentage of cells dividing exhibits initially high, sharp peaks separated by increasingly longer interdivision periods, of which the first lasts 110 min. Since precise doubling of the cell concentration occurs only in the first two bursts of division, analyses of histone and DNA have been focused on these divisions.

The levels of macronuclear DNA and histone per cell systematically fluctuate in cycles that are in phase with each other during synchronous division. Fig. 2 displays the results of a representative synchrony experiment. In part *A* at the top are presented, in order, the stepwise doubling of cell concentration and associated peaks of synchronous

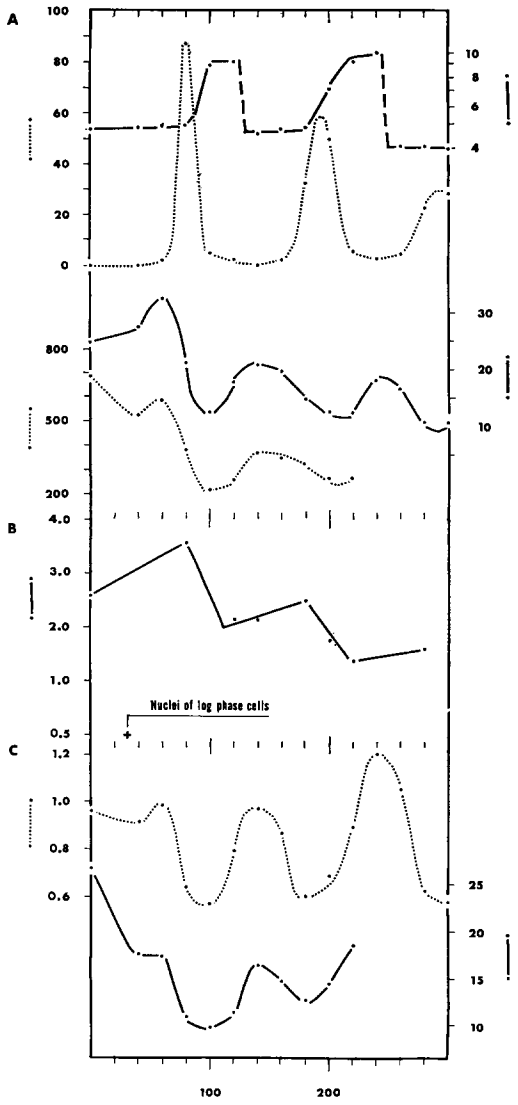

FIGURE 2 Events during synchronous division. Abscissa: minutes after the end of heat treatment. A, Upper curve (solid line) represents cell concentration (cells per ml $\times$ 10^4). Cells were diluted after each division (broken line). Second curve (dotted line) shows per cent of cells dividing. Third curve (solid line) shows macronuclear DNA ($\mu\mu$g per cell). Fourth curve (dotted line) shows macronuclear histone ($\mu\mu$g per cell). B, Average macronuclear volume ($\times$ 1000 μ^3). *C*, Dotted line, ratio of macronuclear DNA to macronuclear volume ($\mu\mu$g per cell per $\mu^3 \times 10^{-5}$). Solid line, ratio of macronuclear histone to macronuclear volume ($\mu\mu$g per cell per $\mu^3 \times 10^{-5}$). Macronuclear volumes for these curves were calculated from the data of part *B*.

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cell division. The third curve shows that the average macronuclear DNA per cell repeats a cycle which reaches a maximum value prior to division and a minimum as cell division occurs. The fourth curve, macronuclear histone per cell, demonstrates similar maxima and minima, which coincide in time with DNA. At the end of heat treatment, the average macronucleus contains 25 $\mu\mu$g DNA and 690 $\mu\mu$g histone. The value for DNA is favorably close to 55 $\mu\mu$g reported for *Tetrahymena pyriformis* W at the same time (17). These DNA and histone values decrease during the first 40 min and maximal levels occur 60 min after the end of heat treatment. During the first division, DNA and histone decrease to 40% of their initial maximal values, and in the 40 min after division both DNA and the macronucleus, but one cannot rule out possible contributions from the cytoplasm.

There are over-all decreases of the values in the undulating histone and DNA curves as successive generations occur (Fig. 2 *A*). Since DNA and histone are halved at each division, the diminishing histone and DNA levels are due to their failure to double during S. (The reduction to slightly less than half which occurs at the first division will be dealt with in the Discussion.) Table I demonstrates that the net amounts of histone and DNA synthesized are approximately 85% of that expected if chromosome duplication were complete. To express it differently, these results would be obtained if about 70% of the macronuclear DNA and histone completed duplication. These proportionate

TABLE I

Incomplete Duplication of Histone and DNA Content during Macronuclear S

		Observed			Expected			
	Generation	Before S	After S	Synthesis during S	After S	Synthesis during S	Expected value after S	Duplication
							%	%
Macronuclear DNA $\mu\mu$g/cell	1	12.5	21.3	8.8	25.0	12.5	85	70
	2	12.2	18.6	6.4	24.2	12.2	76	52
Macronuclear histone $\mu\mu$g/cell	1	213	352	139	426	213	83	65

These data have been obtained from the appropriate maxima and minima of the curves in Fig. 2 *A*.

histone establish new maxima which decrease by one-half during the second division. The DNA and histone repeat this cycle prior to the third division.

The coordinate rises in macronuclear DNA and histone content represent net synthesis because they correspond to known periods of DNA synthesis in *Tetrahymena*. Synthesis occupies the first 40 and 30 min, respectively, after the first and second divisions are completed. Each S phase represents about one-third of the division cycle and is consistent with the portion of a generation spent in DNA synthesis by single logarithmically growing cells (5, 32). In addition, the maximal values for DNA are reached at a time when synchronous *Tetrahymena* cultures are known to be synthesizing DNA within their macronuclei (6). There can be no doubt that the rises of histone content which parallel DNA increases represent net syntheses, and we assume that such syntheses take place in syntheses suggest that histone and DNA may be regulated by the same control mechanism.

Chemical and morphological criteria describe a stepwise reduction in macronuclear material during synchronous division, and indicate that depletions of histone and DNA are not artifacts of the extraction procedures. Fig. 2 *B* demonstrates that the macronuclear volume increases during heat treatment to five times the volume in logarithmic cells before heat treatment. Prior to each cell division, the volume increases about 30% and then decreases to one-half during division. The effect is a stepwise decrease which would require about five synchronous generations to restore the macronucleus to its size displayed during logarithmic growth, provided that changes in smaller nuclei occur in the same proportion as in larger nuclei.

When the values for macronuclear DNA and histone are divided by the corresponding macro-

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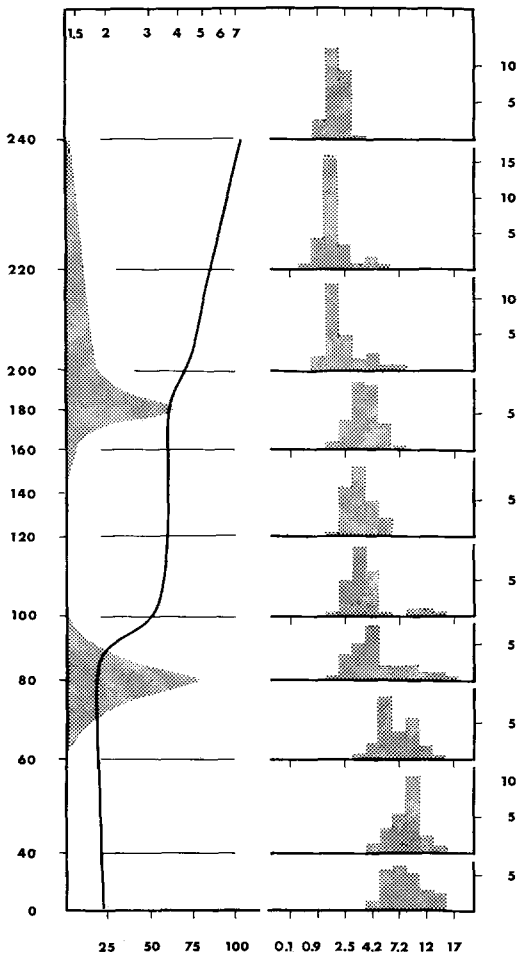

FIGURE 3 Distribution of macronuclear volume during synchronous division. The left half of the figure shows the per cent of dividing cells (shaded area, lower abscissa) and cells per ml $\times 10^4$ (solid line, upper abscissa) during the minutes after heat treatment (ordinate). The abscissa in the right of the figure presents the volumes ($\mu^3 \times 1000$) of 50 macronuclei in samples obtained at times indicated by the horizontal lines. The right hand ordinate shows the number of nuclei.

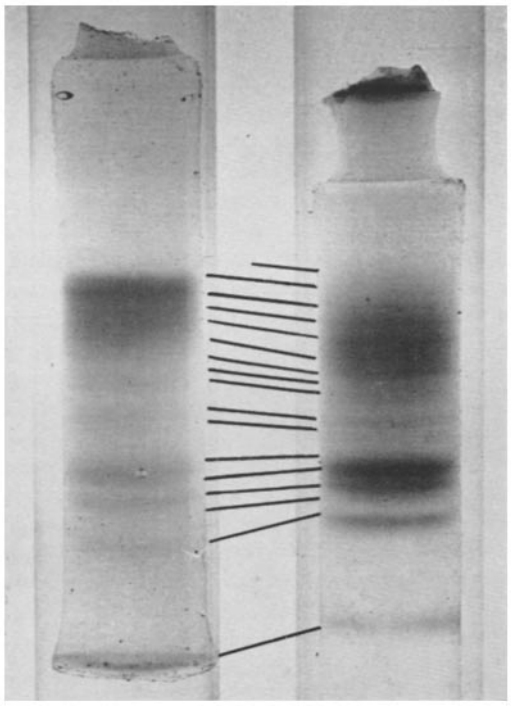

FIGURE 4 Electrophoretic patterns of *Tetrahymena* histones in acrylamide gel. Histone at left is from logarithmically growing cells; at right, from cells in the first synchronous division. Origin is at the top, and cathode at the bottom. Electrophoresis was performed in acetate buffer-urea, pH 4.3, with equal quantities of histone at 3 ma per gel for 45–75 min at room temperature. The most rapidly moving band in each gel is the front of migration.

nuclear volumes (Fig. 2 *C*) and similar phases of the cell cycle compared, DNA and histone contents are in constant proportion to macronuclear volume. The undulating curves for DNA and histone display no net decrease during synchrony. The respective averages for the DNA and histone ratios are 0.83×10^{-5} $\mu\mu g/\mu^3$ and 16.3×10^{-5} $\mu\mu g/\mu^3$, indicating that about 20 times more histone than DNA is present in the macronucleus. The undulations of both curves describe the fact that histone and DNA reach their maxima and minima shortly before those of nuclear volume. For example, during each synchronous division, the DNA content decreases earlier than macronuclear volume, and the ratio falls, approaching a minimum when macronuclear volume begins to decrease. During S phase, DNA content increases more rapidly than volume and a maximum is reached. The ratios always return to the same value at comparable stages of the cycle, except for the decrease associated with recovery from the heat treatment which is observed in the first 40 min of synchrony.

The stepwise reductions of macronuclear volume, DNA, and histone content during synchrony most likely occur within all cells, for it is difficult to explain these events by supposing that chromatin duplication is complete in only a portion of the

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macronuclei at each generation. According to the latter view, two classes of nuclei would be present throughout any S phase. Since DNA and macronuclear volume of comparable stages are proportional, a class representing macronuclei whose chromatin had duplicated during the previous generation should be twice the volume of a class whose chromatin had not duplicated. Fig. 3 demonstrates that during S only one volume class may be found in large quantity. Only shortly before and during division do extensive quantities of more than one class appear. These represent disappearance of large macronuclei and appearance of small nuclei as would occur when a single class of nuclei divides.

Histone from eukaryotes characteristically displays molecular heterogeneity (23),[3] and macronuclear histone from *Tetrahymena* is no exception (12). Fig. 4 displays the pattern of molecular species obtained by disc electrophoresis in acrylamide gel of histone from logarithmically growing cells, and cells in the process of the first synchronous division. Both sources of histone exhibit rich patterns of at least 15 different bands, and these patterns are essentially identical. Histone obtained from other stages of synchronous division also appears to have the same pattern, although quantitative differences may exist in certain bands. Thus, macronuclei of synchronous *Tetrahymena* are equipped with the same repertoire of histones at all stages of their cell cycle.

DISCUSSION

Our studies show that DNA, histone, and the volume of macronuclei decrease in an orderly stepwise manner during the first and second synchronous generations. This decrease is characterized by approximate halvings of macronuclear DNA and histone contents at each division and also by coordinate increases during S phase to about 85% of that expected. Finally, the stepwise reduction is such that the ratios of histone and of DNA to macronuclear volume are constant at comparable stages of each generation.

We view this reduction of the macronucleus as part of the cell's ability to adjust its size in response to changing culture conditions. During logarithmic growth cells are small, and they enlarge during during the stationary phase. The cells will return to smaller size if replaced in conditions permitting logarithmic growth. Something similar occurs during the heat treatment, for although cell division processes are heat sensitive and come to a halt (35), cell volume continues to increase. The macronucleus also enlarges, to accommodate the DNA produced by at least one extra round of replication (17, 35), and histone apparently increases to high levels by the end of heat treatment. Upon release of the heat treatment, cell division resumes at a more rapid than normal pace (35) in the enlarged cells, and produces the stepwise reduction of macronuclear volume and DNA and histone content.

The events during synchronous division pose two major questions: (1) How is the DNA content of the macronuclei reduced? (2) Why should the quantity of histone parallel that of DNA?

Reduction of DNA Content

It is reasonable to consider the fluctuations of DNA, histone, and macronuclear volume as consequences of varying numbers of complete genomic complements in the normally polyploid macronucleus (22). The ability of cells to change macronuclear size over many generations in the synchrony experiments, suggests that the macronuclear complement of genetic information remains fundamentally constant. Although the cells have different macronuclear DNA contents during the first and second generations, the similar lengths of S phases in these generations suggest that all genomic complements undergo duplication in parallel (5). Thus, the reduction of macronuclear materials during synchrony appears to result from failure of certain complements present in one generation to duplicate in the next generation.

Reduction of genetic material is accomplished in two ways. It is well known that in synchronously dividing strain GL (28) extrusion bodies containing DNA are excluded from the macronucleus during division. According to Scherbaum et al. (28), the volume of such bodies represents 2.8% of the macronuclear volume during division, and these bodies occur in 55% of the cells during the first synchronous division. In our experience, the loss of DNA from the macronuclear pellet into extrusion bodies during the first division is 28% of the DNA content of an average daughter macronucleus.[4] This value agrees closely with the corresponding value of 24% calculated for division 1 of Shepard's data (31) for division of *Tetrahymena*,

[3] Einem, G. E., and D. T. Lindsay, 1966. Manuscript in preparation.

[4] Extrusion body DNA of the average cell is one-half of the DNA content before division minus the DNA

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strain W, which had accumulated excess DNA in response to ultraviolet irradiation. Decaying synchrony and probably also reduced amount of extruded macronuclear material (31) lead more closely to apparent halving of the DNA content at the second and third divisions.

The second mechanism by which DNA, histone, and macronuclear volume are reduced expresses itself during S, for the quantity of macronuclear DNA fails to double. Only about 85% of the DNA per cell expected from doubling is found, indicating that only 70% of the DNA in the macronucleus before S completes replication. It is unlikely that the failure to double is due to additional extrusion body DNA and histone lost from the macronuclear pellet during S, nor does it seem likely that so large an amount of material could be lost from the macronucleus during S without its having been recognized by Scherbaum et al. (28), although this possibility cannot be excluded rigorously. Since Fig. 3 provides evidence that doubling of nuclear material takes place in all macronuclei during each generation, we envision the failure to double during S as failure of 30% of genomic complements to duplicate. Thus, one would expect during the next division that a small portion of the genomic complements would be lost as extrusion bodies, and the rest distributed more or less equally to the daughter cells. Of the genomic complements thus received, most duplicate during the subsequent S phase. Provided the complements are transferred intact at division, there will always be at least one full complement of genes in every daughter macronucleus.

The apparent failure of histone and DNA to double completely during the average macronuclear S suggests that their parallel syntheses are mediated by nondiffusible agents absent from certain genomic complements. If this is so, it would be interesting to know whether the decision to duplicate is transferred with each complement or determined anew at the beginning of S. Extrusion body material does not necessarily derive from chromatin which fails to duplicate during S, judging from Shepard's finding (31) that ^{3}H-thymidine incorporated into macronuclei during S may be found in extrusion bodies formed at the next division.

content of the daughter macronucleus, and equals 16 − 12.5 = 3.5. 3.5 is 28% of 12.5. Data from Fig. 2 *A*.

Histone and DNA

The histone described in this paper is electrophoretically heterogeneous basic protein extracted from partially purified macronuclei. Its behavior was not expected to parallel DNA by reason of artifactual binding to DNA. Direct extraction of nuclei with 0.2 N HCl does not require that histone be bound to DNA in order to be extracted. Accordingly, the amount of macronuclear histone is about 20 times more than would be expected if it had derived from DNA fully complexed with histone (15). When steps are taken to prepare such a nucleohistone complex from *Tetrahymena* (18), the histone-to-DNA ratio is 0.7. Lee and Scherbaum (19) have recently shown that this ratio for nucleohistone from highly purified macronuclei decreases from 1.2 to 0.8 during the 1st hr after the end of heat treatment. We tentatively regard the large quantity of histone we observe as a mixture of DNA-bound histone and other basic protein associated with nucleoli or nuclear ribosomes (20) or free in the nucleoplasm. The relative ability of these proteins to form complexes with DNA in the living cell is unknown.

The net synthesis of macronuclear histone in synchronous *Tetrahymena* is discontinuous, occurring throughout S phase, and the amount of synthesis is proportional to the amount of DNA synthesis. The enzymes, β-glucosidase in synchronous yeast (10), histidase, aspartate transcarbamylase, and sucrase of *Bacillis subtilis* (21) display similar discontinuities of synthesis, although their times are considerably shorter than DNA synthesis. Such an abrupt, setpwise increase of enzyme may be ascribed to stepwise synthesis or use of an unstable messenger RNA species whose precise appearance in the cell cycle may depend directly upon polarized transcription of the genome, or some other regulatory system controlling the sequential transcription of cistrons (11). Since *Tetrahymena* histone is heterogeneous, net synthesis of each histone may occur in parallel during DNA synthesis or in brief sequential periods. Lee and Scherbaum (19) have recently provided evidence which suggests that the former case does occur, at least during turnover, for ^{14}C-lysine is incorporated into virtually all bands of electrophoretic patterns of histone during each of the 2 hr which flank the end of heat treatment. Because the net synthesis of histone is in proportion to DNA, we conclude that the decision to synthesize histone is made at the same level as DNA, namely the individual genomic

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complement. Thus, net synthesis of histone appears to be associated with availability of messenger, of which the rate of synthesis or use is controlled by DNA synthesis or some mechansim leading to DNA synthesis.

We agree with Lee and Scherbaum (19) in their suggestion that a loss of rapidly synthesized histone from the nucleus occurs during the first hour of synchrony. It will be recalled that Fig. 2 *A* demonstrates a similar net decrease of macromolecular histone during the same period. Such a loss may represent transfer of histone into the cytoplasm, for one of us (20) has shown recently that, in chicken liver, ribosomes contain histones with the same relative electrophoretic mobilities as chromosomal histones. Furthermore, preliminary experiments[5] indicate that the same is true for *Tetrahymena* and may account for the fast green staining of basic proteins characteristically seen in the cytoplasm at the end of heat treatment (29).

The ability of cells to express specific portions of their genome in a systematic way appears to be inherited with the genome itself. In yeast, net synthesis of two genetically distinguishable β-glucosidases occurs at two characteristic periods during each repetition of the cell cycle (11). In hybrids, each enzyme is synthesized at its characteristic time. Several differentiated types of metazoan cells are capable of dividing repeatedly without losing the ability to express their characteristically differentiated phenotype (4, 7, 13). These variously differentiated cells in a single metazoan are thought to express different portions of identical genomes. A chondrocyte is thought to express that portion of its genome necessary for synthesis of the characteristic cartilage mucopolysaccharide, chondroitin sulfate. On the other hand, the specific phenotype of a skeletal muscle cell does not express this genetic information even though it possesses it, but instead, expresses different information (also present in the chondrocyte) which is necessary for synthesis of the contractile proteins displayed specifically by skeletal muscle. If histones act by inhibiting chromosomally dependent RNA synthesis (14) and by condensing parts of the chromatin into inactive states (8), then coordinate, proportional synthesis of histone and DNA appears to insure sufficient histone for the fu ıctional state of the genome to be built into the new chromosomes formed during each duplication.

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