THE SYNTHESES OF TOTAL MACRONUCLEAR
PROTEIN, HISTONE, AND DNA DURING
THE CELL CYCLE IN *EUPLOTES EURYSTOMUS*

DAVID M. PRESCOTT

From the Department of Anatomy, University of Colorado, Denver. Dr. Prescott's present address is the Institute of Developmental Biology, University of Colorado, Boulder

ABSTRACT

The syntheses of histone, total protein, and DNA during the cell cycle were measured in the macronucleus of *Euplotes eurystomus* by assaying the incorporation of tritiated amino acids and tritiated thymidine in groups of 800 to 1000 synchronized cells. The synthesis of DNA begins at 30% completion of the cell cycle, proceeds at a constant rate, and ends very shortly before the beginning of macronuclear division. Histone labeling is absent during G_1, begins in phase with DNA synthesis, continues at an unchanging rate during the S phase, and ends with the completion of DNA synthesis. The results support the view that the syntheses of histone and DNA are closely coupled events. Label in total protein accumulates at a constant rate during G_1 and appears to shift to a slightly higher rate when histone synthesis begins. At division, radioactive DNA, histone, and total protein are distributed equally between the daughter macronuclei without loss of radioactivity. Radioautographic analysis showed that protein labeling occurs throughout the macronucleus during the entire life cycle. There was no clear difference in the degree of protein labeling between replicated and unreplicated regions of the macronucleus. The distribution of label suggests that most of macronuclear protein labeling during the cell cycle is concerned with the events of transcription rather than replication.

INTRODUCTION

Progress through the cell life cycle is probably centered on a continuum of nuclear events, some of which (particularly in G_1) must be modulated in various ways by the state of the cytoplasm. The only two currently identifiable events which normally form part of nuclear cycle continuity are DNA synthesis and nuclear division. There are several fairly obvious gaps in knowledge about sections of the nuclear cycle, for example, the steps leading to initiation and continuation of DNA synthesis, the events which form the causal relation between the end of DNA synthesis, and the segregation of DNA into separate nuclei. The experiments required to provide direct and specific information on these points are difficult to devise, and it is still necessary to proceed with somewhat

1

more oblique approaches. In this vein, the work in this paper concerns the synthesis of total macronuclear protein, histone, and DNA during the cell cycle in *Euplotes eurystomus*, the turnover of total nuclear protein and histone during several cell cycles.

Euplotes has proven especially suited for this type of study because the cells and macronuclei are large, synchronization of a thousand cells is easily accomplished by selection of dividers, the progress and site of DNA synthesis are marked cytologically by the replication bands, and the macronuclei can be isolated and assayed individually or in small groups.

METHODS AND MATERIALS

Euplotes was cultured at 29°C on *Aerobacter aerogenes* grown in Cerophyll (300 mg/liter) (Cerophyll Laboratories, Inc., Kansas City, Missouri) plus *Tetrahymena*. The generation time of *Euplotes* under such conditions is about 12 hr.

To study nuclear synthesis over the complete cycle, 400 to 500 cells in a late stage of division were picked out of a single, log phase culture with a braking pipette. Such selection requires about 20 min and the synchrony of this population is usually extremely good. Four hours after division, 95% of the cells enter DNA synthesis over a 15 min period, and 100% of the cells begin synthesis over a 30 min period. At the next division all the cells divide between approximately 11 and 13 hr with a major peak of division usually close to 12 hr.

Macronuclear protein synthesis was followed by measuring the incorporation of tritiated amino acids with time in synchronized groups of cells. The radioactivity was introduced by feeding *Tetrahymena* which had been cultured on a mixture of tritiated leucine, arginine, lysine, phenylalanine, tryptophan, alanine, histidine, and isoleucine. All tritiated amino acids were the L form with specific activities ranging between 0.5 and 20 c/mmole. All were obtained from various commercial sources.

DNA synthesis in the macronucleus was followed over a complete cell cycle by feeding the *Euplotes* on *Tetrahymena* previously labeled very heavily with tritiated thymidine (15 c/mmole).

Incorporated radioactivity was measured in groups of 30 to 60 isolated macronuclei using a windowless gas flow counter. The macronuclei were isolated free of cytoplasm individually in a solution of a nonionic detergent, triton X-100 plus spermidine, washed in 70% alcohol, and air-dried on a cover glass. The isolation technique has been described elsewhere (1). The air dried nuclei were washed with an acetone-ether mixture (3:1) at 0°C. Acid-soluble proteins were extracted by a 3 hr treatment with 100 μliter of 0.1 N

H_2SO_4 at 0°C. The extract was neutralized with NaOH, air-dried on a planchet, spread with formic acid, and assayed for radioactivity. The extraction procedure was tested on isolated macronuclei in which the proteins were labeled with tritiated tryptophan only. Consistently, 1 to 2% of the incorporated tryptophan was soluble in acid. This is accepted as reflecting the level of nonhistone protein removed by acid. After a full cell cycle of feeding with mixed tritiated amino acids, about 15% of the incorporated radioactivity is acid soluble, but with the tritiated tryptophan the amount remains at 1 to 2%. This suggests that about 10% of the protein removed by the sulfuric acid is nonhistone. The amount of tryptophan-containing protein removed by 0.1 N HCL was much higher, and the H_2SO_4 procedure was therefore adopted.

The amount of nonhistone protein in acid extracts can, in some situations, be reduced by prior extraction of the material with 0.14 M NaCl (see reference 2). A 12 hr treatment of isolated macronuclei of *Euplotes* with 0.14 M NaCl at 0°C did not reduce the amount of acid-soluble, tryptophan-containing protein, so this step was omitted.

To reduce self absorption, the macronuclei were finally dispersed into a more-or-less even film on the cover glass with a few drops of formic acid. The cover glasses were placed in planchets and assayed in a windowless gas flow counter.

Additional groups of macronuclei were radioautographed in order to determine the relationship between the sites of protein and DNA syntheses over the cycle. For radioautography of tritiated amino acid labeling, the isolated macronuclei were rinsed in 70 and 100% alcohol and air dried. The nuclei labeled with tritiated thymidine were washed in 1 N HCl for 5 min at 25°C to remove unincorporated radioactivity, rinsed in 100% alcohol several times and air-dried. The radioautography was done with NTB3 emulsion by a conventional liquid emulsion technique (3).

RESULTS

Synthesis of DNA

In the macronucleus of *Euplotes*, DNA synthesis occurs in two waves, one wave originating at each end of the elongated nucleus. The positions of the two waves of synthesis are marked cytologically by the presence of the two replication bands in which DNA synthesis is localized (4, 5). The series of radioautographs in Fig. 1 shows, with continuous tritiated thymidine labeling, the progress of DNA synthesis through the macronucleus. No labeling occurs in the region between the two replication bands, but a trail of intense
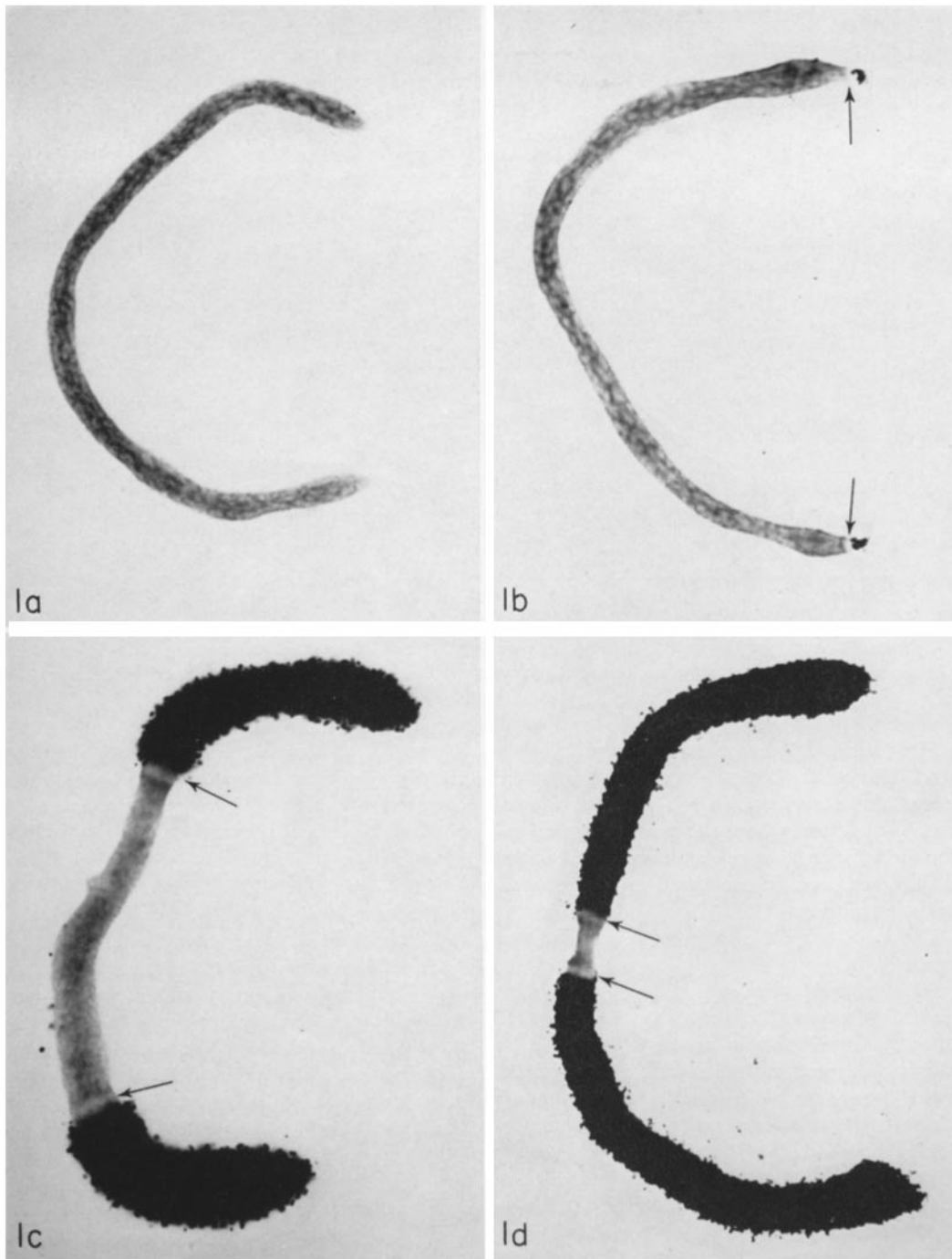
FIGURE 1 A series of radioautographs of isolated macronucleus of *Euplotes* labeled with tritiated thymidine. Arrows indicate DNA replication bands. Fig. 1 *a* A macronucleus from a cell 25% through the cell cycle and still in the G_1 stage. The replication bands have not yet appeared at the tips of the nucleus, and no thymidine incorporation has occurred. Fig. 1 *b* A macronucleus 30% through the cell cycle; the radioautograph shows the first few minutes of DNA synthesis (arrows). Fig. 1 *c* A macronucleus about 50% through DNA synthesis: about 65% cycle completion. Fig. 1 *d* A macronucleus in which the two bands have almost met in the center. DNA synthesis is almost finished: about 90% cycle completion. The exposure time for these radioautographs was 12 hr. Actual length of macronuclei is about 140 μ. $\times$ 1000.
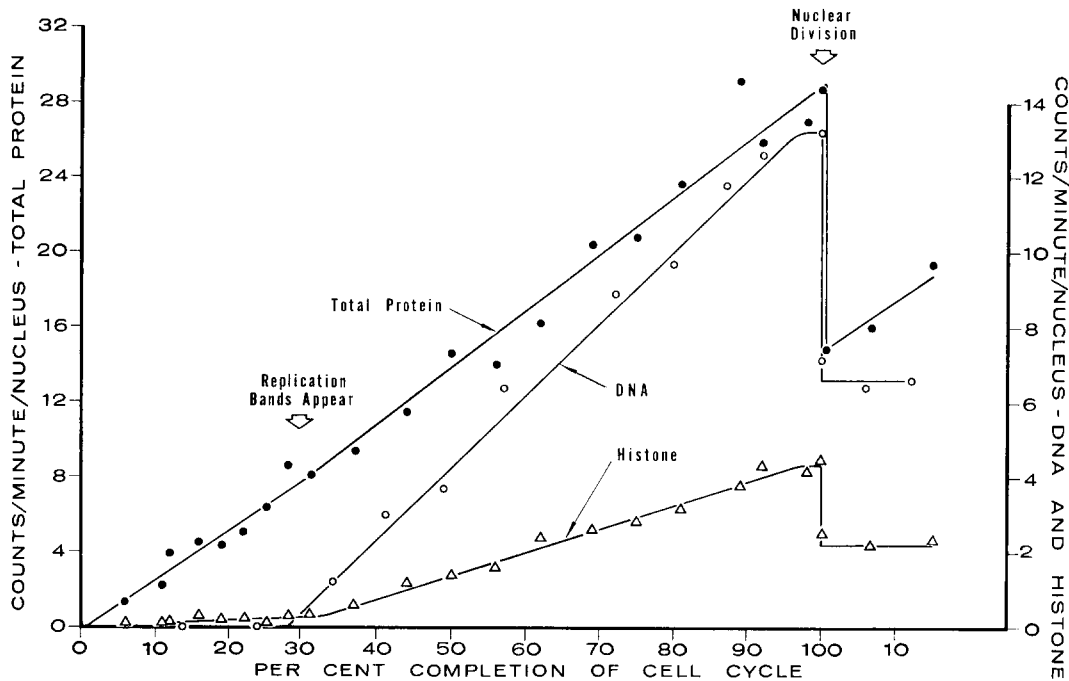
3

FIGURE 2 Incorporation of tritiated thymidine into DNA and of tritiated amino acids into total protein and histone in the macronucleus of *Euplotes* during the full cell life cycle. Each point is the mean count for 30 to 60 nuclei.

labeling occurs in the nuclear region behind each band. The bands ultimately meet and fuse at the center of the nucleus. At this time the macronucleus has already begun to shorten in preparation for amitotic splitting. A measurable G_2 period is absent unless it be considered as the period of amitotic splitting.

The incorporation of tritiated thymidine was measured in groups of 30 to 60 macronuclei isolated from synchronized cells at many points of the cycle. The results are recorded in Figs. 2 and 3. DNA synthesis begins at about 30% completion of the cycle and continues until a few minutes before cytokinesis. These results agree precisely with the cytological and radioautographic detection of the synthesis period. The rate of synthesis shows no deviation from a linear course. Such constancy in the rate of DNA synthesis has previously been described for *Paramecium* (6). Cytological observations of the replication bands in *Euplotes* gave the impression that the rate of DNA synthesis increased toward the end of S, but this proves not to be so. The observations of Ringertz and Hoskins (7) suggest that, under conditions of slower growth, DNA synthesis accelerates toward the end of the S phase. At cell division the DNA

is distributed, by amitosis, to the two daughter nuclei with approximate equality. Two sets of data are given (Figs. 2 and 3) in order to give a measure of the consistency of the results.

Synthesis of Total Macronuclear Protein

The incorporation of tritiated amino acids into total macronuclear protein is shown in Figs. 2 and 3. The incorporation during G_1 is linear, indicating no major changes in the rate of synthesis. At the beginning of S there may be a small increase in rate, and the curves in Figs. 2 and 3 have been drawn with a slight upward shift in slope. Such a small increase might be expected at the G_1 to S transition, either because histone begins to accumulate at this time or because the nucleus may require addition of new protein to support DNA synthesis, or both. The addition of radioactive protein to the macronucleus during S follows a linear course, as it does in G_1. At division, total nuclear protein is divided equally between the two daughter macronuclei. The pattern of increase in labeled protein is somewhat similar to the course of increase of macronuclear proteins measured microspectrophotometrically in *Paramecium* (6). The primary difference is a rapid

increase in rate of protein increase just before division in *Paramecium*.

The radioautographs in Fig. 4 show the distribution of accumulated radioactive protein at two points in the cycle. Labeled protein occurs generally throughout the nucleus during G_1 and S. There is no obvious difference in the concentration of radioactivity between the replicated and the nonreplicated regions of the macronucleus. It would be reasonable to expect a higher concentration of radioactive protein in the region behind the replication band because of addition of new (radioactive) histone at the band. Since histone contributes only 15% of the total nuclear protein in this cell, however, its contribution would not be easy to detect radioautographically against the background of accumulated G_1 labeling.

Synthesis of Histone

A relatively small amount of tritiated amino acids is accumulated into acid-extractable material during G_1 (Figs. 2 and 3). This is believed to represent nonhistone protein because the percentage (1 to 2%) of the total radioactivity that is soluble in 0.1 N H_2SO_4 during G_1 is the same whether labeling is achieved with a mixture of

tritiated amino acids or with tritiated tryptophan alone. Tryptophan has been found to be absent from histones of all cell types so far examined (see reference 2). The acid-soluble radioactivity of G_1 is not extracted by 20% trichloroacetic acid, and therefore does not represent amino acid pool material.

At the G_1 to S transition the rate of incorporation of tritiated amino acids into acid-soluble protein increases sharply. No increase occurs with tritiated tryptophan labeling. The acid-soluble radioactivity is considered to be primarily histone, and the course of this histone labeling is shown in Figs. 2 and 3. As already discussed, the small incorporation into acid-soluble protein in G_1 is interpreted as a contaminant protein, and nuclear histone labeling is concluded to be absent during G_1. At the beginning of DNA synthesis, labeled histone begins to appear in the nucleus. The accumulation of radioactive histone continues at a constant rate, concurrently with DNA synthesis, and continues up to the time of division. At division, histone is segregated equally between the two daughter nuclei. The curves in Figs. 2 and 3 show that the amount of radioactivity in histone remains constant as the cells start through the
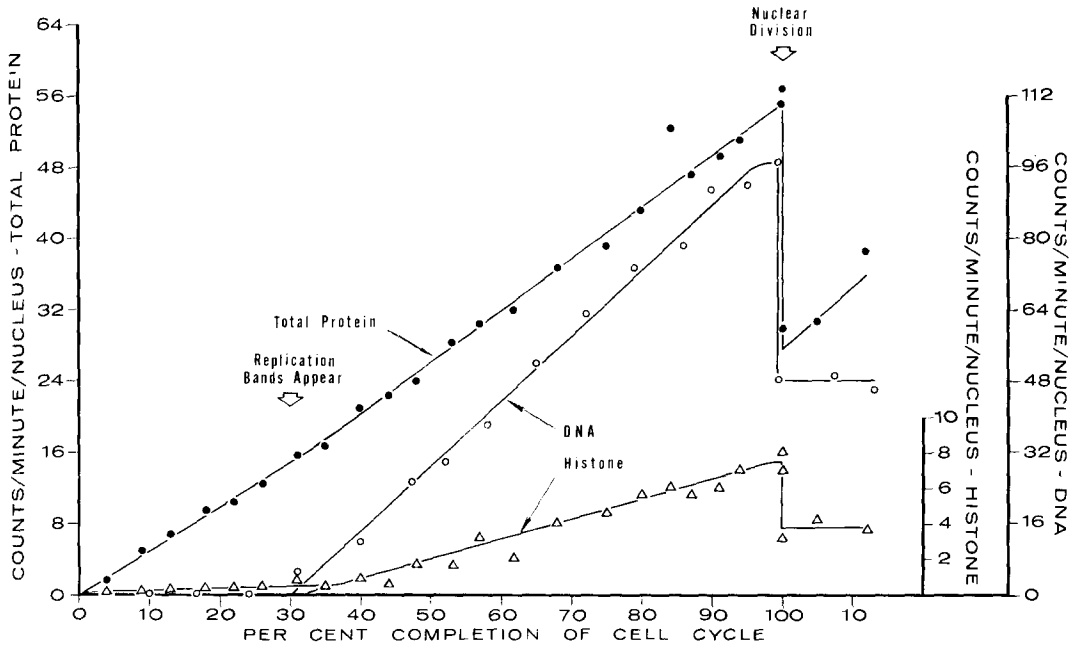

FIGURE 3 This is a repeat of the experiment in Fig. 2. The average incorporation per nucleus of tritiated thymidine into DNA and of tritiated amino acids into total protein and histone are increased over the values in Fig. 2 because a longer labeling period was used for the food organisms (*Tetrahymena*).
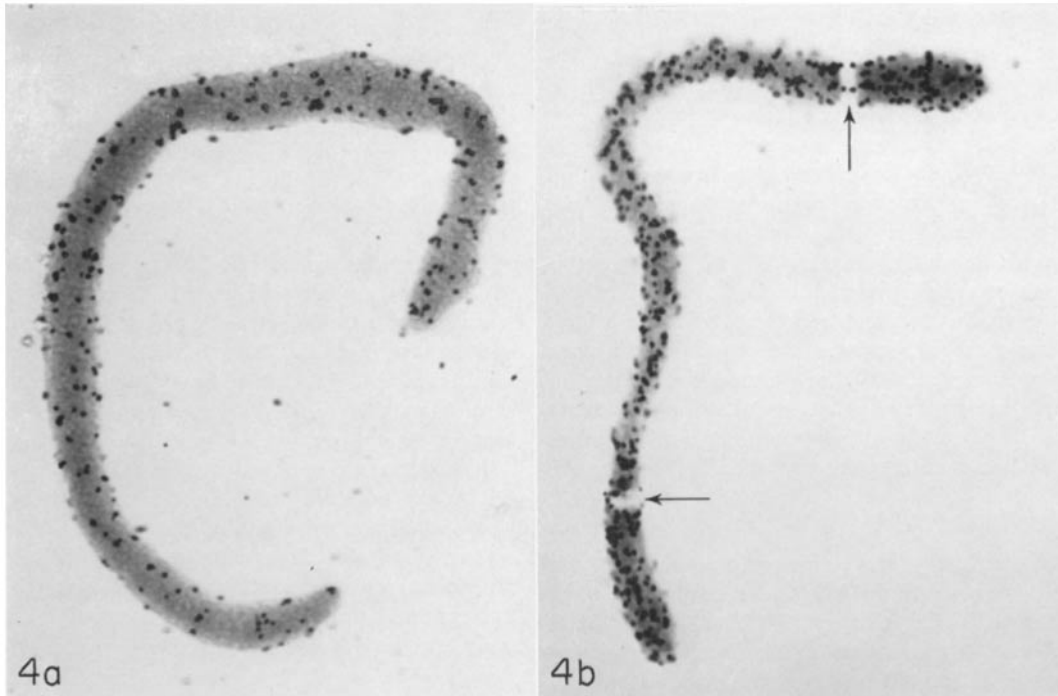
FIGURE 4 Radioautographs of macronuclei of *Euplotes* labeled continuously from the previous division with tritiated amino acids. Fig. 4 *a* A macronucleus 25% through the cell cycle and still in the G₁ stage. Fig. 4 *b* A macronucleus about 30% through DNA synthesis: about 45% cycle completion. Arrows indicate DNA replication bands. The nucleus is generally labeled with no clear difference in concentration of radioactivity between the replicated and nonreplicated regions. The exposure time for these radioautographs was 6 hr. Actual length of macronuclei is about 140μ. × 1000.

next G₁ period. Histone and DNA accumulation, therefore, each proceed with linear courses over the same interval of the cell cycle.

The Fate of Macronuclear Protein During Growth and Division

Euplotes were labeled with tritiated amino acids for one full cycle and at division were washed and cultured on nonradioactive *Tetrahymena*. At intervals over the next two cell cycles, macronuclei were isolated and total labeled protein and labeled histone were determined. The results are plotted in Fig. 5. During roughly the first 25% of the next cycle (first 3 hr of G₁) the radioactivity in total nuclear protein continues to increase. This represents the time required to use up the tritiated amino acids previously ingested. Very shortly before DNA synthesis begins, the amount of radioactivity in total protein ceases to rise. During the remainder of the cycle no change occurs in total

labeled protein, and the radioactivity is divided equally between the two daughter nuclei at division. At this division, synchrony was reimposed by discarding those cells which did not divide over the 30 min period occupied by the majority of divisions. In the next cycle the lack of any decrease in total protein radioactivity suggests conservation of nuclear protein. The labeled protein per nucleus was reduced to one-half with the next division.

Histone radioactivity remained unchanged during the initial G₁ period when the previously ingested radioactive *Tetrahymena* were being used up. When DNA synthesis began, there was still no significant change in the absolute amount of radioactive histone. An increase would be expected if histones had been synthesized in the cytoplasm during G₁ and then transferred, in any substantial amount, to the nucleus during subsequent DNA synthesis. During the remainder of the experiment the amount of histone labeling did

not decrease, except for halving at each of the two subsequent divisions.

DISCUSSION AND CONCLUSIONS

The specific functions of most nuclear proteins are still unknown, limiting the possibilities for relating in any precisely detailed manner, the macronuclear protein labeling over the cell cycle to the two known macronuclear functions of genetic replication and transcription. In addition, interpretations of the data must take into account the fact that labeling of nuclear proteins with tritiated amino acids will detect both turnover and net increase of protein. Turnover may be composed partially of protein breakdown and resynthesis and may also involve an exchange of unlabeled proteins of the nucleus for newly synthesized cytoplasmic proteins. Continuous shifting of protein between nucleus and cytoplasm during interphase has, for example, been demonstrated decisively in ameba by Goldstein (8), and a large fraction of

total nuclear protein is involved in this shuttling movement. At least some of this protein is synthesized in the cytoplasm (9) and migrates into the nucleus. In these experiments on *Euplotes* there is no way of assessing how much of the macronuclear protein is synthesized in the cytoplasm.

It does seem likely that a substantial part of the increase in radioactivity in proteins of the *Euplotes* macronucleus over the cycle represents a net increase since the total protein content of the macronucleus doubles with each cell cycle. It could be reasonably assumed that the labeling of proteins during the S phase represents addition of new enzymes associated with DNA synthesis or new proteins necessary to cope with the increased potential for RNA synthesis stemming from DNA increase. Preliminary measurements on *Euplotes*, however, have failed to show any change in the rate of RNA labeling in the nucleus (D. Evenson, unpublished), and this interpretation cannot be invoked. Nevertheless, the synthesis of messenger
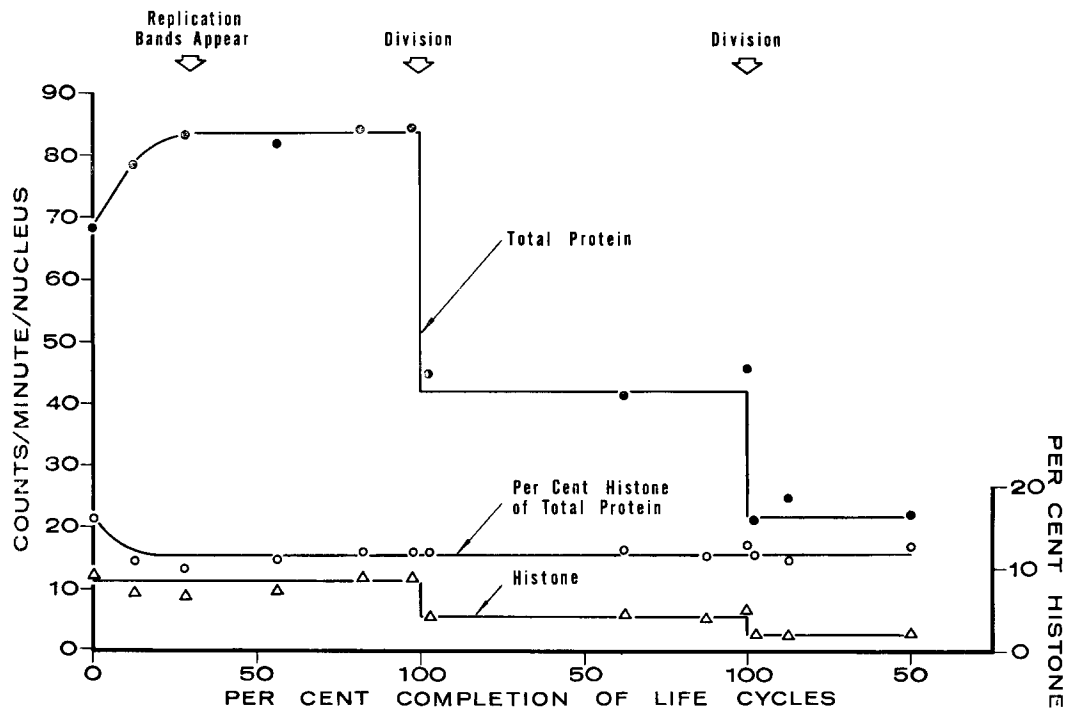

Figure 5 Fate of radioactivity in macronuclear protein of *Euplotes* during growth and division on cold nutrient. Cells were labeled for one cell cycle by feeding *Tetrahymena* cultured on tritiated amino acids. At time zero, the dividing *Euplotes* were washed and the daughter cells cultured on nonradioactive food. Radioactivity in total protein continues to rise until preingested radioactive food is exhausted. No rise occurs in radioactivity in histone, and the amount of total labeled protein and labeled histone per nucleus is halved at each of the next two nuclear divisions. Each point is the mean for 30 to 60 nuclei.

RNA, even at an unchanging rate, might be accompanied by nuclear protein turnover, although the reasons why this should be so cannot be specified at this point. In addition, the synthesis of ribosomal RNA may require addition of new ribosomal protein to the nucleus. Such activities could conceivably account for a major part of labeling of nuclear protein in both the G_1 and the S phase. Part of the S phase protein labeling is clearly due to histone addition, but histone accounts for only 15% of the total macronuclear protein in *Euplotes*.

Presumably, the progress of the macronucleus through G_1 involves qualitative and/or quantitative changes in the macronucleus; some of the protein labeling may be a part of these changes. Advancement out of G_1 into DNA synthesis apparently requires protein synthesis (10, 11), and certainly this protein synthesis must in some degree be associated with the nucleus.

The radioautographs in Fig. 4 show that protein labeling over the cycle occurs generally throughout the macronucleus. Although Gall (4) has shown that histone increase occurs strictly at the site of DNA synthesis (at the replication band), and short pulse labeling with tritiated histidine results in greater radioactivity at the band (5), there is no indication in Fig. 4 of a pronounced increase in protein labeling at the replication site. Since histone makes up only 15% of the total nuclear protein, and since any increase, in the form of histone labeling, takes place in these experiments against a background of extensive G_1 labeling of proteins, histone would not be expected to produce much intensification of the radioautograph at the replication band. The general distribution of labeling throughout the macronucleus in the experiment described here (Fig. 4) might be interpreted as evidence that the bulk of the new protein is associated in various ways with genetic transcription rather than genetic replication.

The histone labeling experiments can be interpreted in a more straightforward manner. Since the paper of Bloch and Godman (12), there have been a number of confirming reports (for example, references 4, 13, 14) that net increase of nuclear histone during the cell cycle occurs only in parallel with DNA synthesis. None of this work, including the original paper of Bloch and Godman, allows a decision about the site of histone synthesis (cytoplasm versus nucleus) or the time of histone

synthesis. These reports do not eliminate the formal hypothesis that some (or even all) histone is synthesized in the cytoplasm during G_1, to enter the nucleus only during S. Bloch and Brack (15) have, more recently, published evidence that special histone is synthesized in the cytoplasm (in the absence of DNA synthesis) and moves into the nucleus during differentiation of sperm cells in the grasshopper. This is one of several reports (16–18) that histone synthesis can be uncoupled from DNA synthesis, but the synthesis of DNA without concomitant histone increase has not been convincingly demonstrated, except of course in systems (bacteria and viruses) which normally lack DNA-associated histones.

The curves in Figs. 2 and 3 confirm that histone is added to the nucleus only during S and show, in addition, that there is at most only a trace of nuclear histone labeling during G_1. Robbins, Borun, and Maizel (19) have obtained very similar results on synchronized HeLa cells. Histone labeling is absent in nuclei during G_1 and begins with the initiation of DNA synthesis. In the *Euplotes* experiments, not only do the periods of macronuclear DNA and histone labeling coincide but both macromolecules are added to the macronucleus at constant rates, suggesting close temporal coordination of the processes.

The experiment in Fig. 5 provides evidence that the histone that is added to the macronucleus during the S phase is synthesized during S itself. Total cell proteins were labeled through one full cell cycle and up to the end of the G_1 phase of the next cycle (first cycle shown in Fig. 5). If any significant amount of histone, destined for the nucleus, had been synthesized and retained in the cytoplasm during the previous cycle or during the G_1 period in question, an increase in macronuclear histone labeling would have been expected during the first S period on nonradioactive food. No increase was detectable, and it is concluded that the addition of histone to the nucleus during S is closely linked to the event of histone synthesis. This conclusion is also supported by previously reported work (5) on histidine labeling at the replication band. These findings indicate that the regulation of histone *synthesis* during the cell cycle must be closely coupled to the synthesis of DNA. There is no evidence yet to indicate whether this regulation of synthesis is at the level of transcription of DNA or translation of RNA into protein.

These experiments on *Euplotes* have no direct

bearing on the question of a nuclear versus a cytoplasmic site of histone synthesis. The most pertinent data on the point are the description of cytoplasmic synthesis during spermiogenesis (15) and the report of histone synthesis in isolated nuclei (20) and in the nucleolus (21), but the problem needs more study.

The data in Fig. 5 for labeled macronuclear proteins beyond the first G_1 period show that total protein and histone are divided equally between daughter nuclei. There is no indication of nuclear protein turnover, but this probably would not be detectable in this type of experiment. Chalkley and Maurer (18) have reported that some histone undergoes turnover in the absence of DNA synthesis but lysine-rich histone becomes labeled only in concert with DNA synthesis.

Finally, as a general conclusion, although the data on *Euplotes* are a step toward describing nuclear progress during the cell cycle, an understanding of the cause and effect continuum of the nuclear cycle will obviously require much more refined and qualitative data on the progressive changes in those proteins involved in DNA replication and transcription and in their regulation.

REFERENCES

1. PRESCOTT, D. M., RAO, M. V. N., EVENSON, D. P. STONE, G. E., and THRASHER, J. D., *in* Methods in Cell Physiology, (D. M. Prescott, editor), New York, Academic Press Inc., **2**, 1966.
2. MURRAY, K., *Ann. Rev. Biochem.*, 1965, **34**, 209.
3. PRESCOTT, D. M., *in* Methods in Cell Physiology, (D. M. Prescott, editor) New York, Academic Press Inc., **1**, 1964.
4. GALL, J. G., *J. Biophysic, and Biochem. Cytol.*, 1959, **5**, 295.
5. PRESCOTT, D. M., and KIMBALL, R. F., *Proc. Nat. Acad. Sc.*, 1961, **47**, 686.
6. WOODARD, J., GELBER, B., and SWIFT, H., *Exp. Cell Research*, 1961, **23**, 258.
7. RINGERTZ, N. R., and HOSKINS, G. C., *Exp. Cell Research*, 1965, **38**, 160.
8. GOLDSTEIN, L., *Exp. Cell Research*, 1958, **15**, 635.
9. BYERS, T. J., PLATT, D. B., and GOLDSTEIN, L., *J. Cell Biol.*, 1963, **19**, 467.
10. LIEBERMAN, I., ABRAMS, R., HUNT, N., and OVE, P., *J. Biol. Chem.*, 1963, **238**, 3955.

11. MAALØE, O., *J. Cell Comp. Physiol.*, 1963, **62**, suppl. 1, 31.
12. BLOCH, D. P., and GODMAN, G. C., *J. Biophysic. and Biochem. Cytol.*, 1955, **1**, 17.
13. ALFERT, M., *Exp. Cell Research*, 1958, suppl. **6**, 227.
14. NIEHAUS, W. G., and BARNUM, C. P., *Exp. Cell Research*, 1965, **39**, 435.
15. BLOCH, D. P., and BRACK, S. D., *J. Cell Biol.*, 1964, **22**, 327.
16. FLAMM, W. G., and BIRNSTIEL, M. L., *Exp. Cell Research*, 1964, **33**, 616.
17. ONTKO, J. A., and MOOREHEAD, W. R., *Biochim. et Biophysica Acta*, 1964, **91**, 658.
18. CHALKLEY, G. R., and MAURER, H. R., *Proc. Nat. Acad. Sc.*, 1965, **54**, 498.
19. ROBBINS, E., BARUN, T. W., and MAIZEL, J. V., *Fed. Proc.*, 1966, **25**, 439.
20. REID, B. R., and COLE, R. D., *Proc. Nat. Acad. Sc.*, 1964, **51**, 1044.
21. BIRNSTIEL, M. L., and FLAMM, W. G., *Science*, 1964, **145**, 1435.