MICRONUCLEAR RIBONUCLEIC ACID
IN TETRAHYMENA PYRIFORMIS

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ABSTRACT
The presence of RNA in the micronucleus of Tetrahymena pyriformis was detected by electron microscope radioautography after incubation with tritiated precursors. The specificity of RNA labeling was shown by ribonuclease digestion. The period of appearance of labeled RNA in the micronucleus is approximately coincident with the DNA synthesis period for the micronucleus. Pulse-chase experiments showed that the micronuclear RNA disappears during the interphase period. The experiments do not distinguish whether the micronuclear RNA is synthesized in situ or acquired by migration from the macronucleus. In either case it is notable that the appearance of labeled RNA is detected in the micronucleus only during the micronuclear S phase.

INTRODUCTION
The contribution, if any, of the ciliate micronucleus to the vegetative life of the cell is not clearly understood. Sonneborn (14) has reported that the micronucleus of Paramecium aurelia is genetically inactive at least with respect to the action of the K locus. In accord with these observations, Kimball (9) failed to detect the incorporation of radioactive precursors of RNA into the micronucleus of P. aurelia. Pasternak (11), however, has reported RNA synthesis in the micronucleus of P. aurelia by radioautography. In the micronucleus of P. caudatum, Moses (10) has detected the presence of RNA by cytochemical methods, and Rao and Prescott (12) have demonstrated the synthesis of RNA by means of radioautography. In yet another ciliate, Stylonychia mytilus, Ammermann (2) has shown by radioautography the presence of RNA in the micronucleus.

The evidence concerning micronuclear activity in Tetrahymena pyriformis has been inconclusive. Wells (17) has provided suggestive evidence of a possible role of the micronucleus during vegetative growth. She found that the growth and survival of amicronucleates produced by irradiation was poor. Alfert and Das (1), on the other hand, failed to detect RNA synthesis in the micronucleus of Tetrahymena by radioautography. Similarly, Gorovsky (6) and Gorovsky and Woodard (7, 8) failed to detect the presence of RNA in the micronucleus by electron microscopic cytochemical methods or the synthesis of RNA by light microscope radioautography.

In the present study we demonstrate the presence of RNA in the micronucleus of T. pyriformis by electron microscope radioautography.

MATERIALS AND METHODS
Culture Methods
T. Pyriformis HSM was maintained axenically at 26°C in synthetic medium (3) supplemented with 0.04% protease peptone. The generation time in maintenance medium is approximately 210 min. The cells were grown in the same medium but without pyrimidines for 8-10 hr prior to experimentation. The cells continue through at least five
cell cycles in pyrimidineless medium with a slightly increased generation time; this growth is supported by the trace of pyrimidines in the proteose peptone, by the small amount of pyrimidines carried over with the inoculum, and by intracellular reserves of pyrimidines.

Labeling Methods

For labeling RNA, either 100 µCi/ml of 5'-uridine-3H (25.8 Ci/mM, Schwarz BioResearch Inc., Orangeburg, N. Y.) or a combination of 25 µCi/ml each of uridine-3H and cytidine-3H (24.0 Ci/µmole, Schwarz BioResearch Inc.) was added directly to the culture. To trace the incorporation of RNA precursors before and during division, dividers were collected at 30 min or at 1 hr after addition of label. Collection of groups of 200-400 dividers was accomplished in 10-15 min with a braking pipette (16).

To determine the incorporation of RNA precursors in interphase cells, dividing cells were collected from an unlabeled culture and then "pulsed" for 30 min with 500 µCi/ml of uridine-3H beginning either immediately after cytokinesis or at 2 hr after division.

In pulse-chase experiments, cultures were incubated with 100 µCi/ml of uridine-3H for 30 min, and two groups of dividers were then collected. One group was fixed immediately (pulse) and the other group was washed free of the radioactivity with synthetic medium containing the usual unlabeled pyrimidines and was kept in this medium for an additional 3 hr before fixation.

Ribonuclease Digestion

To determine the specificity of labeling of RNA the following experiment was performed. From a culture incubated for 1 hr with 25 µCi/ml each of cytidine-3H and uridine-3H, dividing cells were collected and fixed with osmium tetroxide (see below for fixation). The fixed cells were washed several times with 0.01 M phosphate buffer (pH 7.1) to remove excess osmium tetroxide and then divided into two groups. One group was treated with a 1% (w/v) ribonuclease (Worthington Corp., Harrison, N. J.) solution in 0.01 M phosphate buffer at pH 7.1. This solution was boiled for 15 min prior to use in order to eliminate any DNase activity. The enzymatic digestion of Tetrahymena was carried out for 6 hr at 26°C. The enzyme was changed twice during this period. The second group of cells, which served as a control, was incubated with the buffer without added ribonuclease.

Electron Microscope Radioautography

Cells collected in a small drop of culture medium were fixed for 3 min with 4% osmium vapor and embedded in agar according to the technique described by Flickinger (5). The cells in agar were dehydrated in a graded series of ethanol, immersed in propylene oxide, and embedded in Araldite. Sections showing a pale gold interference color were cut with a diamond or glass knife and mounted on uncoated 200-mesh copper grids.

Radioautography was carried out according to the procedure detailed by A. R. Stevens (15). The grids were coated with Ilford L4 emulsion diluted 1:1 with distilled water. Emulsion films exhibiting pale gold interference color were applied to the grids bearing the thin sections. The coated grids were kept in the dark for periods ranging from 2 days to 2 wk prior to development. Following this exposure, the grids were developed for 6 min in Microdol-X and fixed for 10 min in a 15% solution of sodium thiosulfate. The sections were stained for 1 min with a saturated solution of uranyl acetate and for 4 min with lead citrate (13) and examined in a Philips-EM200 microscope operated at 60 kv.

RESULTS

Nuclear Events in T. pyriformis

The following is a brief summary of important nuclear events in T. pyriformis: A detailed account of these events has been published (4). The micronucleus of T. pyriformis has G1, S, and G2 periods and divides by mitosis. The micronucleus lies in a depression in the macronucleus during most of the cell cycle. Late in interphase it moves away from the macronucleus, divides mitotically, and begins DNA synthesis during late telophase; there is no detectable micronuclear G1 period (see Fig. 1). While the micronucleus is dividing, the...
Figures 2-4  Radioautographs of sections of dividing cells labeled for the hour immediately preceding division with 25 μCi/ml each of cytidine-3H and uridine-3H. The micronucleus (MI) is consecutively near the plasma membrane (PM) in Fig. 2, close to the macronucleus (MA) in Fig. 3, and finally situated in a depression of the macronucleus in Fig. 4. The number of silver grains over the micronucleus increases progressively in Figs. 2-4. All the sections were exposed for 2 wk. X 22,750.
macronucleus is still in G₂. Macronuclear division coincides with the micronuclear DNA synthetic period. After macronuclear division, the micronucleus returns to a depression in the macronucleus.

**RNA Labeling**

Our initial experiments with log phase cells suggested that the micronucleus incorporates radioactive RNA precursors around the time of division. To establish more closely the stage at which labeling occurs, cultures were incubated with cytidine-3H and uridine-3H for 1 hr, and dividers were collected, fixed, and processed for electron microscope radioautography. The sample contained early dividers (first appearance of the cleavage furrow), late dividers, and newly divided cells. Fig. 1 shows diagrammatically the stages that were observed in the thin sections of such samples. Figs. 2-4 illustrate sequential stages in division corresponding to stages B, C, and D in Fig. 1. At these three stages, the micronucleus is consecutively near the plasma membrane (B), midway between the plasma membrane and the macronucleus (C), and finally situated in a depression of the macronucleus (D). During and immediately after division, micronuclei show no labeling. Labeling is seen in the micronucleus in stages B, C, and D (Fig. 1), with a progressive increase in the amount of labeling as the micronucleus nears the macronucleus (D). During and immediately after division, micronuclei show no labeling. Labeling is seen in the micronucleus stages B, C, and D (Fig. 1), with a progressive increase in the amount of labeling as the micronucleus nears the macronucleus (Figs. 2-4). Fig. 5 is a radioautograph of an early interphase cell (incubated continuously for 1 hr interval that encompassed predivision, division, and a brief postdivision period). The figure is included to illustrate the general pattern of silver grains over the micronucleus, the macronucleus, the cytoplasm, and background areas.

Cells labeled for the 30 min immediately after cytokinesis showed only slight incorporation. Cells labeled for 30 min beginning 2 hr after division showed virtually no label in the micronucleus (Fig. 6). The macronucleus after both of these incubations was heavily labeled.

A pulse-chase experiment was also performed to determine the amount of labeled RNA that remains in the macronucleus over the course of one generation time. Fig. 7 shows a section of a dividing cell that was pulsed for the last 30 min before cytokinesis. Fig. 8 illustrates a section of a cell that was similarly pulsed but then incubated for 3 hr in unlabeled medium (chased). Most of the micronuclear label incorporated during the period of division has been lost during the 3 hr chase period.

Ribonuclease treatment removed most of the isotopic label from both the macro- and the micronuclei. Fig. 9 shows a control cell and Fig. 10 shows a ribonuclease-treated cell.

The results presented above were based on an evaluation of 20–40 cells in each sample. Although the radioautographic intensity varied somewhat from cell to cell, the results were qualitatively completely consistent.

**DISCUSSION**

**Micronuclear RNA**

Our results indicate the presence (and possible synthesis) of RNA in the micronucleus of *T. pyriformis* during a period that coincides roughly with the period of DNA synthesis. Gorovsky (6) and Gorovsky and Woodard (7, 8) reported that the micronucleus of *Tetrahymena* does not contain or synthesize RNA during vegetative growth. The disparity between their observations and our findings may be due to the following differences in the techniques used:

(a) We have grown the cells in pyrimidineless synthetic medium (supplemented with 0.04% proteose peptone) rather than 2% proteose peptone; use of the pyrimidineless synthetic medium enormously enhances the incorporation of radioactive RNA precursors.

(b) We worked with synchronous populations obtained by mechanical selection of dividing cells instead of asynchronous populations. Use of synchronized cells allows concentrated attention on a particular short section of the cell cycle.

(c) We have used electron microscope radioautography, which reduces by 10-fold any ambiguity concerning the localization of silver grains over the micronucleus. The resolution of light microscope radioautography is about ±1 μ, and of electron microscope radioautography is about ±0.1 μ. This difference in resolution is extremely important because the micronucleus is only 2–3 μ in diameter.

It is possible that the strain difference of the *Tetrahymena* used might be important, although this seems unlikely. Finally, Gorovsky and Woodard (7, 8) used labeling pulses of 8 min (or less), whereas our shortest labeling time was 30 min.
Figure 5 Radioautograph of a newly divided cell labeled with isotopes as in Figs. 2–4 and exposed for only 2 days. Silver grains are present over the micronucleus (Mi), the macronucleus (Ma), and the cytoplasm. X 12,900.

Significance of Micronuclear RNA

The micronuclear RNA demonstrated here, whether transcribed from micronuclear DNA or transported from the macronucleus, appears only in a period roughly coincident with DNA synthesis. In this respect the behavior of *Tetrahymena* micronuclear RNA resembles that of *P. caudatum* micronuclear RNA (12). The apparent absence of RNA in the micronucleus during the other
Figure 6 Radioautograph of an interphase cell labeled for 30 min with 500 µCi/ml of uridine-3H beginning at 2 hr after division. Note the heavily labeled macronucleus (MA) surrounding the unlabeled micronucleus (MI). Emulsion exposure period, 8 days. × 22,750.

Figure 7 Radioautograph of an early interphase cell labeled with 100 µCi/ml of uridine-3H for 30 min during the previous division. Emulsion exposure period, 10 days. MI, micronucleus; MA, macronucleus. × 22,750.

Figure 8 Radioautograph of an early interphase cell labeled as in Fig. 7 followed by 3 hr of incubation (chase) in unlabeled medium. Compare to Fig. 7. Emulsion exposure period, 10 days. MI micronucleus; MA, macronucleus. × 22,750.
portions of the cell cycle must be due to the migration of the RNA out of the micronucleus or breakdown of the RNA within the micronucleus. The following are possibilities (among which we cannot choose at present) regarding the origin and significance of the micronuclear RNA:

(a) Micronuclear RNA is synthesized in situ and migrates to the cytoplasm where it contributes to a cytoplasmic activity. The contribution might be unique, that is, made by the micronucleus but not by the macronucleus, or it may duplicate a contribution made by the macronucleus. It is possible, for example, that the portion of the genome responsible for the synthesis of micronuclear RNA might have been discarded from the macronucleus (during macronuclear development from the anlage) or may be repressed in the macronucleus (12).

(b) Micronuclear RNA is synthesized in situ but functions within the micronucleus rather than migrating out. Such a situation might imply a role of micronuclear RNA in the replication of micronuclear DNA or other (unidentified) intramicro- nuclear activity.

(c) The micronuclear RNA is synthesized in the macronucleus and enters the micronucleus.
during the micronuclear S phase. If such were the case, it might be envisaged that the RNA is involved with some aspect, possibly control, of micronuclear DNA synthesis.

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