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Induction of Nucleolar and Mitochondrial DNA Replication in *Tetrahymena pyriformis*

(nutritional shift-up/CsCl gradients/EM-autoradiography/sucrose gradients)

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ABSTRACT **Selective induction of replication of nucleolar and mitochondrial DNA has been demonstrated in starved-refed cultures of *Tetrahymena pyriformis* by different techniques.**

Labeling of starved cells with [^{3}H]thymidine during a nutritional shift-up and analysis of the DNA in isopycnic CsCl gradients shows that the two initially labeled species of DNA are two species banding on the heavy and light side of the bulk macronuclear DNA. In isolated macronuclei the radioactivity is found only in the high density fraction, which has been shown to be of nucleolar origin. In sucrose gradients the newly replicated mitochondrial and nucleolar DNAs sediment considerably slower than the bulk DNA, as one discrete band corresponding to a molecular weight of about 3 to 4 $\times$ 10^7.

Electron microscope autoradiography of cells labeled with [^{3}H]thymidine as above shows that the peripheral nucleoli of the macronucleus as well as the mitochondria are labeled before any radioactivity is found in the chromatin granules of the macronucleus.

The results clearly indicate that nucleolar and mitochondrial DNA replication are under a control independent of that for the replication of bulk DNA.

In *Tetrahymena pyriformis* the 500–1000 nucleoli are located peripherally in the nucleus (1) and form ribosomes at a rate per nucleolus which is equal to that of HeLa cell nucleoli (2). During starvation the nucleoli fuse into aggregates that again dissociate into multiple peripheral nucleoli during a nutritional shift-up which concomitantly induces rapid acceleration of rRNA synthesis (1).

Since morphological studies show that a rearrangement of the nucleolar material occurs during a nutritional shift-up (1), we undertook this study since we felt that nucleolar DNA might show unusual replicative properties during the dissociation of nucleolar aggregates.

We have previously studied the replication of the rRNA genes during the nutritional shift-up and have presented evidence from DNA·RNA hybridization experiments (3) suggesting that early during the refeeding period the rRNA genes are replicated preferentially. In the study reported here, the same starvation–refeeding system has been employed and it is shown by EM-autoradiography and labeling with [^{3}H]thymidine that the nucleolar DNA as well as mitochondrial DNA are preferentially replicated during the early times of refeeding. The two species of DNA band in isopycnic CsCl density gradients as two discrete radioactivity peaks on the heavy and light side, respectively, of the bulk DNA. In sucrose density gradients the newly replicated nucleolar and mitochondrial DNAs sediment as one discrete peak with a molecular weight of about 3 to 4 $\times$ 10^7.

Abbreviations: EM, electron microscope; rDNA, DNA coding for ribosomal RNA.

MATERIALS AND METHODS

Cultures of *Tetrahymena pyriformis*, strain GL, were grown in a complex proteose peptone medium as described earlier (4). Starvation for 24 hr was carried out as described by Cameron and Jeter (5) except that the nutritional shift-up (refeeding) was performed by adding an equal volume of the complex medium to the starvation medium. DNA in exponentially growing *Tetrahymena* was uniformly labeled by growth for at least eight generations in a medium containing 1 μCi/ml of [^{32}P]orthophosphate (specific activity 50.3 Ci/g from Risø, Denmark). Refeeding was carried out in the presence of 5 μCi of [^{3}H]thymidine/ml (New England Nuclear Chemicals, specific activity >15 Ci/mmol) except in the autoradiography experiments where 83 μCi of [^{3}H]thymidine/ml was used.

For autoradiography experiments, glutaraldehyde-fixed cells were fixed in OsO_4, stained with uranyl acetate and processed as described recently (6).

DNA to be analyzed in CsCl density gradients was prepared from whole cells or isolated nuclei (7) by chloroform–octanol extractions as described by Brunk and Hanawalt (8). The DNA-containing solutions were centrifuged at 45,000 $\times$ *g* for 30 min in order to remove traces of glycogen before CsCl was added.

Each gradient consisted of 7.2 g of CsCl and 6.0 ml of buffer containing about 50 μg of DNA. Alkaline gradients were made as described by Brunk and Hanawalt (8). The gradients were topped with mineral oil and centrifuged for 65 hr at 20° in a 40 fixed angle rotor of a Beckman L-50 centrifuge at 37,000 rpm. About 50 fractions of equal volume were collected from the bottom of the centrifuge tube (polyallomer, Beckman). Buoyant density determinations were made using a Zeiss Abbe refractometer as described (4). Sedimentation analyses of nuclear DNA were carried out by lysing purified nuclei on the top of 5–20% sucrose gradients as described by Miyagishi and Andoh (9), followed by centrifugation at 30,000 rpm at 10° for 1.5 hr in a SW 50 L rotor of a L2-65 Beckman centrifuge. RNA in the collected fractions was removed by

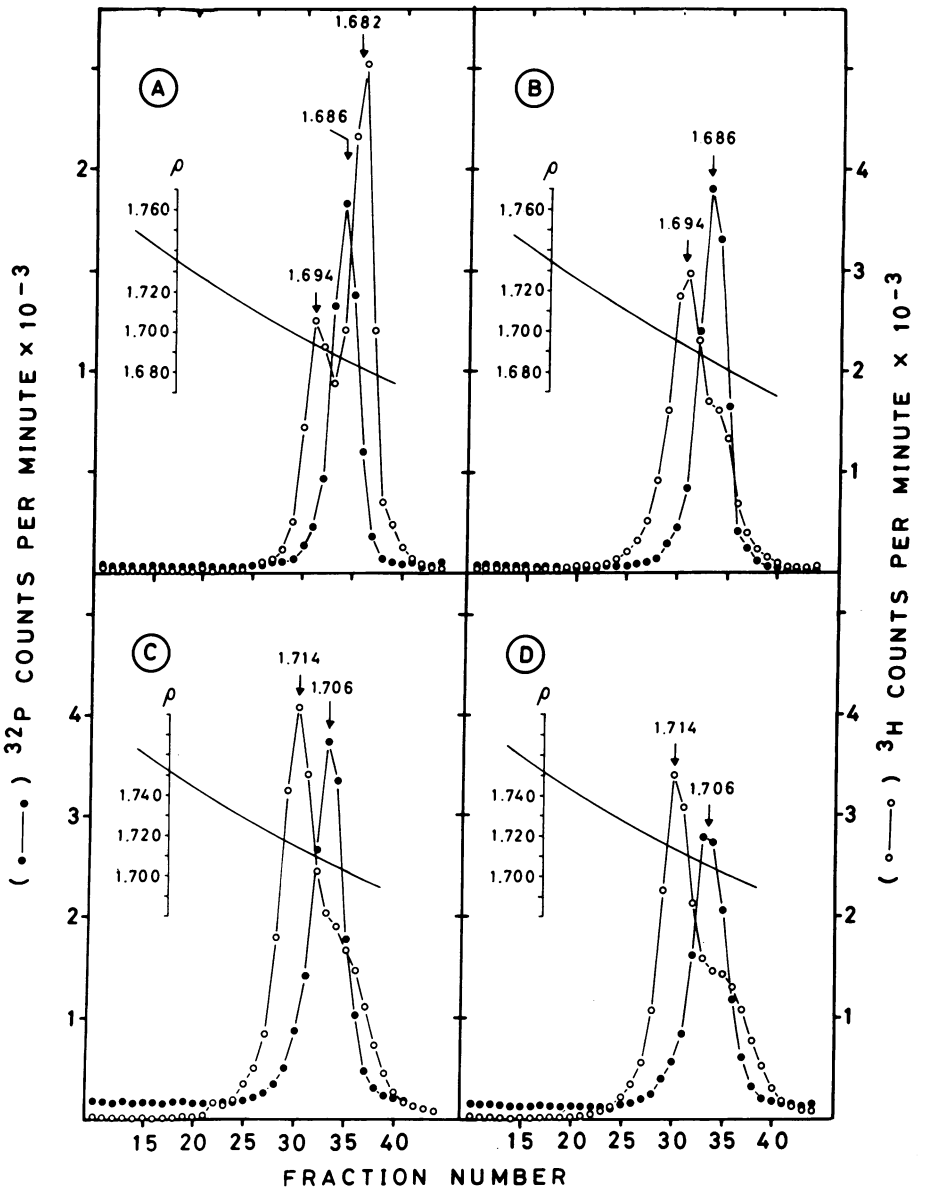

FIG. 1. CsCl isopycnic banding of DNA labeled with [^{3}H]thymidine for 2 hr during the nutritional shift-up. Cells were previously labeled with [^{32}P]phosphate for eight generations to overall label bulk DNA. (*A*) DNA from whole cells banded in a neutral CsCl gradient. (*B*) DNA from isolated macronuclei banded in a neutral CsCl gradient. (*C*) Heat-denatured DNA banded in an alkaline CsCl gradient. Macronuclear DNA (as in *B*) was dialyzed against 0.03 M Na citrate, 0.3 M NaCl, pH 7.0 (2 × SSC), and heat-denatured at 90° for 10 min and cooled rapidly on ice. (*D*) Heat-denatured, RNase-treated DNA banded in an alkaline CsCl gradient. Heat-denatured macronuclear DNA as in (*C*) was digested with 50 μg/ml of pancreatic ribonuclease and 10 μg/ml of T_1 ribonuclease for 1 hr at 37°.

hydrolysis in 0.3 N KOH at 37° overnight. After neutralizing with HCl, DNA was precipitated in ice-cold trichloroacetic acid. Bovine serum albumin (50 μg) was added as carrier and the precipitate was collected on Whatman GF/C filters. The filters were washed, dried and counted as described earlier (4).

RESULTS

In order to characterize the kind of DNA which is replicated first during the refeeding period, cells overall labeled with ^{32}P were starved for 24 hr and then refed for 2 hr with growth medium containing [^{3}H]thymidine. Analysis of the labeled DNA in CsCl isopycnic gradients is depicted in Fig. 1*A* and shows that two minor species of DNA are synthesized preferentially during the first 2 hr of refeeding. The two kinds of DNA band on the heavy and light side of the bulk macronuclear DNA corresponding to buoyant densities of 1.694 and 1.682, respectively. The ^{3}H-radioactivity peak of light density coincides with a satellite DNA which previously has been characterized as mitochondrial DNA (10) and which is found in high concentrations in isolated mitochrondria (Engberg and Leick, unpublished experiments). The high density ^{3}H-radioactivity peak (b.d. = 1.694) coincides with a DNA satellite which has been shown to be of macronuclear origin (10) and which by DNA·RNA hybridization has been shown to contain the genes coding for ribosomal RNA (3). In Fig. 1*B* it is shown that the [^{3}H]thymidine radioactivity peak of heavy density but not the one of light density is found in high concentration in isolated macronuclei.

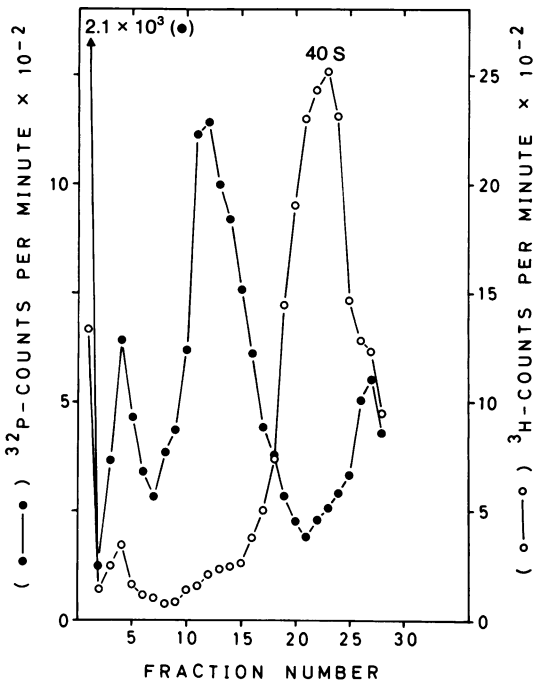

FIG. 2. Sucrose density gradient centrifugation of macronuclear DNA labeled for 2 hr with [³H]thymidine during the nutritional shift-up. Cells were labeled as in Fig. 1. Macronuclei were isolated and lysed on top of the sucrose gradient in sodium dodecyl sulfate as described in *Materials and Methods*. Sedimentation was from left to right in the SW50L rotor at 30,000 rpm for 1.5 hr at 10°.

It has recently been found that newly synthesized polyoma DNA may have associated pieces of RNA which will give a higher density in CsCl of the newly replicated DNA pieces than that of the newly replicated DNA itself (11). In order to make sure that the [³H]thymidine labeled peak of high density in Fig. 1*B* is pure DNA without any RNA associated, the macronuclear DNA preparation in Fig. 1*B* was heat-denatured and treated with pancreatic ribonuclease and RNase T_1. Fig. 1*D* shows that RNase treatment of heat-denatured DNA does not result in a change in density of the newly replicated DNA compared with the bulk DNA and Fig. 1*C* shows the control sample, i.e., an alkaline CsCl gradient of heat-denatured DNA untreated with RNase. Therefore, the newly replicated ³H-labeled macronuclear DNA shown in Fig. 1*B* must be a unique species of macronuclear DNA of high density which is synthesized specifically during the first 2 hr of the nutritional shift-up of starved *Tetrahymena* cells.

In order to get a rough estimate of the molecular weight of the two initially labeled species of DNA, we carried out sucrose density gradient centrifugation of whole cell DNA prepared by a very gentle sodium dodecyl sulfate method, that is, a method giving unsheared *Tetrahymena* DNA of high molecular weight (9). The cells were labeled as in Fig. 1 and isolated macronuclei as well as whole cells were lysed in sodium dodecyl sulfate directly on top of the sucrose gradient in order to avoid or minimize any shearing or DNase cleavage during handling of the DNA. Fig. 2 shows a sucrose gradient of DNA from isolated macronuclei showing the bulk DNA (³²P-labeled) sedimenting in the middle and bottom part of the gradient. The newly replicated DNA sediments slower than the bulk DNA as a discrete peak (S-value about 40S). No, or very little, newly replicated DNA is found cosedimenting with the bulk DNA indicating that the newly replicated macronuclear DNA during the nutritional shift-up is a well-defined species of DNA in terms of molecular weight. When DNA from whole cells was analyzed in a similar way on a sucrose gradient virtually the same profile was found as in Fig. 2 (not shown) indicating that the two newly replicated ³H-labeled DNA species have similar sedimentation properties in sucrose gradients. It is known that linear mitochondrial DNA from *Tetrahymena* sediments around 40S corresponding to a molecular weight of 3 to 4 × 10^7 (10). Assuming that the high density peak of ³H-labeled DNA is noncircular it is reasonable to assume that it has a molecular weight quite similar to mitochondrial DNA. Evidence to support that the two kinds of DNA are not in a very different conformational state comes from the fact that they both shift in parallel to a lighter buoyant density when banded in CsCl gradients containing ethidium bromide (unpublished experiments).

Finally it must be emphasized that the [³H]thymidine labeling of the two minor species of DNA is, indeed, a consequence of the synchrony induced by the nutritional shift-up after starvation. When exponentially growing cells were labeled with [³H]thymidine, the labeling appears in all kinds of DNA at the same time even if very short pulses were used.

In order cytologically to characterize the [³H]thymidine labeled DNA during the nutritional shift-up electron microscope autoradiography was carried out. To localize the DNA synthesized early during refeeding, starved cells were refed in the presence of [³H]thymidine as in Fig. 1. At selected time intervals, cells were sampled and prepared for EM-autoradiography as described in *Materials and Methods*. The autoradiographs in Fig. 3 are typical representatives of the cells sampled at 1.5 hr, 2 hr, and 2.5 hr after refeeding. Table 1, which summarizes the data from the experiments, shows that the silver

TABLE 1. *Cellular localization of* [³H]*thymidine incorporated by refed Tetrahymena cells*

Hours after refeeding	Cells per sample	Silver grains per nucleus	% Nuclear silver grains over nucleoli	% Nucleoli labeled	% Mitochondria labeled n = 5 cells
1.5	10	7.3 (3–19)	77 (33–100)	19 (11–42) TN = 238	29 (25–36) TN = 198
2	15	30.1 (13–52)	84 (63–100)	49 (27–70) TN = 674	59 (49–63) TN = 196

Data are expressed as counts of silver grains over sectioned material. For experimental details see Fig. 3 and *Materials and Methods*. Ranges are given in parentheses. TN = total number inspected.

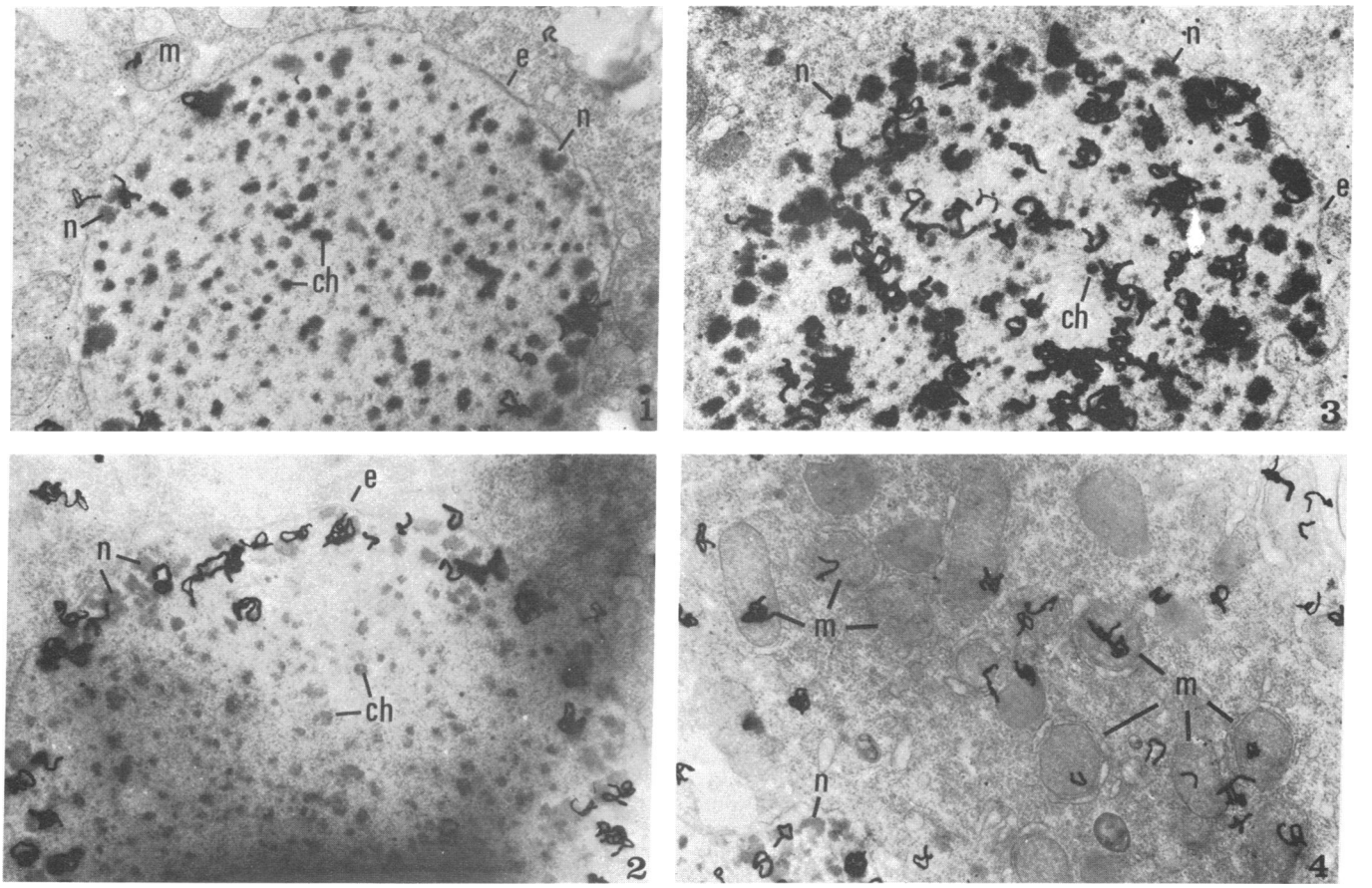

FIG. 3. Electron micrographs of autoradiograms after labeling of cells with [^{3}H]thymidine from time of refeeding (exposure time 6 weeks). (*1*) Representative cell from sample 1.5 hr after refeeding. Curly silver grains are present over nucleolar region (×9,600). (*2*) Representative cell from sample 2 hr after refeeding. Many silver grains are present over nucleolar region (×9,600). (*3*) Cell from sample 2.5 hr after refeeding. The cells (50%) have numerous silver grains over the chromatin region, indicating the onset of bulk DNA replication (×9,600). (*4*) Cytoplasmic region of cell from sample 2 hr after refeeding. Note that many silver grains are associated with mitochondria, indicating mitochondrial DNA replication (×9,600).

Abbreviation used in the electron micrographs: *n*, nucleolus; *ch*, chromatin granules; *e*, nuclear envelope; *m*, mitochondrion.

grains found over the nuclei of all the cells inspected (which were collected at random) were almost exclusively confined to the nucleolar regions. Furthermore, about 50% of the nucleoli in the 2-hr sample were in the process of incorporating radioactive thymidine. Furthermore, a large number of mitochondria also had silver grains over them. The results from the 1.5-hr and 2-hr samples have been included in Table 1. The DNA labeling in the refed cells cannot be an artefact due to slow diffusion of labeled DNA precursor molecules from the cell surface to the center of the macronucleus since pulse labeled exponentially growing cells (10-min pulse) have most of the incorporated label confined to the chromatin region of the nucleus (6).

DISCUSSION

The results presented in this paper suggest that the replication of nucleolar and mitochondrial DNA is controlled by a mechanism which is separate from that which controls the replication of the bulk macronuclear DNA in *Tetrahymena*. Evidence for independent control of ribosomal gene replication has also been obtained in *Drosophila* during the formation of polytene chromosomes (12). However, the *Tetrahymena* system has the uniqueness that a selective replication of nucleolar as well as of mitochondrial DNA can be physiologically induced in a specific way in starved cells which are refed with a complex growth medium. The induction is not a result of a synchronization of the cell cycle during starvation since in normal cells nucleolar DNA is replicated after the bulk DNA (13).

The electron microscope autoradiograms shown in Fig. 3 show that the nuclear DNA synthesized early during refeeding is confined to the peripheral nucleoli of the macronucleus. In an earlier investigation we isolated the DNA that was replicated early during the refeeding period by refeeding starved cells with growth medium containing 5-bromodeoxyuridine (BrdU) and separating the BrdU substituted DNA from nonreplicated DNA by CsCl density gradient centrifugation (3). The BrdU substituted DNA was found by DNA·RNA hybridization experiments to be especially enriched with respect to the rRNA genes (rDNA) indicating that a preferential replication of these genes takes place in response to the nutritional shift-up. The EM-autoradiography experiments

and the density labeling experiments taken together give strong evidence for the hypothesis that nucleolar DNA in *Tetrahymena* contains the genes coding for rRNA.

The newly replicated nucleolar DNA sediments as one discrete peak coinciding with that of mitochondrial DNA under conditions where it is possible to obtain bulk DNA of very high molecular weight (Fig. 2). This finding gives support to the hypothesis that nucleolar DNA in *Tetrahymena* is an autonomous unit with a molecular weight around 30–40 million. We are presently investigating whether rDNA is entirely associated with the nucleolar DNA or if rDNA sequences can also be found in the bulk macronuclear DNA.

In normal vegetative *Tetrahymena* cells the replication of mitochondrial DNA is not restricted to a defined period in the cell cycle (14). However, as for nucleolar DNA, the interesting observation in this paper is that a specific and synchronous replication of mitochondrial DNA can be induced by the nutritional shift-up. Therefore, this system can be a very useful tool to study the induction of replication of these specific cellular DNA species using biochemical methods.

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