*MACRONUCLEAR DNA SYNTHESIS IN STENTOR: REGULATION BY A CYTOPLASMIC INITIATOR**

By Noël de Terra

LABORATORY OF NUCLEAR MEDICINE AND RADIATION BIOLOGY, DEPARTMENT OF BIOPHYSICS AND NUCLEAR MEDICINE, SCHOOL OF MEDICINE, UNIVERSITY OF CALIFORNIA (LOS ANGELES)

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The large ciliate *Stentor coeruleus* lends itself well to microsurgical procedures. By combining these techniques with autoradiography after exposure of cells to thymidine-H^3, it is possible to study mechanisms regulating the occurrence of macronuclear DNA synthesis during the cell growth cycle. Transfer of nuclei between cells which are synthesizing DNA and cells which are not can indicate whether DNA synthesis is determined by the continuous presence or absence of a cytoplasmic factor. Similar experiments involving cell grafting could characterize such a factor as an initiator or an inhibitor. This paper describes these experiments and their results.

Previous work on *Stentor* has suggested that mechanisms regulating the occurrence of several major events in the cell growth cycle are located in the cytoplasm.[3–5] The present results will be discussed in terms of these earlier findings and also in relation to current knowledge about regulation of nuclear DNA synthesis.

Stentor coeruleus is a large heterotrich ciliate; individual organisms often reach a length of 1 mm when fully extended. Figure 1*A* shows the main morphological features of *S. coeruleus*, including the chain macronucleus, the anteriorly placed oral apparatus, and the rows of blue-green pigment granules running longitudinally between the kineties.

Throughout interphase, the macronucleus exists as a chain of nodes spiralling almost the entire length of the organism and situated directly beneath the ectoplasm. At 20°C, amitotic division takes approximately eight hours. Tartar has assigned numbers to the stages of amitotic division.[25] There are eight stage numbers, each covering a successive one-hour interval so that stage 1 designates the first hour of division while stage 8 refers to the eighth hour, during which cytokinesis occurs. The macronucleus undergoes a series of striking morphological changes during amitotic division. At stage 5, the nuclear nodes begin to coalesce until the nucleus is a compact mass in the center of the cell (stage 6; Fig. 1*B*); this subsequently elongates into a thin rod (stage 7) which forms nodes again as it is passively pinched in two by the cleavage furrow (stage 8). The cycle of changes in nuclear morphology is associated with the development of new oral structures. It occurs not only during amitotic division but also during oral regeneration, when a new oral apparatus is formed to replace missing or irreparably damaged mouthparts and during physiological reorganization, when new oral structures replace the pre-existing ones for reasons that are not well understood.

Spectrophotometric evidence indicates that the macronuclei of ciliates are polyploid.[7, 15, 19, 28] Stentors deprived of all but one macronuclear node can regenerate the entire chain and produce viable clones.[26] Each node must therefore contain at least one diploid genome and probably contains over a hundred, since ploidy in another large heterotrich, *Bursaria truncatella*, has been estimated at 2500 × by spectrophotometric measurement of DNA in macro- and micronuclei.[19]

Chromosome-like bodies in the macronuclei of ciliates have been described.[8, 9, 17, 18, 22] In a radiolarian, *Aulacantha scolymantha*, Grell[9] observed long chains of Feulgen-positive rods and postulated that the chromosomes of each genome are strung together end to end to form compound chromosomes ("sammelchromosomen"). Ruthmann[18] has observed what may be similar structures in the chain macronucleus of the ciliate *Loxophyllum mealagris*.

Many micronuclei lie adjacent to the nodes of the macronuclear chain in *Stentor*, but these seem to be of no importance except during conjugation, since emicronucleate strains survive and propagate normally.[21] For the sake of brevity, the macronucleus will be referred to simply as the nucleus from this point on.

Methods.—The "North Carolina" strain of *S. coeruleus*[26] was grown on the smaller ciliate *Colpidium campylum;* a modified Peters' solution served as inorganic medium.[6]

Cells were grafted and nuclei transferred free-hand with a glass needle under a dissecting microscope.[24, 26] Unless otherwise indicated in the text, cultures were raised at 19–20°C and experiments carried out at this temperature.

Macronuclear DNA synthesis was assessed by placing the stentors in Peters' medium containing thymidine-H^3 (Schwartz BioResearch, Inc., 14–15 c/mM) at a concentration of 50–100 μc/ml. Experimental organisms were squashed on microscope slides with a cover slip bearing a small drop of glacial acetic acid. The preparations were frozen on a block of dry ice and the cover slip was removed by lifting the edge with a razor blade. After an additional half hour in ethanol-acetic (3:1) fixative, the slides were stored in 95 per cent ethanol and later passed quickly through a graded alcohol series into distilled water. They were then immediately transferred to calcium-formol fixative[16] to harden the nuclei so that no distortion would occur on drying. Pigment granules were bleached with potassium permanganate and oxalic acid.[21] Macronuclei were stained by the Feulgen technique. Finally, the slides were dipped in Kodak liquid emulsion (NTB2 or NTB3) and exposed at room temperature for one to three weeks.

DNase digestions were carried out in a solution containing 0.2 mg/ml of DNase (Nutritional Biochemicals Corp.), 1 mg/ml of gelatin, and 0.73 mg/ml of $MgSO_4$ in a pH 7.5 buffer prepared by adding 41 ml of 0.1 *M* NaOH to 80 ml of 0.1 *M* KH_2PO_4 and diluting with an equal volume of distilled water.

Results.—Timing of macronuclear DNA synthesis during the cell cycle: (1) *DNA synthesis during amitotic division:* In order to determine whether nuclear DNA synthesis occurs during any part of amitotic division, groups of dividing organisms (stages 3–8) were placed in thymidine-H^3 (100 μc/ml) for 30 minutes. Autoradiography showed that label was always present above the nuclear nodes of cells in early stages of division (stages 3 and 4), seldom present above coalescing nuclei (stage 5), and never present above compacted, elongating, and nodulating nuclei (stages 6–8). These results indicate that DNA synthesis occurs during the first four hours of the eight-hour division period and stops at or near the time when nuclear coalescence begins.

(2) *Initiation of DNA synthesis after division:* In *S. coeruleus*, the cell cycle is of variable length even when cells are grown at constant temperature on the single food organism *Colpidium*. Although stentors removed at the end of division into small dishes containing inorganic medium and *Colpidium* usually divided between

30 and 48 hours later, shorter or longer generation times have been observed under the same conditions. In numerous experiments, stage-8 cells were selected from single cultures and incubated in thymidine-H^3 for successive 2-hour intervals during the first 12 hours of the cell growth cycle. These experiments revealed that the length of the G1 period varied from culture to culture. Most frequently, nuclear label first appeared in autoradiographs between 4 and 8 hours after the beginning of interphase, but in an occasional culture it appeared as early as 2–4 hours or as late as 10–12 hours. It was also found that when stage-8 stentors were removed from the same culture within a one-hour period, they initiated DNA synthesis with excellent synchrony. When label first appeared it was found over the nuclei of all such cells within the same 2-hour time period.

(3) *Continuity of DNA synthesis during the S period:* It is important to know whether the S period, once it has begun, continues without interruption up to stage 5 of division. Dividing cells from three cultures were isolated at stage 8 and placed in dishes containing inorganic medium and *Colpidium*. The resulting daughter cells were transferred in groups of ten to thymidine-H^3 for successive two-hour intervals throughout the cell cycle and prepared for autoradiography. These experiments ended with a 30- to 32-hour point because most of the cells were dividing by this time. Examination of the autoradiographs showed that label was present over the nuclei of all cells from the time that DNA synthesis began up through the 30- to 32-hour point. Once DNA synthesis in *Stentor* has begun, it is evidently continuous up to the time of nuclear coalescence, at least in the presence of an adequate food supply.

These experiments confirm the findings of Guttes and Guttes.[10] Using culture methods very different from those employed in the present study, these workers estimated by autoradiography that DNA synthesis must occur during most of the cell growth cycle in *S. coeruleus*.

(4) Does the failure of the nucleus to incorporate tritiated thymidine during late D (division) and G1 actually represent a cessation of DNA synthesis at this time?

It is possible that the failure of late-D and of G1 nuclei to incorporate thymidine-H^3 is caused by the absence of enzymes involved in phosphorylating thymidine rather than by the absence of DNA synthesis. To investigate this question, dividing cells with compacted nuclei and 18-hour-old cells were incubated for one hour at 24°C in Peters' medium containing 100 μc/ml of thymidine-H^3 triphosphate (Schwartz BioResearch, Inc., 4.8 c/mM). Autoradiography showed label over the nuclei of the 18-hour-old cells but not over the nuclei of the dividing cells.

The failure of G1 and late-D nuclei to incorporate thymidine and thymidine triphosphate into DNA might result from a drastic lowering of thymidine uptake during late stages of division and early interphase. However, cytoplasmic labeling, which was observed at all stages of the cell growth cycle, occurred in many dividing cells with unlabeled nuclei.

In a final experiment, 18-hour-old cells were incubated in thymidine-H^3 (100 μc/ml) for two hours, squashed on slides, fixed in ethanol-acetic fixative, and divided into three groups. Group 1 cells were treated with DNase and group 2 cells with DNase buffer while cells in group 3 received no further treatment. Autoradiography showed that DNase had removed all the nuclear label, while buffer alone had not.

Nuclear vs. cytoplasmic regulation of DNA synthesis: Transfer of nuclei between cells in S and cells in D and G1: If DNA synthesis is regulated by the continuous presence in the cytoplasm of an inhibitor during G1 and late D or of an initiator during S, it should be possible to initiate DNA synthesis prematurely in a late D or a G1 nucleus by transferring it to a cell in S and, conversely, to suppress DNA synthesis in the nucleus of a cell in S by transferring it to a cell in G1.

To investigate this question, the following types of nuclear transfer were carried out: G1 nucleus into S cell; S nucleus into G1 cell; and late D (compacted) nucleus into S cell.

(1) *Experimental procedure:* (A) *Transfer of nuclei:* Compacted (stage 6) nuclei were transferred whole into receiver cells but for technical reasons it was only possible to transfer a few nodes of the interphase nucleus and not the entire chain. However, previous experiments have shown that short chains of nuclear nodes excised from interphase nuclei are not injured by this procedure.[3, 4] The small group of experimental organisms resulting from one hour of microsurgery was placed in Peters' medium for one hour to recover from the operation and then transferred to thymidine-H^3 for an additional two hours.

(B) *Selection of S, G1, and D cells:* To obtain S cells, stage-8 stentors were selected from a single culture within a one-hour period. The resulting daughter cells were placed in Peters' medium, with or without food organisms, and when they reached 18–24 hours of age were used in experiments requiring cells in S. If no *Colpidium* was supplied, food reserves already present in the newly divided cells were generally sufficient for initiation and maintenance of DNA synthesis, even though no growth in mass could occur. If fed, 18- to 24-hour-old stentors were very large but contained so many food vacuoles that the nucleus was completely obscured and could not be removed from the cell. For this reason, starved 18- to 24-hour-old stentors were routinely used as a source of donor nuclei while fed cells were used as receivers because of their greater size. To obtain G1 cells, stage-8 stentors were selected from a single culture within a one-hour period. They were used as a source of G1 nuclei when zero to one hour old. To obtain D cells with compacted nuclei, dividing cells at stage 6 were selected from cultures as needed.

(C) *Controls to determine the state of donor and receiver cells with respect to DNA synthesis during the course of each experiment:* Because of the culture-to-culture variation in length of G1 described earlier, it was impossible to assume that the nuclei of stentors used as G1 donors or receivers would not normally have passed into S during the experiment. However, since cells in late division selected from a single culture within a one-hour period begin the next round of DNA synthesis with excellent synchrony, this possibility could be eliminated by routine use of a suitable control. This was done by selecting out of single cultures more G1 cells than would be needed as donors or receivers; the extra cells were incubated with thymidine-H^3 at the same time as the experimental group and autoradiography of these nonoperated stentors then provided the necessary assurance that G1 donors or receivers would normally have been in G1 throughout each experiment. Similar controls served to ensure that the S cells used as donors and receivers would normally have been in S throughout each experiment; this proved to be a necessary precaution when starved S cells were used as donors, since DNA synthesis occasionally came to a stop in starved cells before they were used in experiments.

(D) *Number of experimental cells observed:* Except where otherwise indicated, the results of each experiment were obtained by observing unambiguous autoradiographs of 20 cells from transfer series which had been properly controlled as described above.

(2) *Transfer of G1 nuclei into S cells:* Chains of nuclear nodes from G1 cells transferred into cells in S could usually be distinguished from the nodes of the host nucleus by their smaller size. In all cells examined, label was present over both host and transferred nodes (Fig. 2). However, when chains of G1 nodes were transferred into G1 cells, no label was ever found over either set of nodes. The appearance of DNA synthesis in G1 nodes transferred to S cells is therefore not due to the effects of the operation.

These data suggest that the occurrence of macronuclear DNA synthesis in *Stentor* depends on the presence or absence of a cytoplasmic factor(s).

(3) *Transfer of D (compacted) nuclei into S cells:* Previous work has shown that the compacted nuclei of dividing cells retain this shape when transferred to interphase cells instead of elongating as they would *in situ*.[3, 4] When these cells were used as donors, the large, compact nuclei were therefore easily distinguishable from the nodes of the host nucleus. Due to the technical difficulty of transferring compacted nuclei, the results of this experiment were obtained by observing autoradiographs of 10 cells instead of 20. Three of these came from transfer series in which the experiments were carried out at 24°C and incubated in thymidine for one hour only. Again, label was invariably found over both host and transferred nucleus (Fig. 3). When compacted nuclei were transferred into G1 cells, no label was ever found above host or transferred nuclei.

These data are consistent with the hypothesis that DNA synthesis is suppressed at the time of nuclear coalescence by reversal of the cytoplasmic event which initiates it at the end of G1. It is also clear from these results that termination of DNA synthesis at stage 5 is independent of the morphological changes occurring in the nucleus at this time.

(4) *Transfer of S nuclei into G1 cells:* When transferred into G1 cells, chains of nodes from S cells could usually be distinguished from the host nodes by their larger size. Autoradiography showed no label over either set of nodes. When chains of nodes from cells in S were transferred into other cells in S, label was always found over both host and transferred nodes. The suppression of synthesis in S nodes transferred to G1 cells therefore cannot be caused by operation injury. These data suggest that the cytoplasmic condition responsible for initiating DNA synthesis is also responsible for maintaining it throughout interphase.

Positive vs. negative control: DNA synthesis in graft complexes between S and G1 cells: Two stentors can be grafted together in any desired orientation.[24] If one of these is synthesizing DNA and the other is not, observation of both nuclei of the graft complex will indicate whether the transition from G1 to S is effected by removal of an inhibitor present in G1 and D cells or by appearance of an initiator present in S cells. Synthesis of DNA by both nuclei of the graft complex would suggest the latter possibility; synthesis by neither nucleus would suggest the former.

In these experiments, a stentor in G1 was grafted to the anterior half of a stentor in S, so that the two members of the graft complex would be of equal size. Preliminary experiments showed that DNA synthesis continued normally in anterior

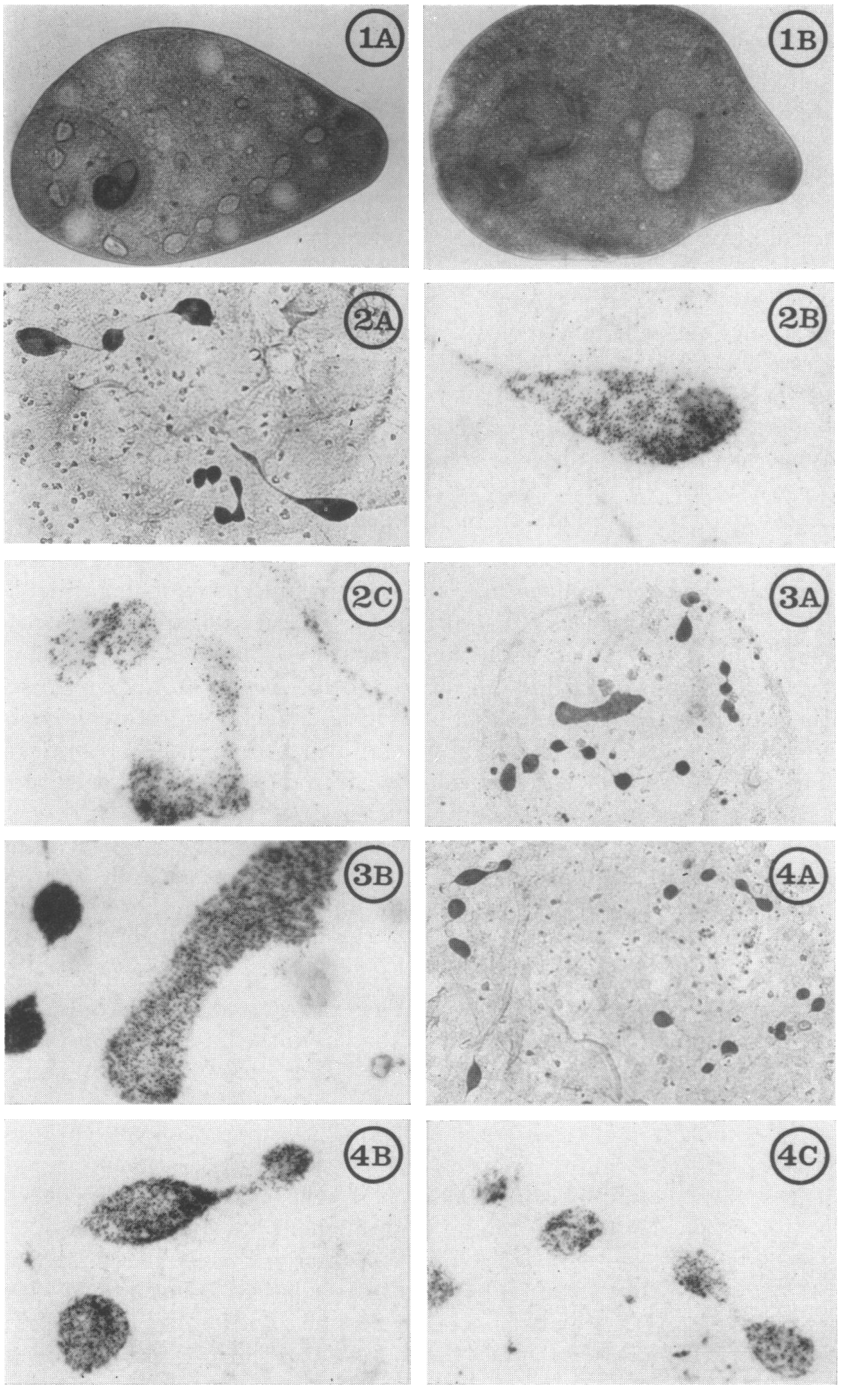

FIG. 1.—Morphology of *Stentor coeruleus*. Living *Stentor* in microcompression chamber. ×75. (*A*) Interphase stentor, showing chain macronucleus. (*B*) Stentor at stage 6 of division, showing compacted macronucleus.

FIG. 2.—Result of transferring nuclear nodes from cell in G1 to cell in S. See text for details of experiment. Feulgen. (*A*) Autoradiograph. ×75. (*B*) Detail of stentor in Fig. 2*A*, showing label over one large node of host nucleus. ×300. (*C*) Detail of stentor in Fig. 2*A*, showing label over transferred G1 nodes. ×300.

FIG. 3.—Result of transferring compacted nucleus from cell in stage 6 of division to cell in S. See text for details of experiment. (*A*) Autoradiograph. ×75. (*B*) Detail of stentor in Fig. 3*A*, showing label over host and transferred nuclei. ×300. →

halves for at least four hours after transection. The two cells were partially fused in various orientations; the only constant factors were the existence of a broad cytoplasmic connection between them and the presence of two intact sets of oral structures. Autoradiography showed that in all the graft complexes examined label was present over both sets of nuclear nodes (Fig. 4). In graft complexes composed of two G1 cells, no label was present over either set of nodes.

These results strongly suggest that cytoplasmic regulation of DNA synthesis mediated by an initiator rather than an inhibitor.

Discussion.—The results of the experiments described in this paper are briefly summarized below:

(1) The occurrence of macronuclear DNA synthesis in *Stentor* appears to be determined by the presence or absence of a cytoplasmic factor since the nuclei of cells in D and G1 initiate DNA synthesis when transferred to cells in S.

(2) This factor is an initiator present in S cells, rather than an inhibitor present in D and G1 cells, since when S and G1 cells of equal size are grafted together, synthesis is initiated in the G1 cell, not inhibited in the S cell.

(3) The initiator appears to be required throughout interphase for maintenance of DNA synthesis since nuclei from cells in S stop synthesizing DNA when transferred to cells in G1.

Regulation of nuclear DNA synthesis has been studied mainly in ciliates, tissue culture cells, and *E. coli*. Kimball and Prescott[12] observed that reorganization bands appear to be synchronous in both macronuclei of binucleate *Euplotes;* this finding suggests that macronuclear DNA synthesis is initiated by a cytoplasmic factor in at least one ciliate other than *Stentor*. In binucleate tissue culture cells, DNA synthesis is asynchronous; however, some cytoplasmic event appears to impose synchrony on the activities of the two nuclei later since they always enter metaphase together.[20] Apparently DNA synthesis can be determined either by cytoplasmic or intranuclear factors, and metazoan cells differ from ciliates such as *Euplotes* and *Stentor* in ways which might well impose considerable differences in regulatory mechanisms. In *Stentor*, where the size of the macronucleus is determined by the size of the cell,[2] cytoplasmic control of DNA synthesis may be obligatory.

In *E. coli*, evidence suggests that DNA synthesis is regulated by a "cytoplasmic" initiator, as in *Stentor*.[11] However, analogies are again complicated by the obvious differences between these cell types. *E. coli* lacks a nuclear membrane and the word "cytoplasmic" consequently cannot have the same meaning as in *Stentor*. *Stentor* has approximately 80 chromosomes of complex, unknown structure, and organization, while *E. coli* has one chromosome consisting of a single DNA molecule. Although DNA synthesis appears to be regulated by a "cytoplasmic initiator" in both cell types, it is impossible at present to say whether this apparent resemblance is superficial or whether it involves any basic similarity of mechanism.

All of the major nuclear events of the cell growth cycle in *Stentor* studied so far appear to be under cytoplasmic control.[3, 4] Previous work has shown that at least two of the changes in macronuclear morphology which occur during division are initiated by cytoplasmic factors which do not act as "triggers" but are continuously required throughout the time that the changes are occurring.[4] It is interesting that the factor initiating DNA synthesis in *Stentor* also appears to act in this manner.

FIG. 4.—Result of grafting a cell in G1 to a cell in S. See text for details of experiment. Feulgen. (*A*) G1-S graft complex. Smaller nodes at right are derived from G1 cell. ×75. (*B*) Autoradiograph over nodes derived from cell in S. ×300. (*C*) Autoradiograph over nodes derived from cell in G1. ×300.

There is no other clue as to how the initiator may function. The findings of many workers have suggested different ways in which DNA synthesis might be regulated. The *Stentor* initiator might act by converting DNA to a primer form,[1] by opening the chromosome at key points for replication,[27] or by changing the structure[13] or location[14] of DNA polymerase. In the ciliate *Tetrahymena* a pool of precursors for DNA synthesis exists in the nucleus during periods of nonsynthesis;[23] if *Stentor* resembles *Tetrahymena* in this regard, it is unlikely that the *Stentor* initiator functions by affecting the accumulation of precursors for DNA synthesis.

Summary.—The techniques of cell grafting and nuclear transfer have been combined with autoradiography in order to study mechanisms determining the occurrence of macronuclear DNA synthesis during the cell growth cycle in *Stentor*. The results suggest that DNA synthesis is regulated by the presence or absence of a cytoplasmic initiator.

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