ACCUMULATION OF REPLICONS IN *TETRAHYMENA*

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1. Introduction

Eukaryotic cells have many replicating units (replicons, for definition see [1] and [2]) in each chromosome as already demonstrated for a number of different species [2]. However, there is as yet little insight into the processes that control the operation of these replicons. During our studies of DNA metabolism we have become interested in this problem and shall here describe a system by which it is possible to accumulate and study the formation of well defined replicons in *Tetrahymena*. We further demonstrate that at least 90% and possibly all of the nuclear DNA exists on a high mol. wt. form ($\geqslant 160 \times 10^6$). This observation is in conflict with the results by Miyagishi and Andoh, who find large amounts of the DNA on a low molecular weight form ($\sim 31 \times 10^6$ dalton) [3]. We also find that the higher molecular weight DNA gets broken down to the smaller fragments during the isolation. Since these fragments are of replicon size, this suggest that there are regions specifically sensitive to nuclease cleavage on the DNA [4,5], and further that these sensitive regions might correspond to the initiation or the termination sites for the replicons.

2. Materials and methods

2.1. *Culture of cells*

Tetrahymena pyriformis, aminonucleated strain GL, was grown at 28°C in a defined medium [6] with low concentrations of phosphates (0.04 mM) and with omission of uridine from the medium. Instead, proteose peptone and yeast extract were added to final concentrations of 0.04% and 0.004%, respectively. In standard experiments the cells were grown in the medium for 18–20 h with an average generation time of about 5 hrs. In most experiments the cultures were prelabelled with [^{14}C]thymidine (0.02 μCi/ml). Prior to pulse labelling of the cells with [^{3}H]thymidine (5 μCi/ml), they were chilled to 0°C, added methotrexate plus uridine (final concentrations of 70 μM and 7 μM, respectively) and quickly transferred to fresh medium by centrifugation (250 *g* for 4 min). The pulse experiments were performed at 28°C for the indicated period of time. At the end of an experiment, the cells were chilled to 0°C, added one volume of NET buffer (500 mM NaCl, 50 mM EDTA and 50 mM Tris, pH 7.2) and harvested by centrifugation at 400 *g* for 3 min.

2.2. *Sedimentation on sucrose gradients*

The harvested cells were quickly resuspended in 100 μl of water and applied on top of an alkaline sucrose gradient (5–20% on top of a 1 ml cushion of saturated sucrose, all in 0.2 NaOH, 800 mM NaCl and 2 mM EDTA). After addition of 100 μl of 5% sarkosyl in 1 N NaOH the gradients were left for 30 min at 4°C and spun in a SW41 rotor at 40 krev/min (4°C) for the indicated period of time. λ DNA was used as a marker for the sedimentation [7].

2.3. *Materials*

All chemicals were purchased from Sigma Chemical Company, St. Louis. [^{3}H]Methyl thymidine (spec. act. 17C/mmol) and [2-^{14}C]thymidine (spec. act. 60 mC/mmol) were obtained from the Radiochemical Centre, Amersham, England. λ DNA containing the deletions G515 and G519 was the

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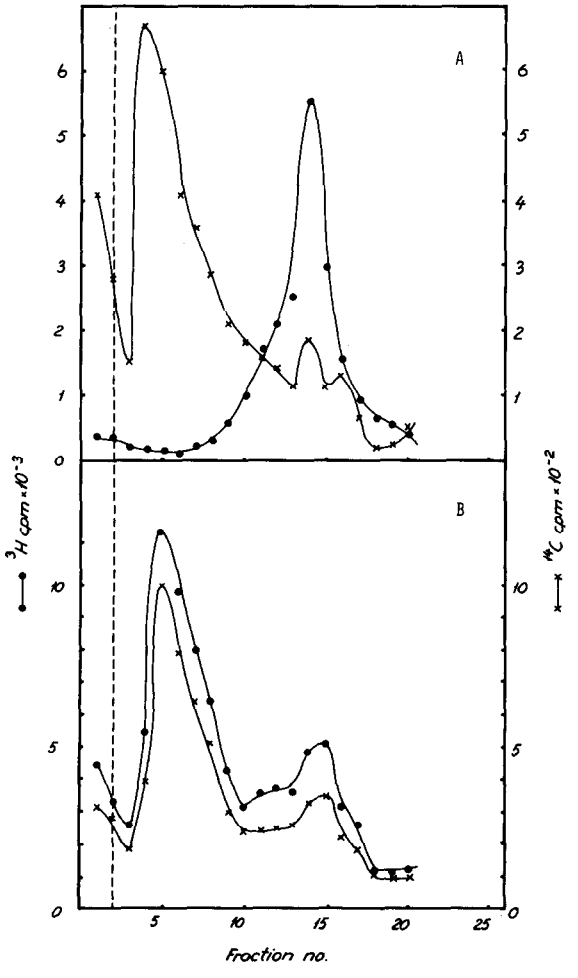
3. Results and discussion

In order to study the replicative DNA intermediates of *Tetrahymena*, we have developed growth conditions by which two of these intermediates can be accumulated. Under these conditions the cells are grown in a medium with nearly limiting amounts of phosphate and uridine and prior to an experiment the DNA replication is slowed down by a short cold shock. Fig.1A demonstrates how [^{3}H]tymidine from a 20 min pulse gets incorporated into DNA sedimenting as a distinct peak with a sedimentation value smaller than the prelabelled [^{14}C]DNA. The pulse labelled [^{3}H]DNA can all or nearly all be converted into the high molecular weight form by a 30–60 min chase as seen from fig.1B. A small amount of the



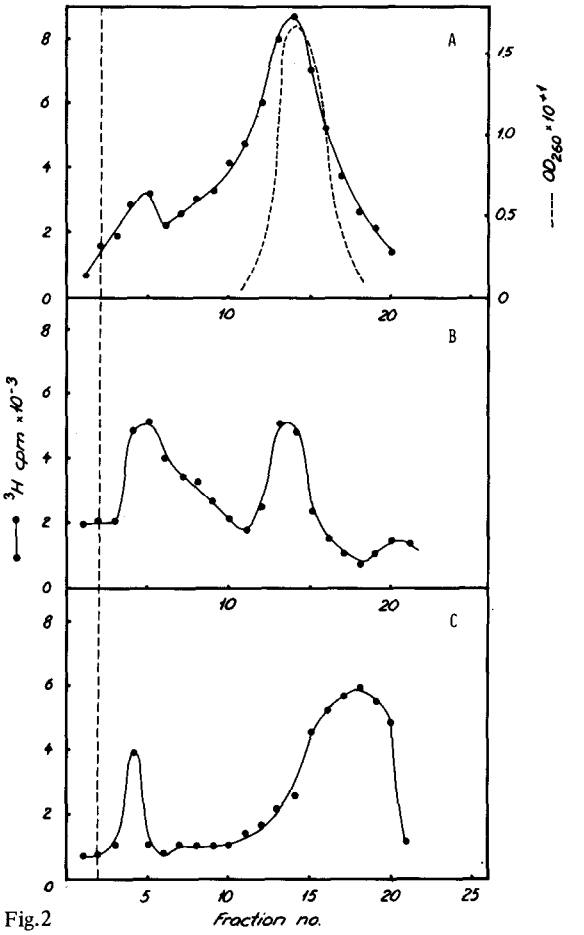


Fig.2

Fig.1. Alkaline sucrose sedimentation of pulse and pulse-chased labelled DNA. The cells were grown and prelabelled with [^{14}C]thymidine as described in Materials and methods. (A) was pulse labelled with [^{3}H]thymidine for 20 min, while (B) was pulse labelled for 20 min and then chased for 60 min with cold thymidine (100 μg/ml). The cells were collected, treated and applied on 5–20% sucrose gradients as described in the Methods. The gradients were spun for 2½ h in a SW41 rotor at 40 krev/min. Fractions were collected from the bottom of the tube and acid precipitated on a Whatman GF/C glass fibre filter. The gravity field is directed to the left. (×——×) [^{14}C]DNA; (●——●) [^{3}H]DNA.

Fig.2. Alkaline sucrose sedimentation of ^{3}H-labelled DNA. The cells were grown and treated as described in Materials and methods. (A) was pulse labelled for 30 min with [^{3}H] thymidine; (B) was pulse labelled as (A) and exposed to a short period of chase (30 min) with cold thymidine, (100 μg/ml); (C) was treated with hydroxyurea (5 mM) for 10 min before the cells were exposed to a 30 min pulse of [^{3}H] thymidine. In a fourth parallel gradient 150 μl of λ DNA (0.9 optical unit at 260 nm) was sedimented. The optical density at 260 nm was read over the marker gradient and is indicated on fig.2A. The labelled DNA was sedimented and counted as described in fig.1. (X——X) [^{14}C]DNA; (●——●) [^{3}H]DNA.

prelabelled DNA will, however, always sediment at the position of the smaller molecular weight DNA. The percentage of this slow sedimenting DNA varies from experiment to experiment dependent upon how carefully the cells have been harvested and lysed. The percentage of the low molecular weight DNA was in a total of 10 experiments (standard experiments, see Materials and methods) found to vary from 8.2–16.5% of the total DNA with an average value of 12.8%. Recent investigations have, however, shown that it is possible to obtain more reproducible values around 10%, if the cells are lysed in the presence of slightly higher concentrations of alkali (i.e. 0.75 N).

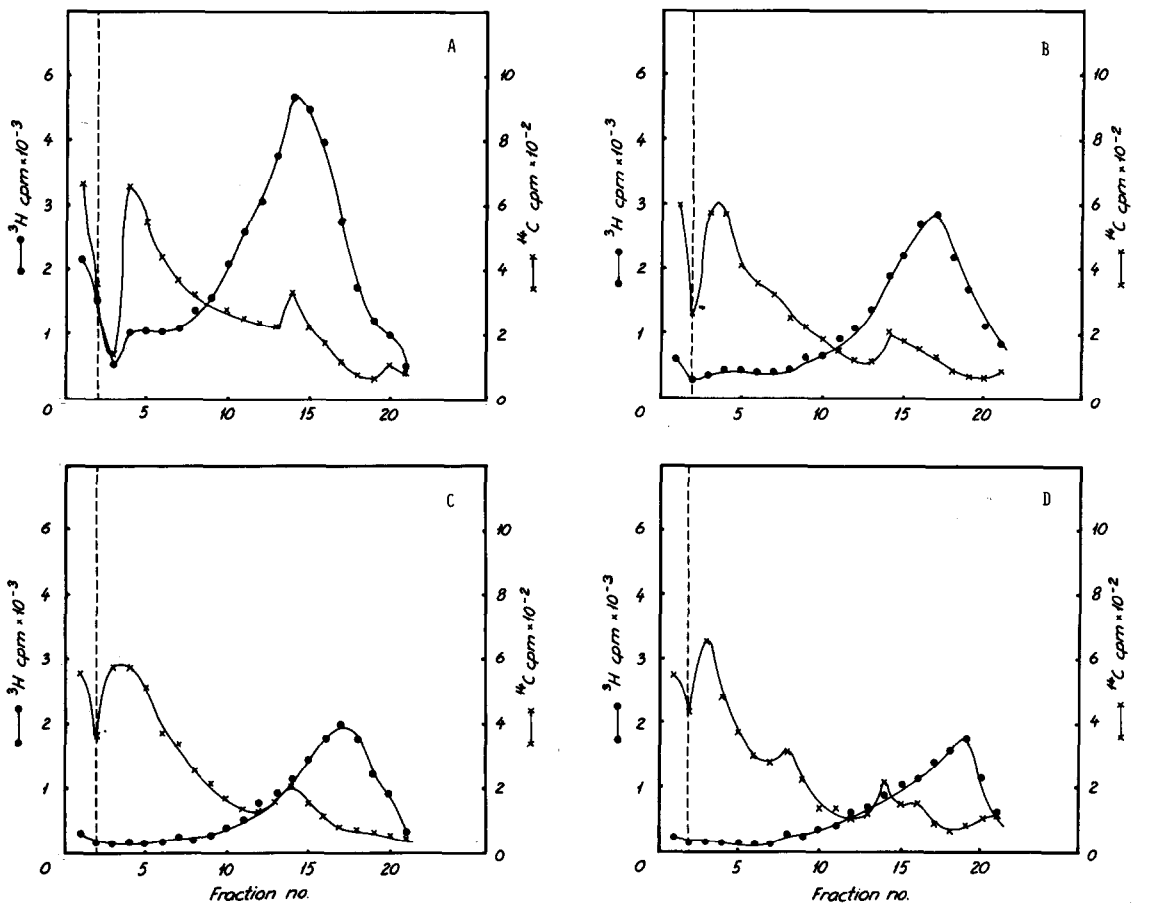


Fig.3. Alkaline sucrose sedimentations of DNA fragments accumulated in the presence of hydroxyurea. The cells were prelabelled with [^{14}C]thymidine, grown and treated as described in Materials and methods. (A) was pulse labelled for 30 min with [^{3}H] thymidine, (B), (C), (D) were treated with hydroxyurea (5, 10 and 25 mM respectively) for 20 min before the cells were pulse labelled with [^{3}H]thymidine for 40 min. The labelled DNA was sedimented and counted as described in fig.1. (X——X) [^{14}C] DNA; (●——●) [^{3}H]DNA.

The fast labelled DNA has a molecular weight of 17×10^6 dalton (41S) in the alkaline sucrose gradient as seen from Fig.2A, where λ DNA is used as a marker. It is not, even after short chase periods, possible to detect any intermediates between the 41S DNA and the high molecular weight form ($\sim 80 \times 10^6$ dalton, 86S) (fig.2B). This implies, that the 41S fragments get converted directly over in the 86S form by ligase joining. When this result is considered together with the fact that the 41S DNA is accumulated as a distinct size class of DNA, it strongly suggests that this DNA represents discrete replicons. Similar values have been obtained for replicons from a number of other eukaryotic cells [2,8–10].

Buoyant density studies of replicons synthesized in the presence of bromodeoxyuridine show that the 41S fragments mainly (> 90%) represent de novo synthesis of full length replicons. Furthermore, it can be shown that pulse labelling of cells grown in a complex medium or of cells which have not been exposed to the short cold shock also reveals synthesis of replicons. These are, however, much less defined in size than described above.

Fig.2C demonstrates that a third class of DNA (Okazaki fragments) can be isolated by treatment of the cells with low concentrations of hydroxyurea for 10 min before exposure to a 30 min pulse of [^{3}H] thymidine. The DNA synthesized in the presence of 5 mM hydroxyurea has a sedimentation value around 10–20S, while the material synthesized after treatment with higher concentrations of the drug, i.e. 25 mM is smaller and has a more defined size around 4–6S (fig.3). The rate of the DNA synthesized in the presence of 5 mM hydroxyurea is inhibited 50% within the first 30 min. This shows, that the cells in this system continue their DNA synthesis, although they are unable to join the Okazaki fragments together with the normal rate [11].

It is evident from the above results that pulse labelled fragments of replicon size can accumulate in *Tetrahymena*. The replicons are extremely well defined in size, although, in other systems have been found to vary in size [2]. The reason for the accumulation of replicons is at the present unclear. One possibility might be that the cells, due to growth in low concentrations of phosphate and uridine, have very low cellular pools of the deoxyribonucleotides and are therefore inhibited in the joining of replicons [11]. This hypothesis is supported by the fact that low concentrations of hydroxyurea inhibit the joining of replicons as well as the joining of Okazaki fragments (Nymann and Westergaard, manuscript in preparation).

Based on the alkaline sedimentation studies presented above, it is clear that at least 90% of the nuclear DNA in *Tetrahymena* exists in the high mol. wt form ($\geqslant 160 \times 10^6$). It is, however, less clear if the remaining 10% of the DNA in vivo is found on a low molecular weight form ($30–35 \times 10^6$ dalton) or if it represents degradation products of high molecular weight DNA [3,13]. Studies of the nuclear DNA on neutral sucrose gradients ([3] Nymann and Westergaard, results not shown) or on agarose gels [14] demonstrate, that more than 80% of the high molecular weight DNA can be degraded to the smaller component during the isolation procedure. As the size of this DNA is similar to that of the described replicons, it is conceivable that nuclease sensitive sites are localized around the initiation or the termination points for the replicons. Experiments are now in progress to investigate if this is a plausible hypothesis. Furthermore, the well-defined system for accumulation of replicons can be used for studies of processes that control the operation of replicons.

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