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Appearance of a late stage during mammalian DNA replication when cells resume formation of 10 kb DNA replication intermediates

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After the joining of human large DNA replication intermediates and before the appearance of mature chromatin DNA, there exists a distinct stage – 'the post-elongation stage'. This stage reappears during recovery of DNA synthesis simultaneously with the reappearance of a large DNA replication intermediate, 10 kb DNA.

DNA synthesis; Post-elongation stage

1. INTRODUCTION

Chromosome replication in mammalian cells is ordered as visualized by the replication of different domains during discrete intervals of the S-phase. The mechanism and regulation of procaryotic DNA replication, and the proteins involved in these events, have been extensively studied. In comparison, knowledge concerning eukaryotic cells is at present sparse.

The formation of human DNA replication intermediates in each replicon is very complex and involves several steps. Principally, small replication intermediates (Okazaki fragments) are joined to larger intermediates and finally adjacent replicon-sized DNAs are joined. We have shown previously that after formation of large replication intermediates one can detect a distinct postsynthetic DNA stage (called 'the post-elongation stage') before the appearance of mature chromatin [1].

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The post-elongation stage has so far been defined by an approach in which we carry out lysis of cells with pulse-labelled DNA in dilute alkali in order to denature the DNA partly. The single-stranded DNA present in the sample is then removed by treatment with nuclease S_1. High molecular mass DNA remains in the sample and represents the post-elongation stage [1]. Recently, we have shown that post-elongation stage DNA contains 10 kb DNA replication intermediates [2] and is dependent on functioning DNA topoisomerase II [3].

We have shown earlier that once the post-elongation stage has formed this DNA gives rise to mature chromatin DNA independent of the presence of moving replication forks [4]. Here, we examine formation of the post-elongation stage in cells recovering from treatment with aphidicolin. Aphidicolin is an inhibitor of DNA polymerases α and δ [5,6]. We find that in cells depleted of 10 kb DNA molecules (large DNA replication intermediates formed by the joining of Okazaki fragments), the post-elongation stage is not detectable. When 10 kb DNA replication intermediates reappear the post-elongation stage is then observable.

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2. MATERIALS AND METHODS

2.1. *Cells and labelling conditions*

Human melanoma cells were grown at 37°C in 5% CO_2 in air as described [1]. The culture medium was Eagle's MEM with Earle's salts, containing 2 mM L-glutamine, 10% fetal calf serum, and antibiotics.

Cells to be used in experiments were seeded in small petri dishes (35 × 10 mm) with 3 ml medium. For pulse labelling of DNA addition of 50 μCi [^{3}H]thymidine (22 Ci/mmol; Amersham) was made to the medium.

2.2. *Cell lysis and treatment with nuclease S_1*

To lyse cells in dilute alkali, medium was drained from the petri dish and 2.25 ml of 0.03 M NaOH was added [1,7]. After 30 min at 0°C in the dark, the solution was neutralized by the addition of 0.9 ml of 0.067 M HCl/0.02 M NaH_2PO_4. The sample was then either digested with nuclease S_1 or immediately made 0.5% with regard to SDS.

To digest the DNA with nuclease S_1, 300 μl of a solution comprising 300 mM sodium acetate (pH 4.6), 0.5 mM zinc acetate, and 750 mM NaCl was added to the petri dish. 200 IU/ml of nuclease S_1 (Sigma) was then added and the mixture incubated for 30 min at 37°C. Digestion was stopped by making the solution 1% SDS-0.02 M EDTA.

2.3. *Agarose gel electrophoresis*

The DNA was separated in 0.75% agarose gels using an LKB Multiphor electrophoretic system. Gels were cut into slices of 1 mm thickness and the radioactivity was measured in a toluene-based scintillation fluid containing 3% Soluene 100, using a Packard scintillation counter.

3. RESULTS

3.1. *Approach*

To delineate the post-elongation stage DNA the following approach was employed [1]: Cells with labelled DNA were lysed in dilute alkali to induce partial DNA denaturation. Denaturation was initiated at gaps present in the replicons. The solution was then neutralized in order to allow renaturation of the large DNA. Next we removed all single-stranded DNA replication intermediates by digestion of the DNA with nuclease S_1, which digests single-stranded but not double-stranded DNA.

Double-stranded DNA remained in the solution which could be subdivided into two stages. One stage represents mature chromatin DNA: this DNA was fragmented by digestion with nuclease S_1 to form DNA fragments with a size of 70–200 bp single-stranded DNA. Before the DNA appears as mature chromatin DNA, however, one can detect a stage where nuclease S_1 does not fragment the DNA (the post-elongation stage) and the DNA appears as high molecular mass material.

Fig.1A shows gel electrophoretic separation of the post-elongation stage DNA detectable in cells pulsed with thymidine for 3 min (high molecular mass DNA located in slices 3–6). In cells containing mature chromatin one detects instead DNA fragments in slices 20–35.

Fig.1B shows gel separation of pulse-labelled DNA (3 min labelling) containing the 10 kb DNA replication intermediate (slices 23–32). The 10 kb DNA is formed by joining of the primary replication intermediates (Okazaki fragments) (located in slices 40–50) and is not present in cells with mature chromatin [8]. In these experiments digestion with nuclease S_1 was omitted.

3.2. *Pulse labelling of DNA immediately after treatment with aphidicolin*

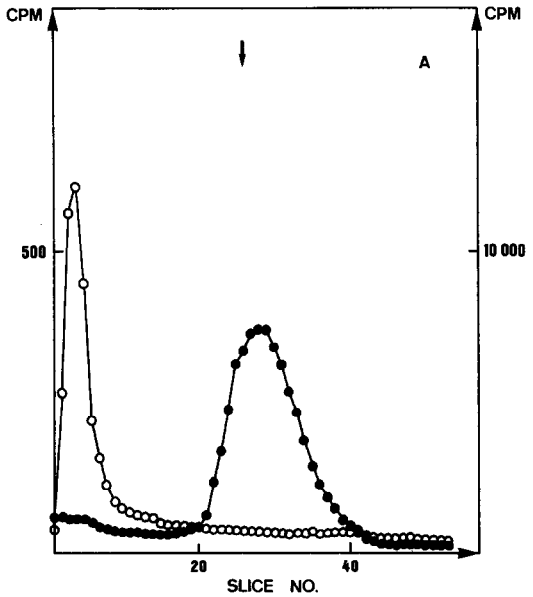
To deplete the cells of large DNA replication intermediates, we treated cells with aphidicolin for 60 min. Aphidicolin is a known inhibitor of DNA polymerases α and δ. We have previously shown that this treatment arrests DNA synthesis and prevents labelling of DNA replication intermediates. The large 10 kb DNA intermediates that are formed before addition of aphidicolin will join and form large DNA. These cells therefore would be depleted of 10 kb DNA molecules [8].

Cells were incubated for 60 min with aphidicolin, the medium then changed to fresh medium supplemented with [^{3}H]thymidine and incubation continued for 45 s or 3 min. Fig.2A shows that in neither sample was it possible to detect the post-elongation stage. Only a small amount of mature chromatin DNA fragments was demonstrable.

Furthermore, detection of the 10 kb DNA replication intermediates was not possible when digestion with nuclease S_1 was omitted. Instead the gel separations showed large amounts of Okazaki fragments (fig.2B). 10 kb DNA was located in the gel in slices 23–32 and Okazaki fragments in slices 40–50.

3.3. *Pulse labelling of DNA during the recovery of DNA synthesis*

Subsequently, we performed experiments where the cells were incubated in fresh medium for 30 min after treatment with aphidicolin for



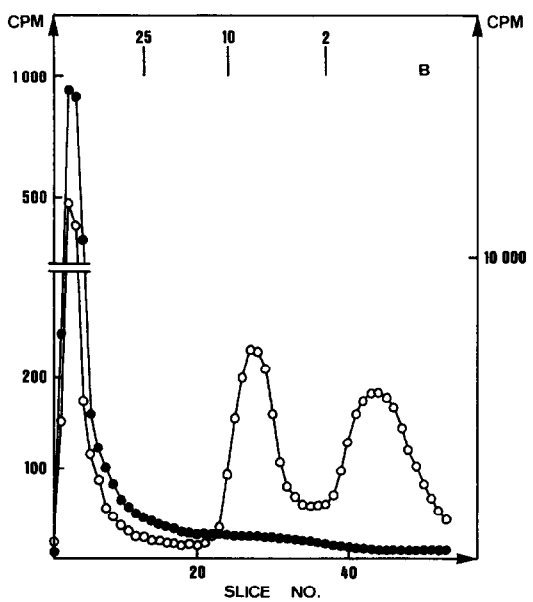
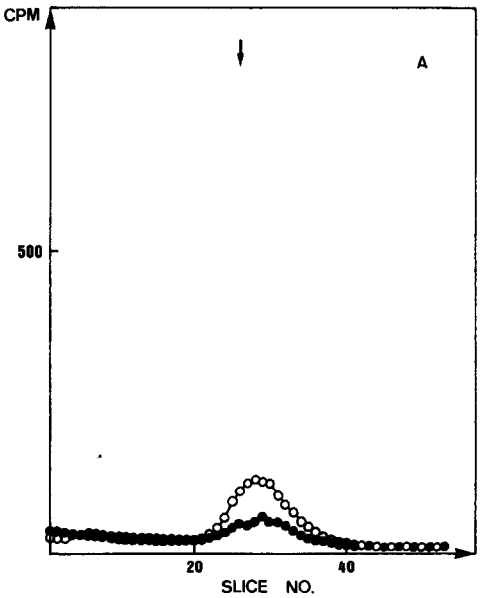


Fig.1. The post-elongation stage. (A) Human melanoma cells were labelled with thymidine for 3 min (○—○) or 24 h (●—●). Cells were lysed in dilute alkali, the DNA digested with nuclease S_1 and the remaining double-stranded DNA then separated in 0.75% agarose gels. Arrow denotes the location of a 2 kb single-stranded DNA marker. The scale to the left refers to (○—○), that on the right to (●—●). (B) Cells incubated as in (A). Cells were lysed in dilute alkali and the DNA (without prior digestion with nuclease S_1) separated in 0.75% agarose gels. Arrows denote the location and size (in kb) of single-stranded DNA markers.



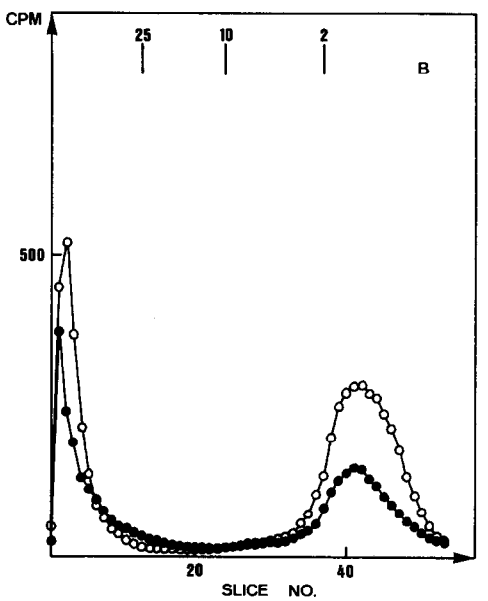


Fig.2. Cells examined immediately after treatment with aphidicolin. (A) Cells were treated with aphidicolin (10 μg/ml) for 60 min and then labelled with thymidine for 45 s (●—●) or 3 min (○—○). Cells were lysed in dilute alkali, the DNA digested with nuclease S_1 and then separated in 0.75% agarose gels. Arrow denotes the location of a 2 kb single-stranded DNA marker. (B) Cells incubated as in (A). Cells were lysed in dilute alkali and the DNA (without prior digestion with nuclease S_1) then separated in 0.75% agarose gels. Arrows denote the size (in kb) and location of single-stranded DNA markers.

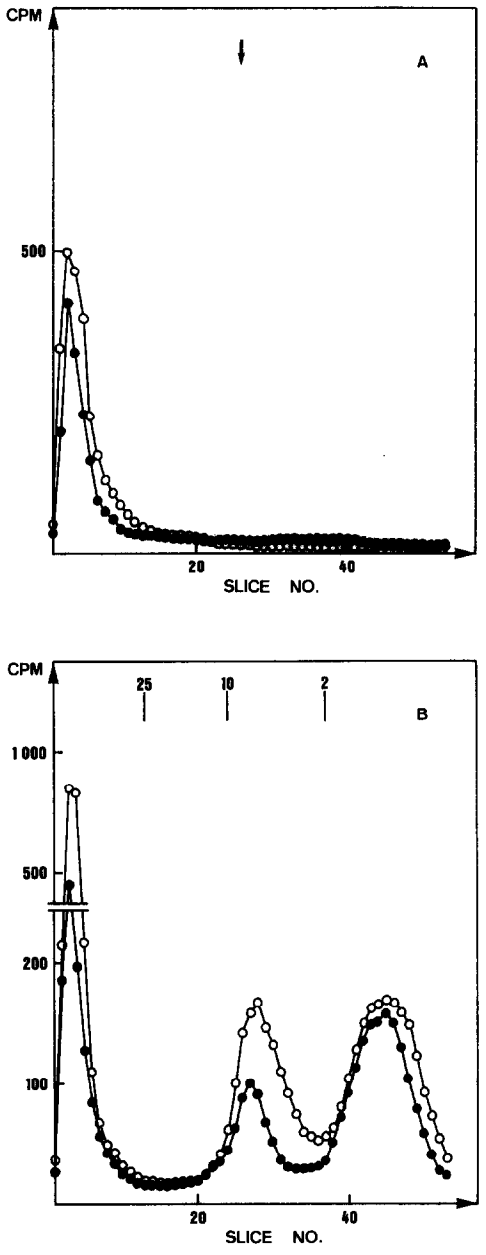


Fig.3. Cells examined during recovery of DNA synthesis. (A) Cells were treated with aphidicolin (10 μg/ml) for 60 min, washed free of the drug and incubated for 30 min in fresh medium. The cells were then pulsed with thymidine for 45 s (●—●) or 3 min (○—○). Cells were lysed in dilute alkali, the DNA digested with nuclease S_1 and then separated in 0.75% agarose gels. (B) Same protocol as in (A). Cells were lysed in dilute alkali and the DNA (without prior digestion with nuclease S_1) separated in 0.75% agarose gels. Arrows denote the size (in kb) and location of single-stranded DNA markers.

60 min. [^{3}H]Thymidine was then added to the medium for 45 s or 3 min.

Fig.3A shows that the post-elongation stage was indeed detectable in these cells. Furthermore, the presence of 10 kb DNA replication intermediates and Okazaki fragments was demonstrated when digestion with nuclease S_1 was omitted (fig.3B).

4. DISCUSSION

During cell proliferation, not only must the DNA be accurately reproduced, but the protein structure of chromatin must also be duplicated. It has been reported that chromatin assembly probably takes place through a two-step reaction [9]. Initially, nucleosome formation occurs uncoupled from their spacing on DNA. In the second step DNA topoisomerase activity is involved. Both topoisomerases I and II have been implicated in the process [9,10]. It seems very likely that the post-elongation stage participates in the process of chromatin assembly.

Several lines of evidence indicate that detection of the post-elongation stage during DNA synthesis is dependent on the presence of 10 kb DNA molecules [2]. To provide further data in support of this, we examined the reappearance of the post-elongation stage in cells initiating DNA synthesis after depletion of 10 kb DNA molecules.

To deplete cells of 10 kb DNA molecules we performed incubations of cells with the inhibitor of DNA polymerases α and δ, aphidicolin. The cells were then assayed for the presence of the post-elongation stage immediately after the treatment with aphidicolin or during the recovery of DNA synthesis.

Our results showed that when the cells were depleted of 10 kb DNA molecules the post-elongation stage was undetectable; however, it became evident on the reappearance of the 10 kb DNA molecules. In cells where only Okazaki fragments were demonstrable, we were unable to detect the stage DNA. This was in agreement with the following pathway: the initially formed Okazaki fragments give rise to 10 kb DNA intermediates which lead to the post-elongation stage. Finally, mature chromatin DNA appears.

The formation of post-elongation stage DNA depends on several factors. The presence of 10 kb DNA intermediates is one of these requirements,

other factors being the nucleosome repeat length and the presence of functional DNA topoisomerase II [3].

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