The supply of exogenous deoxyribonucleotides accelerates the speed of the replication fork in early S-phase

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SUMMARY

Earlier studies have established that the average speed of a replication fork is two to three times slower in early S-phase than in late S-phase and that the intracellular 2'-deoxyribonucleoside 5'-triphosphate pools grow during S-phase. In this study, the effect of the exogenous 2'-deoxyribonucleoside 5'-triphosphate (dNTP) supply on the average replication speed in a synchronised population of human HeLa cells was tested. The speed of replication fork movement was measured on extended DNA fibers labelled with 2'-deoxythymidine analogues 5-chloro-2'-deoxyuridine and 5-ido-2'-deoxyuridine. We show that the introduction of exogenous dNTPs accelerates the replication process at the beginning of DNA synthesis only. In late S-phase, the administration of additional dNTPs has no effect on the speed of replication forks. The availability of 2'-deoxynucleotides seems to be a rate-limiting factor for DNA replication during early S-phase.

Key words: Replication, Speed of replication fork, Deoxynucleotide, Chlorodeoxyuridine, Iododeoxyuridine

INTRODUCTION

The speed of replication fork movement has been studied extensively over the past three decades. Most of the relevant data were obtained using autoradiographic analyses of extended DNA fibers (Edenberg and Huberman, 1975; Liapunova, 1994). In recent years, similar experiments can be carried out through immunofluorescence detection of newly synthesised DNA with halogenated 2'-deoxythymidine analogues (Jackson and Pombo, 1998). Previously published results have shown that the average replication fork rate in mammalian cells is not constant throughout the entire synthetic period (S-phase) of the cell cycle (Housman and Huberman, 1975). In early S-phase, during which the active genes are replicated (Goldman et al., 1984; Hatton et al., 1988), the replication fork rate is 2-3 times slower than in late S-phase, when a replication of the heterochromatin sequences takes place (O’Keefe et al., 1992).

A previous biochemical study (Walters et al., 1973) has reported extremely low 2'-deoxyribonucleoside 5'-triphosphate (dNTP) pools in G1-phase, being increased just prior to the initiation of DNA synthesis and growing further throughout S-phase. Nordenskjöld et al. (1970) have shown on mice embryos that the rate of DNA synthesis is tightly correlated with the intracellular 2'-deoxyribonucleotide pools during embryonic development. In order to establish whether or not the supply of exogenous intracellular 2'-deoxynucleotides is a manner through which the slow rate of DNA replication in early S-phase can be accelerated, the cells were loaded with additional dNTPs artificially and the replication rate was assessed afterwards. When supplied with additional exogenous 2'-deoxynucleotides, replication factories were able to produce with virtually full power from the beginning of their ‘working hours.’ Our results have thus indicated that the supply of the replication machinery with 2'-deoxynucleotides apparently represents a rate-limiting factor for DNA synthesis during early S-phase.

MATERIALS AND METHODS

Cell culture and synchronisation

Human HeLa cells were cultured on circular glass coverslips derivatized with 3-amino-propyltriethoxy-silane as described (Wang et al., 1996) at 37°C in Eagle’s minimal essential medium (MEM) (Sigma, USA) containing 10% fetal calf serum (FCS), 1% glutamine, 1% penicillin, 1% streptomycin and 0.85 g l⁻¹ NaHCO₃ in an atmosphere supplemented with 5% CO₂. Unless specified otherwise, the cell cultures were synchronised at G1/S border by a double 2'-deoxythymidine (dT) block: the cells were incubated in MEM + 10% FCS containing 3 mM dT for 16 hours, then in a fresh medium for 12 hours and again for 16 hours in a medium with 3 mM dT. After 100 minutes of incubation in a fresh medium, more than 90% of the cell population started DNA synthesis. An alternative synchronisation approach was also used: a partially synchronised cell culture after one 16 hour long dT block was grown in a fresh medium for 9 hours, and then nocodazole (Sigma, USA) was added in the final concentration 50 ng ml⁻¹ for the next 3 hours. Cells in mitosis were shaken off, re-suspended in a fresh medium, and regrown on circular coverslips. After 9 hours of incubation, the culture reached the G1/S border. The
degree of synchrony was monitored by flow cytometry analysis (kindly provided by Dr M. Dostál), in which the DNA content of the synchronised cells was measured following propidium iodide staining. In an alternative approach, the synchronisation to early or late S-phase was checked on the basis of characteristic replication patterns in situ after immunocytochemical visualisation of the labelling with halogenated 2′-deoxyuridine (see e.g. O’Keefe et al., 1992). In order to achieve a higher degree of synchrony after entering S-phase, a protocol using double dT block was used.

### Hypotonic shift conditions and DNA labelling

In order to deliver the additional 2′-deoxynucleotides into the cells, a method of hypotonic shift (Koberna et al., 1999) was applied: the cells were briefly rinsed in KHB buffer (10 mM HEPES, pH 7.4, 30 mM KCl), overlaid with KHB supplemented by a 20 μM equimolar mixture of dATP, dUTP, dTTP and dCTP, incubated in a moisture chamber at 37°C for 10 minutes, washed with MEM, incubated in MEM with 10% FCS at 37°C in a CO2 incubator for 5 minutes and subsequently labelled. In a control cell population, exactly the same procedure was performed with the following exception: KHB was loaded into the cells without the nucleotide mixture. The experiments were performed at the beginning of DNA synthesis (early S-phase) and also 6 hours later (late S-phase).

In order to label the newly synthesised DNA for fluorescence imaging, the cells were incubated in a medium containing 20 μM CldU (5-chloro-2′-deoxyuridine, Sigma, USA) for 5 minutes, then IdU (5-iodo-2′-deoxyuridine, Sigma, USA) was added to the final concentration 20 μM for the next 5 minute incubation. In order to distinguish the replication from other possible effects of nucleotide incorporation such as repair of damage caused by the treatment, the cells were synchronised to G1 phase of the cell cycle and supplied with CldU and IdU. No significant fluorescence signal was observed either in situ or on extended DNA fibers stained with YOYO-1 (1,1′-[1,3-propanediyl]bis[(dimethyliminio)-3,1-propanediyl]bis[4-[(3-methyl-2(3H)-benzoxazolylidine) methyl]triiodide, Molecular Probes, USA; see e.g. Koberna et al., 1999; data not shown).

In order to measure the total amount of newly synthesised DNA, the cells were loaded with 30 nM [α-32P]2′-deoxyctydine 5′-triphosphate (3000 Ci mmol⁻¹, ICN Biomedicals, Inc., USA), which was included in the solutions loaded into the cells during the hypotonic shift. Then, the cells were incubated in MEM+10% FCS for 10 minutes in order to label the growing DNA. After labelling, the cells were chilled on ice, washed three times in cold phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 9.7 mM Na2HPO4, 1.1 mM KH2PO4, pH 7.4), resuspended in 0.2 ml of cold PBS and counted. The suspension containing 10^6 cells was precipitated with 10%TCA for 20 minutes on ice. The precipitate was filtered onto a Whatman glass fiber filter, washed twice with 10% cold TCA, then twice in ethanol, and subsequently air-dried. The level of radioactivity was determined in a Beckman LS5000TD liquid scintillation counter.

### Fluorescence visualisation of replication fork movement

DNA spreads were prepared as described (Taylor and Hozier, 1976) with small modifications: the cells grown on circular coverslips were overlaid with lysis solution (1% sarcosyl, 0.01 M EDTA, 0.01 M Tris-HCl, pH 8.0) for 30 seconds. The glass coverslip was tilted to allow the stream of the cell lyzate to run down the slide, and then air-dried. The spreads were washed in two changes of 5% ice-cold trichloroacetic acid for 10 minutes, and rinsed in two changes of 95% ethanol. For in situ replication mapping, the cells were fixed by 2% formaldehyde in PBS and permeabilised by 0.2% Triton X-100 in PBS. The labelled DNA was detected using a standard procedure (Aten et al., 1992). The samples were treated with cold 4 M HCl for 20 minutes prior to the first antibody reaction. The fluorescence was detected by a cooled CCD camera (PXL with a KAF 1400 chip, Photometrics, USA) coupled to an Olympus AX70 microscope. On fiber fluorograms, the number of 100-150 replication tracks were analysed (in each of the three independent experiments) in order to provide the mean value and the standard error in each sample.

### Results

#### Changes in replication speed during S-phase

We employed the method of fiber fluorography (Berezney et al., 2000) in order to monitor the changes in the replication fork speed in human HeLa cells through S-phase propagation. 5-chloro-2′-deoxyuridine (CldU) and 5-iodo-2′-deoxyuridine (IdU) were used as markers of the newly synthesised DNA (Aten et al., 1992) in two labelling pulses of different duration. DNA tracks produced by individual replication forks were

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**Fig. 1.** Fluorographic replication patterns. Labelling patterns of newly synthesised DNA tracks as visualised on a single DNA fiber are shown (green, DNA labelled for 10 minutes with CldU; red, 5 minutes IdU labelling). Two isolated elongating forks (arrows) and a short replicon with initiation and termination sites marked with arrowheads (A); two adjacent forks initiating (B) and terminating (C) during the labelling period. Bars, 5 μm.

**Fig. 2.** Assessment of the degree of synchrony in studied cell populations. The results of in situ mapping of the 5-bromo-2′-deoxyuridine incorporation (A, B) and flow cytometry analyses (C, D) are shown. The shape of flow cytometry histograms (cell number vs DNA content) as well as the characteristic in situ replication patterns (see e.g. O’Keefe et al., 1992) testify to the high degree of synchrony in early (A, C) and late (B, D) S-phases.
Exogenous nucleotides accelerate early replication

observed by fluorescence microscopy on the spreads of lysed cells (Fig. 1). In order to analyse the different labelling patterns in such a specimen, we accepted that the replication process is bidirectional in mammalian cells and that it is terminated exclusively at the meeting of the two adjacent forks (Edenberg and Huberman, 1975; Huberman and Tsai, 1973). Postulating this, the use of two overlapping labelling pulses enabled us to distinguish between the forks elongating for the entire labelling period (Fig. 1A) and those being initiated or terminated during this short time window (Fig. 1B,C). The complete replication units were also observed in this manner (Fig. 1C). In order to detect the changes in the average replication fork speed at different periods of S-phase, only the length of the elongating tracks was taken into account (Huberman and Tsai, 1973; Housman and Huberman, 1975). In early and late S-phases, the degree of synchrony was monitored (Fig. 2). Through employment of this approach, the average values of replication fork speed were 0.22–0.53 \( \text{m m minute}^{-1} \) (replication speed), hours after passing the G1/S border (time).

Replication speed after the addition of nucleotides

As dNTPs do not pass through the cellular membrane spontaneously, we used the method of hypotonic shift described previously (Koberna et al., 1999) in order to deliver them into the cells. This approach, in contrast to the microinjection technique, allowed us to supply the entire cell culture with a sufficient amount of nucleotides at once. The synchronous application of nucleotides was necessary in order to maintain a defined response of the whole cell culture to a short labelling pulse. Immediately after the application of the hypotonic shift, the DNA synthesis decreased to 75% of the levels in untreated cells, but these changes are fully reversible and do not affect cell viability (Koberna et al., 1999).

The hypotonic treatment decreased the level of DNA synthesis to 0.17±0.01 \( \text{µm minute}^{-1} \) or 0.37±0.02 \( \text{µm minute}^{-1} \) in early and late S-phases, respectively. When a dNTP mixture was added into the applied hypotonic solution, the replication accelerated to 0.31±0.01 \( \text{µm minute}^{-1} \) in early S-phase, while the in situ replication pattern remained unchanged (Fig. 4). On the other hand, the speed of replication in late S-phase cells remained unchanged (0.37±0.01 \( \text{µm minute}^{-1} \)), regardless of whether or not dNTPs were added (data not shown). The late S-phase replication patterns were the same in cells supplied with dNTPs and in control cells (not shown). As a standard synchronisation procedure, a double thymidine block was used. In order to exclude the possible effect of 2’- deoxythymidine on nucleotide metabolism in early S-phase, a mitotic shake-off synchronisation was also used, which produced the same results (data not shown).

In order to confirm the fluorescence microscopy observations, radioactive labelling of the newly synthesised DNA was performed. The cells were synchronised by a single 2’-deoxythymidine pulse and the total radioactivity level in the DNA precipitate was measured after labelling in early S-phase. A radioactive label was added directly into the hypotonic solution in a concentration, which could not significantly affect the intracellular nucleotide pools (see Materials and Methods). Again, the cell population loaded with additional nucleotides

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**Fig. 3.** Measurement of the average replication speed during S-phase. Changes in replication speed during different periods of S-phase. In order to assess the speed of a single replication fork, the parts labelled with green, but not red fluorescence on the tracks produced by a single elongating fork were measured. These represent the product of 5 minutes of continuous DNA synthesis. Units: \( \text{µm minute}^{-1} \) (replication speed), hours after passing the G1/S border (time).

**Fig. 4.** The effect of deoxyribonucleotides supply on the replication speed. Early S-phase cells after the loading of the hypotonic solution without (A,C) or with (B,D) the equimolar mixture of deoxyribonucleotide triphosphates. In order to quantify the acceleration of the replication fork movement, only the tracks being elongated for the whole labelling period were analyzed on DNA spreads (A,B). Replication patterns in situ, typical for early S-phase, were also detected. As the fluorescence signals in both channels overlap almost completely, only the CldU channel is shown in C and D. Bars, 10 \( \text{µm} \).
showed a significantly higher level of DNA synthesis (145%) in comparison with the control, despite the fact that the specific activity of the probe was reduced while additional exogenous ‘cold’ dNTPs were loaded into the cells.

DISCUSSION

Using fiber fluorography, we found that the replication speed increases by a factor of about two to three from early to late S-phase. The high spatial resolution of this method (in comparison to autoradiography) enables an accurate analysis of replication patterns even after a sequence of short labelling pulses. Our results were in agreement with the previously published autoradiographic results of Housman and Huberman (1975).

In this study, we showed that the addition of 2'-deoxyribonucleotides accelerated the replication process in the early, but not in the late S-phase cells. However, the possibility cannot be excluded that the appearance of the replication tracks after the nucleotide addition during early S-phase could also indicate a premature activation of late replicating DNA sequences, which are known to be replicate more rapidly. Since the essays used were not sequence-specific, the late-replicating DNA sequences could not be distinguished in fiber fluorograms. Though we cannot rule out this possibility, we consider it unlikely. The cells in the early and late S-phases are easily identified on the basis of their characteristic in situ replication patterns (O'Keefe et al., 1992). The in situ replication pattern after the stimulation with additional nucleotides was fully compatible with that of the untreated early S cells and differed from the late S patterns, indicating that mainly early-replicating sequences were detected. These data, together with the results of Walters et al. (Walters et al., 1973), who reported low dNTP concentrations in the early S-phase cells and their increase through the S-phase propagation, point to the direct effect of the 2'-deoxyribonucleotide supply on the speed of replication fork movement at the beginning of S-phase.

The radioactive evaluation of the DNA synthesis and the previously published analyses of fluorescence microscopy (Koberna et al., 1999) clearly show that additional exogenous dNTPs were introduced into cells. Keeping this in mind, we need to ask whether the increased speed of the replication forks is directly related to the increased pool of dNTPs. It is not likely because it is well known that pools of 2'-deoxyribonucleoside 5'-triphosphates are tightly regulated in cultured cells (e.g. Reichard, 1985; Oliver et al., 1996). The imbalance in dNTP pools precedes the down-regulation of enzymes controlling the de novo and salvage pathways of dNTP synthesis, ribonucleotide reductase and 2'-deoxythymidine kinase, respectively. Indeed, we could not establish any increase in the dNTP pools after 10 minutes of treatment (unpublished observations). Thus, the rapid restoration of normal dNTP pools is of primary relevance. At most, the increase of the dNTP pool might correspond to the ‘pulse’ increased pool lasting a short time only, with dNTPs being rapidly metabolised to their precursors. We are of the opinion that the accelerated speed of the replication fork is the response to the enhanced pools of dNTP metabolic pathway rather than just the pool of dNTPs.

It is tempting to speculate that the onset of replication occurs prior to the necessary amount of nucleotides being available for DNA synthesis and active gene sequences, enriched in euchromatin, are thus replicated slowly. The question can be raised as to whether or not this indicates a deeper developmental reason – a selective advantage of those cells, which conserve this most important part of the genetic information carefully, with a better chance of repairing possible errors.

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