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Visualization of bidirectional initiation of chromosomal DNA replication in a human cell free system

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ABSTRACT

Initiation of DNA replication is tightly controlled during the cell cycle to maintain genome integrity. In order to directly study this control we have previously established a cell-free system from human cells that initiates semi-conservative DNA replication. Template nuclei are isolated from cells synchronized in late G_1 phase by mimosine. We have now used DNA combing to investigate initiation and further progression of DNA replication forks in this human *in vitro* system at single molecule level. We obtained direct evidence for bidirectional initiation of divergently moving replication forks *in vitro*. We assessed quantitatively replication fork initiation patterns, fork movement rates and overall fork density. Individual replication forks progress at highly heterogeneous rates (304 ± 162 bp/min) and the two forks emanating from a single origin progress independently from each other. Fork progression rates also change at the single fork level, suggesting that replication fork stalling occurs. DNA combing provides a powerful approach to analyse dynamics of human DNA replication *in vitro*.

INTRODUCTION

Replication of eukaryotic DNA is tightly controlled to maintain genome integrity during the proliferative cell division cycle. During S phase, chromosomal DNA replication occurs at discrete sites in the cell nucleus called replication foci (1,2). On average, the chromosomal DNA present in each replication focus contains between one to ten individual replicons [reviewed in (3)]. Each replicon encompasses between 30 to 450 kb or more of DNA, each of which is replicated from its internal origin (3). The replicons present in one replication focus are often clustered and become activated in a coordinated manner. Activation of the earliest firing origins therefore leads to the activation of the first cohort of replication foci, thus marking the onset of S phase. Individual replication foci are active for about 45–60 min (1,4–6). Activation of new foci during S phase is asynchronous and occurs throughout the remainder of S phase (4,5,7,8).

A key step in the control of the progression of the cell division cycle, therefore, is the initiation of chromosomal DNA replication at the G_1 to S phase transition. In order to study this control at a molecular level directly, we have established and refined in the past years a human cell-free system that initiates chromosomal DNA replication under cell cycle control in an origin-specific manner (9–11). This system uses template nuclei, which are isolated from human cells synchronized in the late G_1 phase of the cell division cycle by the iron-chelating compound mimosine (12). Chromosomal DNA replication in these nuclei, which are licensed and competent to replicate, is initiated upon addition of a cytosolic extract from actively proliferating human cells, containing essential soluble initiation factors (10,13), and is under the additional control of cyclin-dependent protein kinases (10,14). Initiation results in the formation of DNA replication foci, which have patterns typical for very early S phase. Sites of nascent DNA in the template nuclei have been found to coincide with early-firing DNA replication origins, such as the lamin B2 origin, but not with later replicating domains such as the ribosomal DNA cluster (11).

The evidence in support for initiation of chromosomal DNA replication in this human system is derived from indirect fluorescence microscopy operating on a per-nucleus basis. Percentages of replicating nuclei are scored after incubation in a replication elongation buffer, which is supplemented with a cytosolic extract as a source for initiation factors (9,10,13). Negative controls consist of identical incubations performed in the absence of the cytosolic extract, resulting in the detection

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of the proportion of contaminating S phase nuclei that support run-on replication at pre-existing DNA replication forks (10,12,15). The difference between these two values is calculated as the proportion of nuclei that initiate chromosomal DNA replication *in vitro*. Importantly, a visualization at molecular resolution of the initiation step of bidirectional chromosomal DNA replication forks *in vitro* has not been reported to date.

The firing of replication origins and the further progression of replication forks can be visualized at single molecule level by molecular combing and fluorescence microscopy. In this technique, nascent DNA is first labelled *in situ* by incorporation of modified nucleotides. Individual DNA fibres are then isolated and stretched uniformly at 2 kb per μm in a parallel orientation on glass coverslips (16,17), and the labelled DNA replication tracts are visualized by fluorescent microscopy (18,19). Initiation of DNA replication forks, and their direction and rate of progression can be determined by the consecutive use of two differently modified nucleotide analogues. This approach has been successfully applied to the study of replication dynamics in extracts of *Xenopus laevis* eggs *in vitro* (18–20) and in *Escherichia coli*, yeast and mammalian cells *in vivo* (21–25).

We have therefore adopted DNA combing to characterize the initiation and further progression of replication forks on human chromosomal DNA fibres *in vitro*. We provide direct evidence for an *in vitro* initiation of bidirectional DNA replication forks in late G_1 phase template nuclei upon incubation in cytosolic extract. After initiation, individual replication forks within one replicon progress at heterogeneous rates, indicating that they are uncoupled from each other.

MATERIALS AND METHODS

Cell culture and synchronization

Human EJ30 cells were cultured as monolayers and synchronized in late G_1 by adding 0.5 mM mimosine to proliferating cells for 24 h (12), and in S phase by releasing cells after a 24 h-treatment with 2 mM thymidine into fresh medium for 2 h, as described previously (9).

Cell synchronization was monitored by flow cytometry of isolated nuclei. One million nuclei were directly stained with propidium iodide [5 μg/ml in phosphate-buffered saline (PBS) containing 0.4% Triton X-100] and analysed by FACScan (Becton Dickinson) using the Lysis II-software. Data are presented as histograms showing relative DNA content (*x*-axis) and cell number (*y*-axis).

DNA synthesis reactions and analysis of reaction products

Template nuclei and cytosolic extract were prepared exactly as described previously (9,10). Standard DNA replication initiation reactions contained the following components: cytosolic extract from asynchronously proliferating HeLa cells (100 μg of protein), a buffered mix of rNTPs and dNTPs and 2×10^5 nuclei from synchronized EJ30 cells (10). Nascent DNA was labelled by addition of 5 μM digoxygenin-11-dUTP and/or 25 μM biotin-16-dUTP (Roche) at the indicated times. The final reaction volume was adjusted to 50 μl with replication buffer [20 mM K-HEPES (pH 7.8); 100 mM K-acetate; 1 mM $MgCl_2$ and 0.1 mM DTT]. Incubation time was 3 h.

Proportions of replicating nuclei were determined by fluorescence confocal microscopy exactly as described previously (10,13). For a preparation of chromosomal DNA fibres for DNA combing, the standard reactions were scaled-up 4-fold to a final volume of 200 μl. The nuclei were sedimented after the reaction and resuspended in 0.5% low-melting point (LMP) agarose in PBS at 37°C and encapsulated in solid LMP-agar blocs on ice. LMP-agar blocs were incubated two times in ESPK solution (0.5 M EDTA, 1% sarcosyl and 2 mg/ml proteinase K) at 55°C for 24 h, two times in TE buffer [10 mM Tris-Cl (pH 8) and 1 mM EDTA] at 55°C for 1 h and stored in 0.5 M EDTA at 4°C until analysis by molecular combing.

Molecular combing and detection by fluorescent antibodies

DNA was combed on silanised glass coverslips as described (19). Biotin was detected with TexasRed-conjugated avidin (Vector Laboratories, diluted 1:50), followed by two layers of alternating anti-avidin antibodies (Vector Laboratories, diluted 1:50) and TexasRed-conjugated avidin. For detection of the digoxigenin label, a mouse FITC-conjugated anti-digoxigenin antibody was used (Jackson Laboratories, diluted 1:25), followed by Alexa Fluor 488 conjugated rabbit anti-mouse and goat anti-rabbit antibodies (Molecular Probes, diluted 1:50). Total DNA was counterstained with YOYO-1 (Molecular Probes) at 1:10 000 after antibody incubations and washed three times with PBS before mounting in Vectashield (Vector Laboratories).

RESULTS

Initiation of chromosomal DNA replication *in vitro*

To study initiation of chromosomal DNA replication forks *in vitro* on single DNA fibres, we first had to achieve an efficient synchronization of human cells in G_1 phase with as little as possible contaminations of S phase cells. Treatment of asynchronously proliferating human cells for 24 h with a 0.5 mM concentration of the plant amino acid mimosine arrests them reversibly in late G_1 phase (12,26). Treatment with 2 mM thymidine inhibits DNA replication fork progression *in vivo*, and a release of thymidine-treated cells for 2 h results in a population of S phase cells (9). Using these treatments, we synchronized human EJ30 cells in late G_1 phase and in S phase of the cell division cycle and confirmed successful synchronization by flow cytometry (Figure 1A).

The presence of DNA replication forks in nuclei isolated from these synchronized cells was determined using nuclear run-on replication assays *in vitro*. Incubation of template nuclei in a buffer containing ribo- and deoxyribonucleotide triphosphates including biotinylated and/or digoxigenylated dUTP leads to elongation of DNA replication and labelling of nascent DNA with the modified dUTP at existing forks formed *in vivo*, before isolation of the nuclei (12). Less than 1% of the G_1 phase nuclei contained active replication forks, whereas about 90% of the control S phase nuclei did (Figure 1B). Addition of a cytosolic extract from

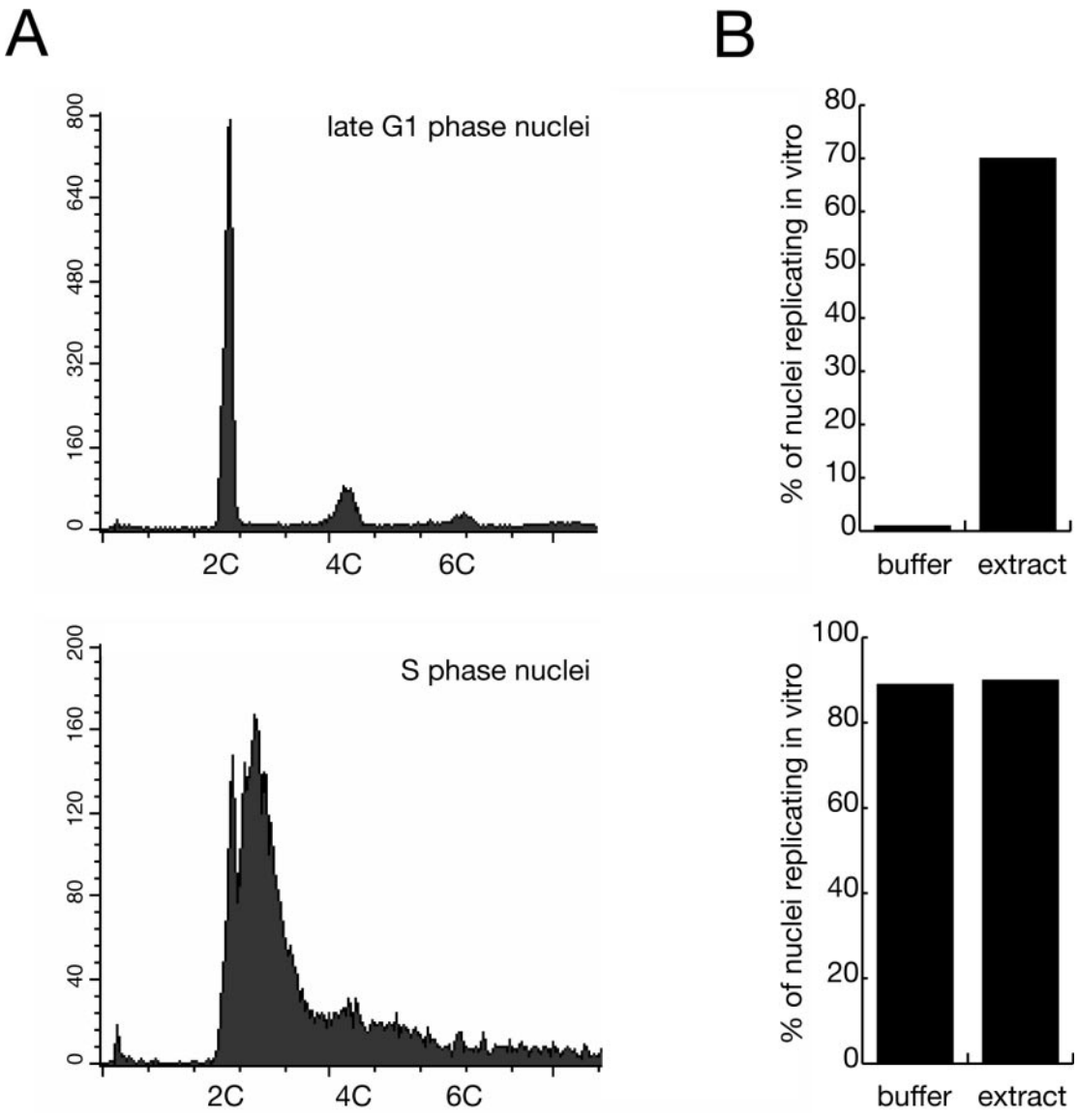

Figure 1. Replication of chromosomal DNA in G_1 and S phase nuclei. (**A**) Control of cell synchronization. Human EJ30 cells were synchronized in late G_1 phase and in S phase. The DNA content of isolated nuclei was determined by flow cytometry. Relative positions of diploid (2C) and polyploid genomic DNA content (4C and 6C) are indicated. (**B**) Determination of the proportion of replicating nuclei *in vitro*. Isolated nuclei from late G_1 phase and S phase cells were incubated in elongation buffer to label nuclei containing replication forks established *in vivo* at the time of preparation [buffer, see: (12)]. To visualize nuclei initiating chromosomal DNA replication, these nuclei were also incubated in elongation buffer supplemented with cytosolic extract *in vitro* [extract, see: (10)]. The proportions of replicating nuclei were determined by confocal microscopy.

asynchronously proliferating cells triggers initiation of new DNA replication forks in late G_1 phase template nuclei *in vitro* (10,11,13,27). Consistent with these reports, we found that up to 70% of the late G_1 phase nuclei initiated chromosomal DNA replication upon addition of cytosolic extract, whereas the proportion of replicating control S phase nuclei essentially remained constant (Figure 1B). Therefore, more than 98% of the late G_1 phase nuclei from EJ30 cells replicating in the presence of cytosolic extract have initiated chromosomal DNA replication *in vitro*, whereas <2% are S phase contaminants. We conclude that these template nuclei provide a suitable signal-to-noise ratio to allow single fibre analysis of initiation and further progression of DNA replication forks *in vitro*.

Visualization of DNA replication foci *in vitro*

To allow visualization of DNA replication fork progression on single DNA fibres, we used an experimental approach of consecutively adding two distinct labels (Figure 2A). Digoxigenylated dUTP was added at the beginning of the incubation and biotinylated dUTP was added after 1 h. Therefore, DNA replicated during the first hour of the incubation only contains digoxigenin, which is detected by a green fluorescent probe. DNA replicated during the following 2 h contains both biotin, which is detected by a red fluorescent probe, and digoxigenin. It is thus rendered yellow because of the overlap of green and red signals (Figure 2A).

We first visualized incorporation of these two labels into their physiological intranuclear sites. All G_1 phase nuclei

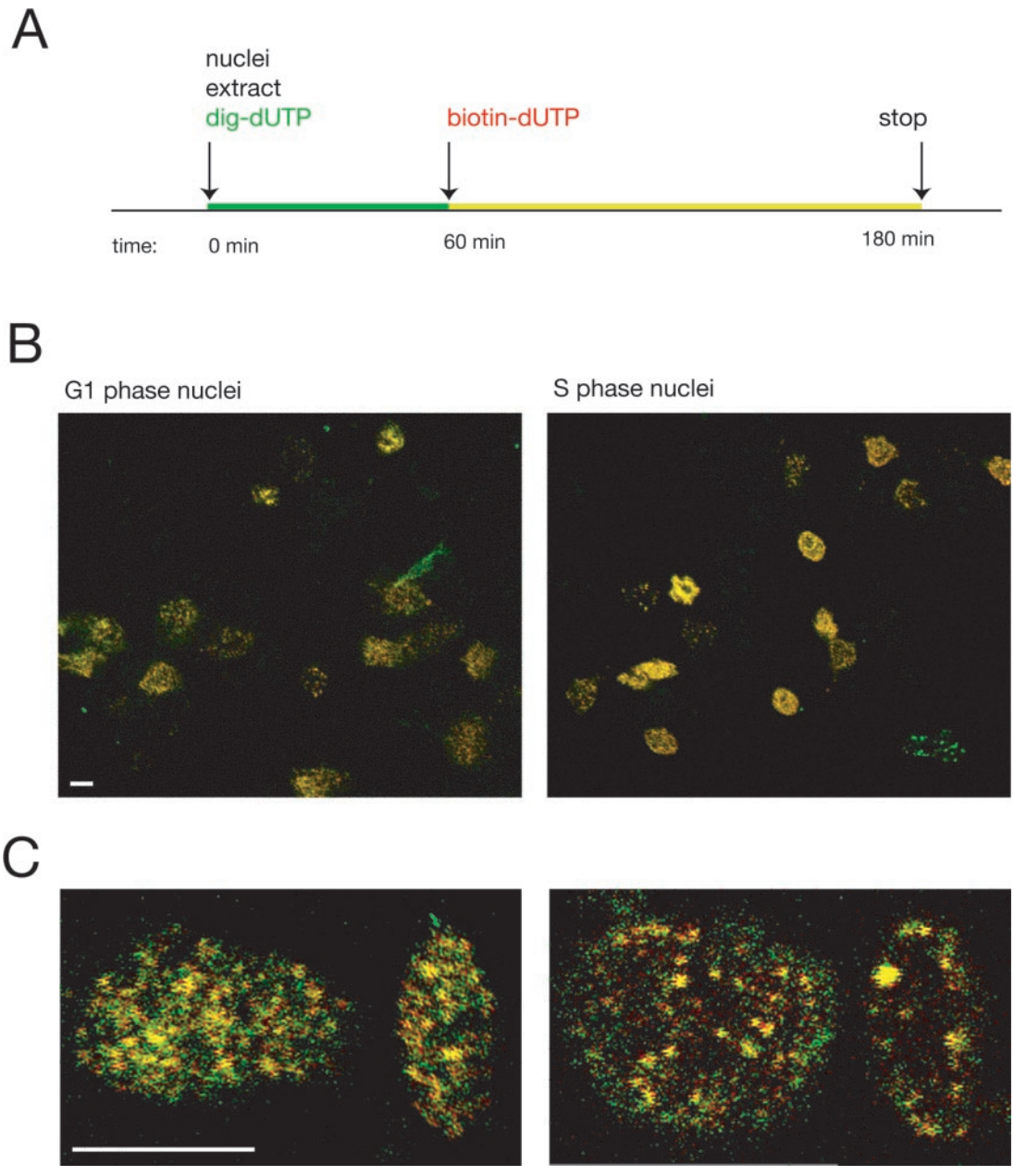

Figure 2. Visualization of replication focus dynamics in late G_1 and S phase template nuclei *in vitro*. (**A**) Labelling protocol for nascent DNA. Template nuclei, cytosolic extract, digoxygenin-dUTP (dig-dUTP) and biotin-dUTP (bio-dUTP) were added at the indicated times and the reaction was stopped after 180 min. (**B** and **C**) Intranuclear sites of replicated DNA were detected by fluorescence confocal microscopy. Incorporation of dig-UTP into nascent chromosomal DNA was detected by fluorescein isothiocyanate (FITC)-conjugated anti-digoxygenin F_{ab} fragments (green), and of bio-dUTP by TexasRed-conjugated streptavidin (red). Merged images of the red and green channels are presented showing co-localization in yellow. (B) Representative overview fields of replicating nuclei. (C) Visualization of individual DNA replication foci in representative nuclei. Scale bars, 5 μm.

that initiated chromosomal DNA replication in the extract contained patterns of early replication foci incorporating both labels (Figure 2B). Because initiation in late G_1 phase nuclei occurs already after a few minutes of the incubation (10,27), we conclude that all nuclei that have initiated replication during the first hour of the incubation continue to replicate in the subsequent 2 h. By comparison, S phase nuclei also incorporated both labels, but in patterns of replication foci typical for early, mid and late S phase [Figure 2B, see reference (2)], consistent with their synchronization profile (cf. Figure 1A). In addition, less than 2% of the S phase nuclei terminated replication during the first hour of the incubation with a typical late S phase pattern of a few heterochromatic replication foci [Figure 2B, green nucleus at bottom right of S phase panel, see reference (2)], indicating that they have exited S phase and entered G_2 phase *in vitro*.

When viewed at higher magnification, >95% of individual replication foci that were either initiated in late G_1 phase nuclei or maintained in S phase nuclei incorporated both labels, indicating that they remain active *in vitro* for more than 1 h (Figure 2C and data not shown).

These nuclei therefore appeared suitable for single molecule analysis of DNA replication initiation and elongation in this cell-free system.

Visualization of DNA replication fork progression by molecular combing

Using the protocol outlined in Figure 2A, we labelled replicating chromosomal DNA *in vitro*, and used DNA combing to reproducibly stretch individual DNA molecules in a linear, parallel fashion with a constant stretching factor (1 kb = 2 μm). Replicated DNA tracks were then detected on combed DNA molecules with appropriate fluorescent probes. The expected labelling patterns indicating initiation, elongation and termination events of uni- and bidirectionally moving DNA replication forks are shown schematically in Figure 3A.

Using G_1 phase nuclei as templates, we detected well defined patterns of replicated DNA tracks indicating initiation of two divergently moving bidirectional forks during the first hour of the incubation (Figure 3B, pattern a). We also detected patterns of replication forks suggesting initiation after 1 h of incubation (Figure 3B, pattern b). Pattern c may result either from unidirectional movement of a fork initiated *in vivo*, initiation of only one unidirectionally moving fork *in vitro*, or from bidirectional initiation *in vitro* where one fork was immobile or stopped progression during the first hour of the incubation. Finally, we also observed termination patterns of two convergent forks after 1 h (Figure 3B, pattern d), or termination or fork stalling patterns during the first hour of the incubation (Figure 3B, pattern e). The latter pattern could in principle also arise from both initiation and arrest of two diverging forks before 60 min. Identical patterns were detected on combed chromosomal DNA from S phase nuclei (data not shown). Note that patterns b, c and e may also be interpreted as breakage of fibres which originally contained pattern a.

In order to interpret these replication fork patterns quantitatively, we compared the frequency of each type of pattern found in G_1 and S phase template nuclei incubated in cytosolic extract (Table 1). To exclude problems arising from breakage of digoxigenylated DNA fibres, we counterstained the whole DNA fibres after combing with YOYO-1 and restricted our quantitative analysis to replication tracks on unbroken fibres. Initiation and bidirectional divergent fork movement within one continuous track (pattern a) was observed at an average frequency of 19.8% of replication patterns in initiating late G_1 phase nuclei, but only at a frequency of 3% in S phase nuclei. Late initiation pattern b were seen at a frequency of 25.6% in late G_1 nuclei, but only at 14.4% in S phase nuclei. These pattern b could also be explained, in principle, by a restart of a collapsed replication fork after 1 h of incubation. However, when we added the second label already at 30 min instead of 60 min, we found more pattern b (34.5% at 30 min compared to 25.6% at 60 min), at the expense of fewer bidirectional pattern a (7.0% at 30 min compared to 19.8% at 60 min), strongly suggesting that pattern b represent true late initiation events. Taken together, and given the very small contamination of G_1 nuclei with S phase nuclei (Figure 1B, and see below), we conclude that at least 45.4% of all replication patterns in late G_1 phase nuclei represent true initiation events that have taken place *in vitro*, as opposed to 17.4% in S phase nuclei. This result very strongly supports at the single molecule level the conclusion that late G_1 phase template nuclei efficiently initiate chromosomal DNA replication in this *in vitro* system.

In addition, we observed a major population of pattern c showing unidirectional fork movement. This population was represented in both initiating G_1 phase nuclei and elongating S phase nuclei at high frequencies of 53.9 and 51.4%, respectively (Table 1). We observed only 0.7% of termination events in late G_1 phase nuclei, whereas S phase nuclei displayed a total of 31.2% (Table 1, patterns d and e). This latter observation allows us to estimate that the extent of contamination of replication tracks from G_1 phase nuclei with tracks from contaminating S phase nuclei is not >2%. Therefore, the vast majority of unidirectional fork movements detected in G_1 phase nuclei cannot be explained by elongating structures derived from S phase contaminations. They may arise from unidirectional initiation events in G_1 phase nuclei, or from bidirectional initiation events where one fork has stalled before the addition of the second label.

We conclude from these experiments that late G_1 phase template nuclei predominantly initiate and further elongate new DNA replication forks *in vitro*, whereas S phase nuclei predominantly elongate and terminate replication *in vitro* at existing forks that had been established *in vivo*.

Localization of DNA breaks

Previous experiments have shown that nuclei from cells arrested in late G_1 phase by mimosine contain DNA double strand breaks (27). Furthermore, chromosomal areas of damaged DNA marked by phosphorylation of histone H2AX overlapped with intranuclear sites where replication initiated when these nuclei were used as templates *in vitro* (27). We therefore tried to localize DNA strand breaks on combed DNA in the vicinity of DNA replication tracks. Unreplicated DNA fibres were visualized by counterstaining with the dye YOYO-1 after combing. We found that 84% of replication tracks contained a double strand break within the labelled track. We observed double strand breaks at the ends of the digoxigenin signal where DNA replication has begun only in a very few cases. In contrast, the vast majority of DNA breaks was located at the growing end of replication forks incorporating both biotin and digoxigenin signals (Figure 4). These broken structures may be due to collapsed forks that have encountered a DNA single or double strand break that has arisen as consequence of mimosine treatment *in vivo* (27). In support of this hypothesis, we observed 26% more DNA breaks within replication tracks in the DNA preparation from mimosine-treated G_1 phase cells than from thymidine-treated S phase cells. The distance between the start of the replication track and the break at the growing end varied greatly between a few up to more than a 100 kb (data not shown). These data suggest that DNA breaks caused by the mimosine treatment *in vivo* may be located some significant distance away from activated origins, but within the activated replicon. However, we cannot exclude the trivial possibility that many breaks occur mechanically at replication forks during sample preparation or DNA combing. We have previously observed that combed DNA containing stalled or ongoing replication

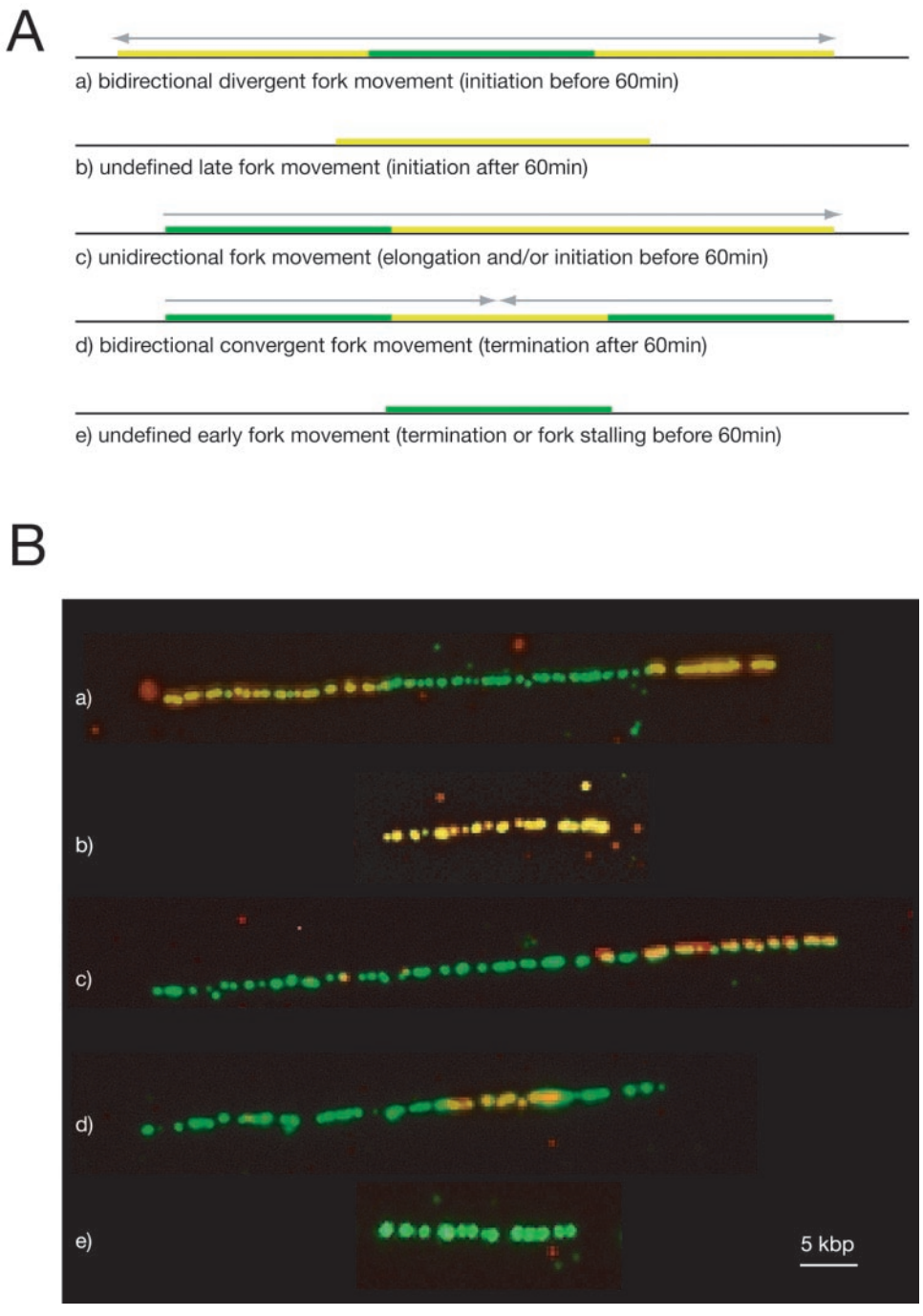

Figure 3. Visualization of replication fork movement on single human chromosomal DNA fibres by molecular combing. (**A**) Expected labelling patterns of DNA replication fork movements on single DNA fibres. DNA replicated during the first 60 min interval of the incubation outlined in Figure 2A (dig-dUTP incorporation only) is highlighted in green and DNA replicated during the second 60–180 min interval (dig-dUTP and bio-dUTP) is highlighted in yellow (i.e. merge of green and red signal). The directionality of defined replication fork progression is indicated by grey arrows. (**B**) Examples of actual labelling patterns observed by molecular combing. Incorporation of dig-UTP into nascent chromosomal DNA is detected by FITC-conjugated anti-dig antibody followed by Alexa488-conjugated secondary and tertiary antibodies (green signal), and incorporation of bio-dUTP is detected by TexasRed-conjugated avidin followed by two alternating layers of anti-avidin antibodies and TexasRed-conjugated avidin (red signal).

forks is broken into smaller fibres than unreplicated or fully replicated DNA (unpublished data).

We conclude that DNA breaks resulting from mimosine treatment (27) do not prevent initiation of DNA replication, but may interfere with fork progression *in vitro*.

Origin spacing and replication fork density

Replication origins in mammalian cell nuclei are spaced several hundred kilobases apart, but tend to be activated in synchronous clusters (3,24,28). To investigate the spacing of activated chromosomal DNA replication origins in human

Table 1. Quantification of DNA replication fork progression patterns

Replication fork pattern	G_1 phase nuclei (%)	S phase nuclei (%)
Initiation and bidirectional divergent fork progression (pattern a)	19.8	3.0
Late initiation and undefined fork progression (pattern b)	25.6	14.4
Unidirectional fork progression (pattern c)	53.9	51.4
Terminations (pattern d)	0.35	29.7
Terminations or stalling (pattern e)	0.35	1.5

Mean values of two independent experiments are shown.
More than $n = 60$ replication tracks were scored per experiment.

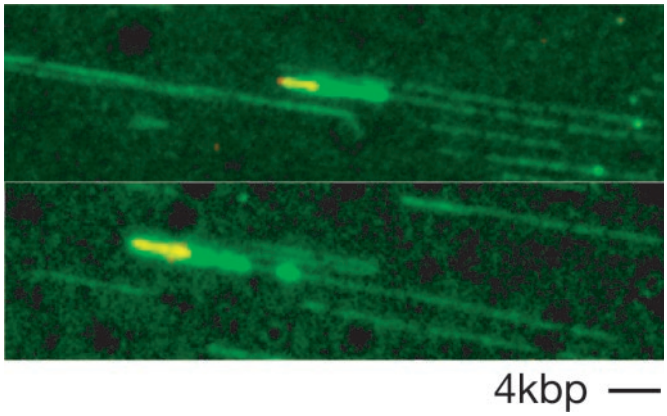

Figure 4. Visualization of DNA breaks in the vicinity of replication tracks. Total DNA was visualized by staining with YOYO-1 (faint green) and replicated DNA was visualized as detailed in the legend to Figure 3. Two representative fields are shown. Note that DNA breaks are observed at the growing yellow end of a replication track, but not at the green end where replication *in vitro* initiated.

late G_1 phase nuclei *in vitro*, we counterstained whole DNA fibres with YOYO-1 after combing and determined the average inter-origin spacing. Even though we were able to observe long unbroken fibres up to 450 kb, we were not able to detect two or more origins of bidirectional replication on the same DNA fibre.

In order to determine the overall fork density by molecular combing, we measured the length of replicated and unreplicated DNA and counted the number of replication forks (Table 2). We calculated the extent of replicated chromosomal DNA in late G_1 phase template nuclei after the 180 min incubation *in vitro* as 0.67% and derived an average replication fork density of one per 1858 kb (Table 2). This value is consistent with, albeit slightly below values previously obtained by [^{32}P]dATP incorporation experiments (10). In comparison, we found that ~12 times more DNA was synthesized in the same time interval in S phase template nuclei *in vitro*, which was due to a higher fork density, but not to higher fork speeds (Tables 2 and 3). These relative fork densities may also explain why so few termination events of two converging replication forks are detected in G_1 compared to S phase nuclei (cf. Table 1).

Table 2. Extent of DNA replication in G_1 and S phase template nuclei *in vitro*

	G_1 phase nuclei	S phase nuclei
Total length of measured DNA	11 149 kb	7975 kb
Replicated DNA	74.6 kb	617.4 kb
Proportion of replicated DNA	0.67%	8.9%
Number of replication forks	6	47
Replication fork density	1/1858 kb	1/152.7 kb

Data from one representative experiment are shown.

Table 3. Quantification of DNA replication fork progression in G_1 phase and S phase template nuclei *in vitro*

	G_1 nuclei	S nuclei
Mean track length of total digoxygenin signal (±SDM) (0–180 min)	54.8 ± 29.2 kb	54.9 ± 26.1 kb
Mean track length of digoxygenin only signal (±SDM) (0–60 min)	19.4 ± 14.1 kb	17.7 ± 12.0 kb
Mean track length of biotin signal (±SDM) (60–180 min)	35.4 ± 24.1 kb	37.2 ± 21.3 kb
Average fork speed (±SDM) (0–-180 min)	304 ± 162 bp/min	305 ± 145 bp/min
Average fork speed (±SDM) (0–-60 min)	323 ± 235 bp/min	295 ± 201 bp/min
Average fork speed (±SDM) (60–180 min)	295 ± 200 bp/min	309 ± 178 bp/min

Data are normalized per single DNA replication fork on unbroken DNA fibres. Mean values and SDs of one representative experiment are shown.
A total of $n = 36$ and 41 replication tracks for G_1 and S phase templates, respectively, were analysed. Replication tracks of patterns d and e (terminations) have been removed from these statistics.

Replication fork dynamics *in vitro*

The molecular combing approach allowed us to characterize the dynamics of replication fork progression in G_1 phase template nuclei at the level of individual replicons following initiation *in vitro*. We restricted our analysis to double-labelled replication patterns, which were not broken at their ends and measured the overall track lengths of both digoxigenin and biotin signals (Table 3). Whereas an average of 54.8 kb was synthesized per replicon during the entire 180 min of the *in vitro* incubation (digoxigenin signal), 35.4 kb were synthesized during the last 120 min (biotin signal) and 19.4 kb during in the first hour. The average fork speed during the incubation remained nearly constant at 304 bp/min, ranging from 323 bp/min during the first hour of DNA synthesis per single fork to 295 bp/min during the remaining 2 h of the incubation. Note that both these values might be underestimated because replication did not necessarily initiate at the beginning, nor continue for the entire duration of the incubation. We compared next the fork speeds calculated from the two individual labelled tracks (Figure 5A). The plot shows that there is no strong correlation between the fork progression rate of the first and the second label ($R = 0.14$, $P = 0.42$). This could be due to asynchronous initiation and perturbed fork progression, or a combination of both.

However, replication was not uniform amongst different replicons *in vitro*. The standard deviation (29.2 kb) from

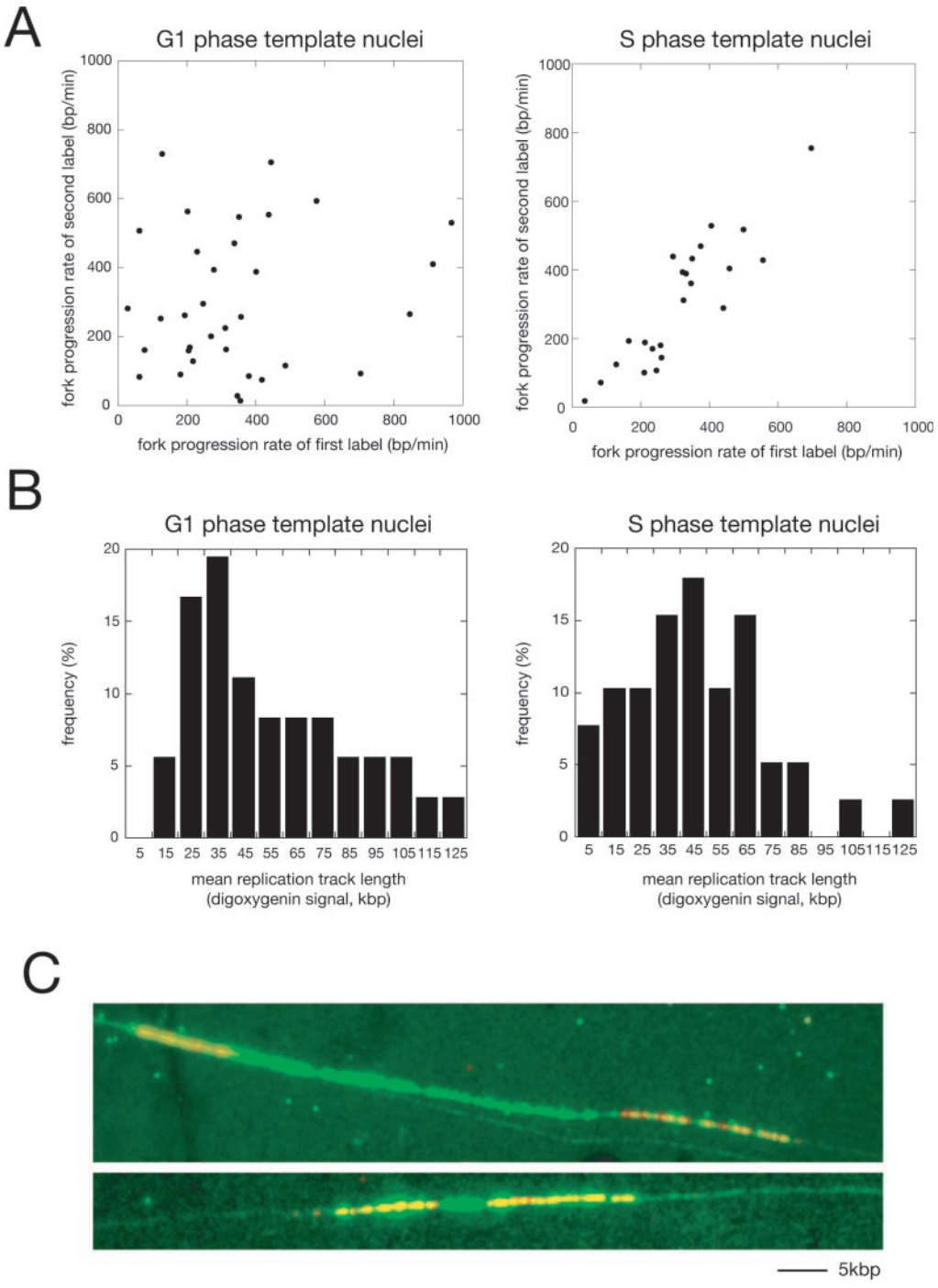

Figure 5. Heterogeneity of replication fork progression *in vitro*. (**A**) Heterogeneity of individual replication fork progression rates during the incubation *in vitro*. Progression rates of individual forks calculated from replication track lengths obtained during the first digoxigenin label and during the second biotin label were plotted against each other. Data from G_1 phase and S phase template nuclei are shown on the left and right panels as indicated. (**B**) Replication track length distribution. The lengths of all measured digoxigenin labelled DNA replication tracks were divided into 10 kb classes and their frequency of occurrence was plotted. Data from G_1 phase and S phase template nuclei are shown in the left and right panels, respectively. (**C**) Visualization of divergently moving bidirectional replication forks in G_1 phase template nuclei. Representative patterns of replicating unbroken DNA fibres counterstained with YOYO-1 (faint green) are shown. Note that asymmetric fork progression is detected by different yellow track lengths for every pattern.

the mean replication track length (54.8 kb) is very high throughout the 3 h incubation (Table 3). The ratio of standard deviation to the mean did not change during the incubation, suggesting that fork stalling does not increase *in vitro*. The distribution of individual replication track lengths of the digoxigenin signal confirms that they are very heterogeneous in size, ranging from only a few kb up to 125 kb (Figure 5B). This drastic heterogeneity can be explained by highly variable fork progression rates, by non-synchronous initiation and termination or fork stalling and by a combination of those.

In order to determine whether the variable fork rates are specific to G_1 phase nuclei prepared from mimosine-treated cells, we also analysed the replication fork dynamics in isolated S phase nuclei *in vitro*. We observed in S phase nuclei similar mean replication track lengths, fork rates and track length distributions as in G_1 phase nuclei (Table 3, Figure 5A and B). However, we found a strong positive correlation between fork rates of the first and the second label (Figure 5A; $R = 0.88$, $P = 2 \times 10^{-5}$). This strongly suggests that the replication fork rates in S phase template nuclei are not perturbed by staggered individual initiation events and fork stalling as found in G_1 phase nuclei.

In a recent report, an analysis of replicating chromosomal DNA in the prokaryote *E.coli* has demonstrated that divergent forks move independently from each other (21). We were therefore interested if replication fork progression in human cell nuclei is coordinated between two bidirectionally progressing forks within a single replicon after their initiation *in vitro*. A tight coordination of their progress rates would result in symmetric labelling patterns. To our surprise, we found that one fork had progressed to a different extent than the other on unbroken DNA fibres in almost all cases, based on the track lengths of the biotin signals in G_1 phase nuclei (Figure 5C, and data not shown). This difference in the track lengths of the biotin signals could be attributed to differences in fork speed or to asymmetric fork stalling. Taken together, we conclude that the two replication forks which were initiated from one human chromosomal origin may progress independently from each other *in vitro*.

DISCUSSION

This study represents the first detailed study of replication dynamics in a human cell-free system. It provides a quantitative whole genome view of DNA replication *in vitro* at single molecule resolution through the employment of DNA combing. The main advantage of such single molecule studies is the detection of individual and non-averaged replication events in comparison to bulk population studies.

We have differentially labelled nascent DNA *in vitro* by supplementing the *in vitro* reaction first with digoxigenin-dUTP, followed by the later addition of a second label, biotin-dUTP, to analyse spatio-temporal initiation patterns and fork progression. The main conclusions from this work are 2-fold. Firstly, we have visualized for the first time directly that this cell-free system initiates chromosomal DNA replication in human cell nuclei in a bidirectional manner. Secondly, we have shown that individual DNA replication forks emanate from activated replication origins *in vitro* with highly heterogeneous progression rates.

Direct evidence for bidirectional initiation *in vitro*

Previous studies have provided indirect evidence for *in vitro* initiation by fluorescence microscopy on a per-nucleus basis (9,10,13–15) or biochemically by bulk analysis of origin activation (11). Here, we show for the first time bidirectional DNA replication initiation events on the whole human genome *in vitro*, by using single DNA fibre analysis. We have observed uninterrupted replication tracks synthesized during the first hour of the *in vitro* incubation that are flanked on both sides by uninterrupted tracks synthesized in the subsequent 2 h (pattern a in Figure 3 and 5). Upon closer inspection, one can detect very short unlabelled sections within these tracks. These gaps are not sections of chromosomal DNA where initiation had started before the addition of label, i.e. *in vivo*, before preparation, because they occur at multiple random positions on every single replication track, whether labelled with biotin or with digoxigenin (Figures 3–5). These small gaps are therefore interpreted as discontinuities in detection rather than discontinuities in labelling, as observed previously (5,18–20,24,25). Our results therefore show that the human cell-free system initiates chromosomal DNA replication in late G_1 phase template nuclei in a bidirectional manner. We have previously established that DNA synthesis in these template nuclei *in vitro* is due to semi-conservative DNA replication (9,10) that initiates in an origin-dependent manner (11). This current study therefore completes our direct demonstration that the human cell-free initiation system recapitulates *in vitro* the fundamental aspects of human chromosomal DNA replication observed *in vivo*.

We compared the frequency of bidirectional initiation patterns in late G_1 phase nuclei to those in S phase nuclei directly. We found that the percentage of the divergent bidirectional fork patterns was 6-fold higher in late G_1 than in S phase nuclei upon incubation in cytosolic extract. In addition, we observed replication tracks that were initiated after the addition of the second label. These late initiation patterns were again more frequent in late G_1 than in S phase nuclei after incubation in cytosolic extract. Therefore, these observations provide direct evidence that late G_1 phase nuclei are valid templates for true initiation of chromosomal DNA replication in this cell-free system.

On the other hand, S phase nuclei still contain relatively low percentages of bidirectional early and of late initiation patterns, indicating that they are also templates for limited initiation events *in vitro*. Replication origins fire throughout S phase *in vivo*, only 10–15% of replicons are active at a given time (3), and initiation of new replication foci depends on the prior completion of previously active foci (8). Therefore, in principle, the cell-free system is also capable of initiating later firing origins in established S phase nuclei, albeit at reduced efficiency compared to earliest firing origins activated at the G_1 to S phase transition.

A rather surprising observation was the high percentage of apparently unidirectional forks in late G_1 phase nuclei. A small fraction of these patterns in G_1 nuclei may arise from the few S phase contaminants (<2%) present in the preparation of nuclei (cf. Figure 1). It might therefore be argued that these patterns could indicate elongation *in vitro* at existing replication forks that were initiated in nuclei from mimosine-treated cells *in vivo* and that mimosine would thus arrest the cells in early S phase and not in late G_1 phase. However, this interpretation is not supported by our nuclear run-on replication data of Figure 1, nor by the published literature on this system in which existing forks were not detected in nuclei from human cells after treatment with 0.5 mM mimosine, in contrast to nuclei from S phase cells (10–12). Therefore, the major proportion of these unidirectional replication track patterns in G_1 phase nuclei most likely result from true initiation events *in vitro*. This implies that these patterns represent either unidirectional initiation events, as has been observed *in vivo* (25),

or bidirectional initiation events, where one fork has stalled before the addition of the second label, as discussed in detail below.

Replication fork progression *in vitro*

We determined an average replication fork speed of about 300 bp/min in human cell nuclei *in vitro*. This value is about 2–5 times lower than fork speeds determined by DNA fibre analysis in human cells *in vivo* (5,25). We observed a high degree of variability of individual DNA replication fork speeds *in vitro*, ranging from a few nucleotides/min up to 1 kb/min. Replication fork speeds varied to a similar extent also in human cells *in vivo*, within the defined region of ribosomal RNA genes, and between different parts of the genome (25). We therefore conclude that our cell-free system reflects an inherent heterogeneity of replication fork speed found in mammalian cells *in vivo*, rather than inducing an artefactual variation experimentally.

A recent study in rodent cells showed a dependence of DNA replication fork speed and origin usage on the concentration of available nucleotide pools (24). In our *in vitro* experiments nucleotide pools are set at an optimal concentration required for DNA synthesis (9,10). However, the standard protein concentration used in our *in vitro* experiments is significantly below the estimated protein concentrations found in intact nuclei *in vivo*. We therefore suggest that lower average fork speeds observed *in vitro* may be due to the diluted supply of DNA replication factors, rather than to sub-optimal nucleotide pools.

Although the average fork speed did not change significantly during the incubation period, a direct comparison of the two neighbouring tracks of one single fork (i.e. digoxigenin only and biotin) revealed that the progression rate of an individual fork was not constant throughout the 3 h time course of a standard *in vitro* reaction. In the extreme case, this can be explained by asynchronous, staggered initiation at one end and fork stalling at the other. Asynchronous initiation is directly supported by the visualization of two kinds of replication tracks: one that was initiated within the first hour of the incubation (cf. pattern a in Figure 3) and one that was initiated after 1 h (cf. pattern b in Figure 3). In further support of asynchronous initiation, an earlier addition of the second label increased the proportion of type b pattern whilst decreasing the proportion of the bidirectional pattern a. Replication fork stalling is supported by our observation that most bidirectional forks on unbroken DNA fibres progress in an asymmetric way. Alternatively, and not mutually exclusive with this extreme case, the two replication forks emanating from a single origin might travel at different and variable rates. Therefore, the apparently unidirectional replication track patterns we observed at a high frequency (cf. pattern c in Figure 3) could present an extreme form of an asymmetric bidirectional initiation event. In this case, one fork stalled before the second label was added and the other fork continued to progress after addition of the second label. Unidirectional fork patterns have also been reported in a recent study on fork progression in the human rDNA locus *in vivo* (25), which would be consistent with our *in vitro* data.

Lastly, replication fork progression speeds may be influenced by the presence of damaged DNA templates. In our study, template nuclei were prepared from human cells synchronized at the G_1/S border by treatment with the iron chelator mimosine (12), which was shown to cause DNA strand breaks as a consequence (27). Alkylation damage and nucleotide depletion by treatment with hydroxyurea have shown to provoke fork slowing and stalling in Hela cells (29). Thus, one possible explanation for impaired DNA replication fork progression observed in the *in vitro* system could be due to DNA single and double strand breaks, which cause replication fork stalling or collapse. This explanation is supported by the higher variability of replication fork progression rates in G_1 phase nuclei than in S phase nuclei (Figure 5), and by the increased occurrence of breaks at the growing end of replication tracks in G_1 phase nuclei compared to S phase nuclei. Importantly however, the presence of these DNA strand breaks did neither prevent continuation of other replication forks on the same fibre, nor abolish initiation and further progression on unbroken DNA fibres.

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