Determination of DNA replication kinetics in synchronized human cells using a PCR-based assay

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ABSTRACT

Studies on the temporal order of DNA replication are difficult due to the lack of sensitivity of methods available for replication kinetic analysis. To overcome problems associated with the current techniques, we propose a PCR-based assay to determine the replication time of any single-copy DNA sequence in complex genomes. Human cells labeled with 5-bromodeoxyuridine (BrdU) were flow sorted, according to their DNA content, at different times after synchronous release from the G1/S phase boundary. The selective removal of newly-replicated BrdU-substituted DNA was achieved by UV light irradiation followed by S1 nuclease treatment. The timing of replication of selected DNA sequences (housekeeping, tissue-specific, and non-coding loci) was determined by polymerase chain reaction (PCR) amplification using appropriate primers. DNA sequences localized in inactive replication units allowed amplification whereas those that have replicated will not be amplified by PCR. Using this sensitive and quantitative assay the replication kinetic analysis of a number of different DNA sequences can be performed from a single sorting experiment.

INTRODUCTION

The DNA replication in eukaryotic cell nucleus is limited to a discrete portion of the cell cycle denoted as the DNA synthetic phase (S phase). During this period the entire genome must be effectively and accurately duplicated, offering the opportunity either to maintain or to alter patterns of gene expression. The replication of DNA in eukaryotes initiates at multiple sites called origins in an ordered pattern where different regions of chromosomes replicate in a characteristic temporal sequence (1–4). In mammalian chromosomes, it has been established that some regions replicate early while others are late replicating, following a somatically heritable fashion. These observations suggest that the time at which DNA synthesis initiates from each origin appears to be programmed and is reproducible from cell cycle to cell cycle (5–7).

Early work with DNA fiber autoradiographic technique and electron microscopy revealed that origins are spaced at intervals of 15–300 kb, suggesting that there must be 20,000 – 50,000 origins of replication in a mammalian genome (1,2). Initiation of DNA replication in mammals is coordinated in groups of adjacent origins termed replicons (1–3). Human chromosomes are organized into a series of replication time bands averaging 1,300 Kb each (8,9). Each band represents a cluster of 10–20 replicons, wherein all replicons in a cluster synchronously initiate and terminate DNA synthesis (1,2,10). The understanding of the process of DNA replication initiation requires the identification and characterization of such replicons, which, in turn, necessitates techniques to easily and rapidly measure DNA replication timing.

We have developed a PCR-based assay for analyzing replication kinetics of any DNA sequence in mammalian cells. In this approach, flow cytometry has been used to sort synchronized cells, based on their DNA content, at different time intervals after release from G1/S arrest. Selective removal of newly-replicated BrdU-substituted DNA is accomplished by UV-light irradiation followed by S1 nuclease treatment, as described previously by D’Andrea et al (11). The replication kinetics of selected sequences were analyzed by PCR amplification since single-copy sequences in replication intermediates from mammalian cells are virtually undetectable by Southern hybridization. This PCR-based assay has been used here to determine the temporal order of replication of several loci in human DNA including housekeeping, tissue-specific and, non-coding DNA sequences.

MATERIAL AND METHODS

Cell culture

Two human lymphoblastoid cell lines, GM06246 [47,XX, +inv dup(15)(15q13)] and DS71 (46,XY), were cultured at 37°C in RPMI 1640 medium (BRL Gibco) supplemented with 10% fetal calf serum (FCS, Hyclone). For cell cycle blocking experiments, confluent cells were seeded at roughly $3 \times 10^5$ cells/ml. Following a 14–16h incubation with the DNA synthesis inhibitor ciklopirox olamine (CPX, Sigma) (12) at a final concentration of 12μM, 2μM fluorodeoxyuridine (FrdU, Sigma) and 25μM

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5-bromodeoxyuridine (BrdU, Sigma) were added. After an additional 15 min of incubation, the cells were harvested by centrifugation and resuspended in fresh drug-free RPMI 1640 medium containing 10% dialysed FCS and 25 mM BrdU for release experiments.

Human peripheral blood lymphocytes from healthy normal individuals were isolated by Ficol-Hypaque (Pharmacia) gradient density centrifugation. The lymphocytes were stimulated by the addition of phytohemagglutinin (1 mg/ml, Sigma), treated with 10 mM CPX, cultured in drug-free medium and released from inhibitor as described previously (12, 13).

At the end of the release period, cells were stained with a combination of ethidium bromide (EB) and Hoechst 33258 (HO258) following the procedure developed by Kubbies and Rabinovitch (14). Briefly, cells were resuspended in the staining buffer (0.1 ml/10^6 cells) containing: 0.1M Tris—HCl pH 7.5, 146 mM NaCl, 0.2% FCS, 1 mM CaCl_2, 0.5 mM MgCl_2, 0.1% Nonidet P-40, 50 µg/ml RNAse A, 2.4 µg/ml HO258 and 5 µg/ml EB. After 2h incubation on ice, the samples were subjected to flow cytometric analysis.

**Flow cytometry and extraction of high molecular weight DNA**

Bivariate flow cytometric analysis was performed with a Becton-Dickinson dual-laser beam FACS IV instrument operating at the combined 351–364-nm line (to excite HO258 fluorescence) and with a 5-W argon ion laser (Innova 90-5) operating at 488 nm (to excite EB fluorescence). Cells (0.2–1×10^6) were flow-sorted, based on their BrdU-DNA content (14), at different time intervals after release from the G1/S phase boundary.

Cells from each flow sorted fraction were centrifuged, resuspended in extraction buffer (50 mM Tris—HCl pH 7.8, 150 mM EDTA, 0.75% SDS with 200 µg/ml proteinase K) and incubated 16h at 37°C. The lysates were extracted three times with one volume of phenol and twice with chloroform. The DNA so treated was dialysed 16h at 4°C in 1× TE. The concentration of DNA in each samples was determined by the plastic wrap/spot method (15).

**UV-irradiation and S1 nuclease treatment**

DNA isolated from flow-sorted fractions were added at a concentration of 5 µg/ml to the nicking solution: 100 mM Tris—HCl, pH 7.6, 100 mM NaCl, 20 µg/ml yeast tRNA, 20 µg/ml HO258, and 50 µg/ml PBS-VII as internal control (described below). A brief treatment with the fluorescent dye HO258, greatly increases the photosensitivity of BrdU-containing DNA (16). Fifty µl (250 ng DNA) was transferred to a quartz cuvette (Coleman) and positioned directly at the surface of a 366 nm UV hand lamp (BLAK-RAY UVL-21, UVP inc.) and irradiated with a UV dose of 8.0×10^4 erg/mm^2. The lamp was calibrated with a UV radiometer (Blak-Ray, J-221, UVP inc.). After irradiation, the DNA solution was transferred to a microcentrifuge tube, the volume was completed to 100 µl with 1×TE buffer and extracted once with one volume of chloroform:isooamyl alcohol (24:1 vol/vol). The DNA was then ethanol precipitated in presence of 40 µg/ml of glycogen (Boehringer Mannheim) as a carrier, washed with 75% ethanol, air dried and resuspended in 25 µl of S1 nuclease buffer (30 mM Sodium Acetate pH 4.8, 50 mM NaCl and 1 mM ZnCl_2). Twenty units of S1 nuclease (BRL) were added, and digestion carried out at 37°C for 5 min. The reaction was terminated by addition of 75 µl of 25 mM EDTA, followed by two phenol/chloroform and one chloroform:isooamyl alcohol (24:1 vol/vol) extractions. The DNA so treated was ethanol precipitated in presence of 40 µg/ml glycogen, washed with 75% ethanol, air dried, resuspended in 50 µl of 1× TE, and stored at 4°C shielded from light.

**PCR amplification**

PCR reactions were carried in a total volume of 40 µl containing 20 ng of flow-sorted DNA, 0.4 µM each primer and 1 U Taq DNA polymerase (Perkin-Elmer/Cetus) in 10 mM Tris—HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl_2; 0.01% gelatin; 100 µM each dNTP; 7.5 µCi 32P-dCTP (3000 Ci/mmol, NEN-Dupont). All PCR assays were carried out in an automatic thermal cycler (Model 480, Perkin-Elmer) under the following conditions: 1 min initial denaturation at 95°C followed by 26 cycles of denaturation at 94°C, 30s; annealing 55–60°C, 30s; extension at 72°C, 60s; completed by a final extension cycle at 72°C for 7 min. Appropriate oligodeoxynucleotides for each genomic loci were synthesized and used as PCR primers generating products in the size range of 132–750bp.

The 32P-dCTP labeled PCR products were fractionated on polyacrylamide gel in 1×TBE buffer (89 mM Tris-borate, 89 mM boric acid, pH 8.0, 8 mM EDTA) under non-denaturing conditions. The dried gel was exposed to an X-ray film (Kodak AR) at room temperature.

**RESULTS**

Experimental strategy

The PCR-based assay to determine the replication time of single copy DNA sequences is schematically illustrated in Fig. 1. An asynchronously growing cell population is reversibly arrested at the G1/S boundary using the DNA synthesis inhibitor CPX (12) to achieve an efficient synchronous entry into the S phase. The cells are then treated with a nontoxic concentration of the thymidine analog 5-bromodeoxyuridine (BrdU) in order to label DNA which is being replicated at the time of incubation (17). Since BrdU decreases the fluorescence (quenching) of HO258, cells with newly-replicated sequences that have incorporated BrdU into their DNA show a reduced HO258 fluorescence intensity relative to those cells with non-substituted DNA (14, 18). The progression of the cell population that has incorporated BrdU for different periods of time after release from the G1/S phase boundary can be followed using the fluorescence-activated cell sorter (FACS). The cells are flow sorted on the basis of DNA content, as determined by EB fluorescence intensity, which increases as the cells progress through S phase and on the basis of BrdU incorporation into these cells which is detected as reduced HO258 fluorescence. The DNA from the flow sorted fractions is isolated and the photodegradable property of BrdU is used for the selective removal of BrdU-containing newly-replicated DNA. The single-strand nicks introduced in the BrdU-DNA sequences are converted into double-strand breaks by the action of the S1 nuclease. The replication kinetics of selected sequences is determined by PCR amplification using appropriate flanking primers. After gel electrophoresis, a comparison of the relative intensity between the PCR products defines the time at which a DNA sequence is being replicated. In this assay, DNA sequences localized in inactive replication units allow amplification whereas those that have replicated will not be amplified by PCR.
Figure 1. Schematic illustration of the PCR-based assay to study replication timing after selective removal of newly-replicated DNA sequences. Details are given in the results section. B-DNA, BrdU-substituted DNA; T-DNA, native DNA; F, forward PCR primer; R, reverse PCR primer.

Figure 2. Flow histograms of fractionated lymphoblastoid cell line DS71. The cells were grown as described in Materials and Methods, stained with Hoechst 33258 (HO258) and ethidium bromide (EB), and subjected to a bivariate flow cytometric analysis. The stained cells were then fractionated on the basis of their BrdU-DNA content, at different release times from the G1/S arrest, by using the cell sorter. As the cohort of cells released from G1/S progresses through S, the EB fluorescence intensity (which is proportional to DNA content) increases (A through F). The HO258 fluorescence intensity does not vary since the fluorescence is quenched by BrdU and BrdU uptake increases with DNA content. This double staining protocol permits the isolation of only cells that have incorporated BrdU and are off the diagonal containing S phase cells which have not taken up BrdU (compare F and G). This procedure minimizes contamination of non-substituted DNA in the flow sorted populations. Non-overlapping sorting windows (open boxes in A through F) were used to separate the cells at the increasing times after release from G1/S phase boundary thus ensuring that only cells from the leading edge of the synchronous cohort were collected for DNA preparation. The ordinate is a linear scale of HO258 fluorescence intensity and the abscissa is a linear scale of EB fluorescence. CPX, ciclopirox olamine; REL, release.

Bivariate flow cytometric fractionation of BrdU-labeled cells through S phase

The seven representative flow histograms in Fig. 2 show the progression of the synchronized cell population through S phase. These histograms reveal that the growth conditions employed allow an efficient arrest at the G1/S boundary by the inhibitor CPX (Fig.2a) indicating that this compound is effective in affecting synchronous release into S phase. The progression of the synchronized cell population through S phase is readily observed having reached the end of S phase by 7h post-release (Fig. 2 a–f), but a fraction of G1/S arrested cells fails to enter S phase as shown by the persistent cell population at the G1 fluorescence channel. The simultaneous measurements of EB and HO258 fluorescence allows discrimination between native DNA and BrdU-quenched HO258 fluorescence in cells during S phase.
Figure 3. Effect of UV irradiation and S1 nuclease treatment on BrdU-substituted DNA. A) Lymphoblastoid cell line GM06246 DNA was isolated from flow sorted fractions collected before the G1/S release (CPX block) and at 6h post-release. The presence of β2-microglobulin (β2MG) PCR products in non-replicating DNA (CPX block) S1/UV treated samples, but not in 6h rel DNA indicates that the UV-light and S1 nuclease effects are specific for replicating BrdU-substituted DNA. B) Replication timing of β2-microglobulin in lymphocytes. Mitogen-stimulated lymphocytes DNA isolated from different flow sorted fractions were treated as indicated and subjected to PCR amplification using primers specific for the β2MG locus. The PCR products were fractionated on a 6% non-denaturing polyacrylamide gel. Rel, release time; S1, S1 nuclease; UV, Ultraviolet light; CPX, ciclopirox olamine.

Quantitative PCR
In order to use a PCR approach to visualize DNA being replicated, it is necessary that any quantitative decreased PCR amplification be due to newly-replicated DNA and not to the PCR conditions. Since quantitative analysis requires measurements in the exponential phase of amplification, initial experiments were designed to determine adequate quantitative conditions. For these experiments, the retinoblastoma (RB1) and the β2-microglobulin (β2MG) loci were amplified for 14—30 cycles and the PCR products were fractionated by polyacrylamide gel electrophoresis and the radioactivity in the amplified bands was quantified (data not shown). We observed that the amplification signal increases from almost undetectable at 14 cycles to maximum at 30 cycles. Up to 27 cycles, the amount of PCR products appeared to double with each additional cycle, corresponding to the linear portion of the amplification. Approaching the 29th cycle, the rate decreased and reached a plateau. An important consequence of reaching plateau is that initially low concentration of DNA template may continue to amplify. Since PCR products are not detectable by ethidium bromide staining under such conditions, we used radioactive substrate in 26 cycles of PCR amplification to ensure a linear amplification.

Selective removal of BrdU-substituted DNA with UV-irradiation and S1 nuclease treatment
In this procedure, a preferential degradation of BrdU-substituted DNA should be observed when DNA samples are subjected to UV irradiation and S1 nuclease digestion (11). DNA treated this way should hamper the PCR amplification of DNA sequences being replicated. The validity of the PCR approach is illustrated in Fig. 3a. Both, non-substituted and BrdU-substituted, DNA are equally resistant to SI nuclease when untreated with UV light.

Table 1. Replication time of different loci

<table>
<thead>
<tr>
<th>Locus Name</th>
<th>Expression*</th>
<th>Replication Time**</th>
<th>Blood</th>
<th>GM06246</th>
<th>DS71</th>
</tr>
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<tbody>
<tr>
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<td>H</td>
<td>E</td>
<td>E</td>
<td>E</td>
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</tr>
<tr>
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<td>D15S10</td>
<td>?</td>
<td>-</td>
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<td></td>
<td>-</td>
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</table>

*H; Housekeeping T; Tissue-specific ?; Unknown
**E; Early (0—3h) M; Middle (3—5h) L; Late (5—7h) —; Not tested
cells at the G1/S boundary. In this regard, aphidicolin, an inhibitor of DNA polymerase \(\alpha\) activity, allows a significant amount of thymidine (or BrdU) incorporation and appears to be preferentially sensitive to UV-induced degradation. These results indicate that UV irradiation of S1 treated DNA samples prior to PCR amplification greatly reduces DNA substituted with BrdU in one strand.

**Replication timing analysis in various cell lines**

The applicability of the method to detect DNA sequences being replicated in different cultured mammalian cells was tested. Fig. 3b shows that the \(\beta\)MG PCR signal intensity decreases during the interval 2–4h relative to the 0h and 2h times. This result indicates that \(\beta\)MG locus amplified by these primers starts replicating in mitogen-stimulated human lymphocytes between 2–4h after release from the G1/S phase boundary. The same replication kinetics for \(\beta\)-microglobulin was also observed in two different lymphoblastoid cell lines, DS71 and GM06246 (data not shown).

**Temporal ordering of different loci**

To test the usefulness of our PCR-based assay for defining replication timing, different loci in addition to \(\beta\)MG were analyzed in various cell types (Figs 3 and 4). Our results may be summarized as follows (Table 2): The \(\beta\)MG, the GABRB3 exon8, RB1, CD7 and CO12A1 loci are early replicating in the lymphoblastoid cell line GM06246, as shown by the decreased PCR signal between 2–4h (Figs 3 and 4). GABRA1, IGF2 and D15S10 DNA sequences start their replication in the middle S phase (data not shown), whereas the \(\alpha\)-satellite sequences D16Z2 shows a late replicating pattern (Fig. 4). The reproducibility of the approach is illustrated by the similar replication pattern obtained for GABRB3 and \(\beta\)-microglobulin loci in cell lines DS71 and GM06246, as well as blood cells.

**DISCUSSION**

We have described a sensitive and rapid PCR-based assay for analyzing replication timing of any DNA sequence in human cells. This PCR assay was developed to overcome the problem of sensitivity associated with the replication timing analysis of single copy sequences in complex eukaryote genomes. The PCR technology allows a specific \(10^2–10^6\) fold amplification of single-copy sequences, thus eliminating the requirement for large amounts of DNA to study DNA replication kinetics. For instance, our procedure allows the detection of sequences being replicated from 20 ng of DNA (ca. 2 \(\times\) 10\(^3\) cells), as compared with other procedures requiring much more DNA (20,21) and therefore not useful in flow sorting experiments given the small amount of DNA available.

The cell cycle synchronization step in our procedure is needed to enrich the cell populations undergoing S phase traverse. CPX, an antifungal agent, is used to reversibly arrests cells before the onset of active DNA replication (12,23). CPX and another compound, mimosine which also blocks entry into S phase, are members of a new class of more efficient drugs for synchronizing cells at the G1/S boundary. In this regard, aphidicolin, an inhibitor of DNA polymerase \(\alpha\) activity, allows a significant amount of thymidine (or BrdU) incorporation and appears to be less useful in such synchronization protocols (23,24). While there are disadvantages of using drugs to perturb cell cycle for measuring replication kinetics, our results indicate that the procedure is extremely useful to determine differences in replication timing between DNA sequences in a single sorting experiment (Figs 3, 4 and table 1). In this regard, the very late replication time of the \(\alpha\)-satellite sequence 16Z2 is consistent with previous studies that have shown that DNA sequences associated with centromeric regions of eukaryotic chromosomes from higher organisms replicate late in S phase (4), and fluorescence in situ hybridization experiments (22) confirmed that CD7, GABRB3 and RB1 loci all replicate early (unpublished results).

In order to define the time of replication for DNA sequences in S phase, the FACS has been used to isolate discrete cell populations based on their BrdU-DNA content. The BrdU-substituted DNA has a differential sensitivity to UV light relative to the unsubstituted DNA which permits selective removal of the newly-replicated sequences (11,19). The rate of BrdU-DNA double strand breaks is more than 25 fold greater than is the rate of native DNA double strand breaks and a 90% recovery of intact non-substituted DNA sequences is expected by this approach (11).

This procedure for selective removal of newly-replicated DNA is very efficient, specific and reproducible as shown in this report and previous studies (11,25). The sensitivity of this assay could however be dependent on the DNA context of the target sequence. It has been shown that the ‘consensus’ sequence RCTTG/T is present at the sites of strong and medium damage (19). The sites of no significant damage share the same ‘consensus’ sequence NGTRR. According to these observations, resistant regions could be encountered, but this potential problem could be overcome with an appropriate selection of primers from DNA sequences neighbouring the region of interest.

Newly-replicated DNA that has been BrdU-labeled can be fractionated on CsCl gradients (26). Such density gradients require a large number of cells and provide imperfect separation relative to the selective BrdU-DNA photodegradation which allow a more precise fractionation of cells during S phase traverse and is particularly useful when working with small quantities of DNA such as the flow sorted samples with DNA yields in the 1–2 \(\mu\)g range. The same flow-sorted fractions can be used for analyzing different DNA sequences. In one experiment, 250ng of DNA (~1/6 of flow sorted fraction) can be processed as described above and stored for later use, resulting in enough material for 10–12 PCRs from different loci. By analyzing a certain number of genomic loci, including early and late replicating sequences, it is possible to determine the replication temporal order of these sequences in a particular cell line. The data reported in this paper define the replication temporal order of a number of genomic loci from different cell lines. Our results show that all tested housekeeping genes replicate early, whereas the pattern is more variable for tissue-specific and non-coding DNA sequences. To date, every DNA sequence analyzed has a distinct replication time within the cell cycle: Housekeeping genes replicate early in S phase of many cell types and the same is observed in ~25% of all tissue-specific genes (7), consistent with the results presented here (Figs 3, 4 and table 1). However, some tissue-specific genes show a different pattern of replication denoted by an early replication in expressing cells, but late in non-expressing cell types (3,7,27). A very powerful application of this approach is the possibiility of analyzing the replication timing of a pair of natural alleles by using microsatellite polymorphisms detected by PCR. Such polymorphisms exist for a great number of genes and random loci (28). It is known that

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differences in organization between homologous chromosomes may affect the replication timing of a gene. The late-replicating inactive X chromosome in female mammals is a classic example in which homologous chromosomes differ in structure and function (25, 29). In this regard, the replication patterns of the active and inactive X chromosome could be studied separately using these polymorphisms. Differences between homologous autosomes have also been observed. Recently, differences were detected in replication timing between homologs of human chromosome 15 at a specific region subject to genomic imprinting (30). Another interesting application of this assay is the analysis of the organization of replicons in extended chromosomal regions in order to define the boundaries of timing zones. In this regard, there have only been a few studies of the temporal order of replication over extensive regions of chromatin (22, 31, 32). The technology described here should allow additional studies of changes in replication time over larger chromosomal regions as well as further investigations of the relationship between gene expression and replication time.

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