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High-Resolution Analysis of DNA Replication in Released Chromatin Fibers Containing 5-Bromodeoxyuridine

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ABSTRACT

A strategy has been devised to physically map replication sites in released chromatin of mammalian cells. When added to the culture medium, 5-bromodeoxyuridine (BrdU) is incorporated into replicating DNA, partially replacing thymidine. BrdU pulses as short as one minute can be visualized on preparations of straightened chromatin fibers from protein-extracted nuclei by means of monoclonal anti-BrdU antibody. Short BrdU pulses (<10 min) appear as strings of fluorescent signals that are 50–300 kb in length. This corresponds to the estimated size of individual replication units. Pulse chase experiments reveal that replicating DNA is tightly associated with the residual nuclear matrix, whereas newly replicated DNA is positioned on the released loop chromatin of nuclear halo preparations. Simultaneous fluorescence in situ hybridization (FISH) on BrdU-substituted released chromatin fibers suggests that replication can initiate at multiple sites anywhere within an α -satellite array of several Mb.

INTRODUCTION

DNA replication is initiated at parental DNA sequences called origins of replication, followed by bidirectional elongation of the replication bubble (7,10). Identification of replication origins in higher eukaryotes has proven much more difficult than in yeast, and most attempts have either been unsuccessful or unreproducible. We still do not know whether DNA replication initiates at specific sites in mammalian chromosomes. Hence, we need practical assays to map and study replication sites in complex genomes.

Mitotic chromosomes that have incorporated the halogenated base analog 5-bromodeoxyuridine (BrdU) during the preceding S phase show conven-

tional bands, each of which must contain several dozen replication units (replicons). Even the use of very short BrdU pulses did not reveal any additional order of replication below that of the structural banding pattern (15). Fluorescence in situ hybridization (FISH) on yeast artificial chromosome (YAC) DNA fibers has revealed structures resembling replication intermediates (bubbles and forks) in human DNA cloned in yeast (14). However, DNA synthesis in a human cell nucleus may not necessarily initiate at the same origins.

Different mechanisms have been used to release chromatin from mammalian cell nuclei and extend it (2, 3,5,9,11–13,17). DNA fibers prepared by detergent and high-salt lysis and stretching of nuclear chromatin across the surface of a glass slide can be hybridized over at least several Mb with resolution of <10 kb (5). A novel approach to study replication of specific DNA sequences in the context of the entire human genome has been developed by coupling fiber FISH with immunofluorescent detection of BrdU incorporation. This in situ replication assay provides a temporary snapshot of the ongoing replication during a very short time interval of the S phase. The technique allows one to visualize single replication units directly in released chromatin.

MATERIALS AND METHODS

BrdU Pulse Labeling

BrdU (Sigma Chemical, St. Louis, MO, USA) was added to 10 mL of growing cell culture (i.e., fibroblasts or lymphoblasts) at a final concentration of 10 μ g/mL. After 1–10 min of BrdU incorporation, the cell culture was washed with medium containing 100 μ g/mL thymidine (10 times the concentration of BrdU) in order to eliminate possible storage or pool effects. Cells were then harvested immediately according to standard procedures and resuspended in phosphate-buffered saline (PBS; 136 mM NaCl, 2 mM KCl, 10.6 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.3). Since it takes only 10–20 min from the time of adding

BrdU to the preparation of chromatin fibers, essentially all incorporated BrdU represents nascent replicating DNA.

For pulse-chase experiments, cells were grown in the presence of 10 μ g/mL BrdU for 5 min, washed and then cultured in medium containing 100 μ g/mL thymidine for several hours. After 3–6-h chase times, the pulse-incorporated BrdU represents newly replicated DNA.

Preparation of Chromatin Fibers

Approximately 10^5 cells in 0.5 mL of PBS were centrifuged onto a clean microscope glass slide at 800 rpm for 4 min using a Shandon Cytospin® 2 (Shandon, Pittsburgh, PA, USA) and allowed to air-dry. The resulting slide

should carry a monolayer of unfixed cells about 5 mm in diameter. Thereafter, the cells were lysed immediately by inserting the slide vertically into a Coplin jar partially filled with a solution of 2 M NaCl, 25 mM Tris-HCl, pH 7.8, 1% Triton® X-100 (Sigma Chemical) (5). The cell monolayer should be completely immersed in the high salt-detergent solution. Long incubation times (>10 min) cause detachment of the DNA loops from the nuclear matrix and produce highly elongated chromatin fibers (Figure 1c). The nuclear matrix is largely destroyed and the slide covered with an irregular network of straightened chromatin fibers. Short extraction times (<2 min) remove histones and other soluble proteins but leave the matrix attachment sites intact,

resulting in DNA loops that surround the residual nuclear matrix in a halo-like fashion (Figure 1, a and b). It is still possible to define the borders of individual cells.

After 2–10 min of high salt-detergent treatment, an equal volume of ethanol was added to the releasing solution. To avoid cell loss, the first milliliter of alcohol was pipetted dropwise into the high salt-detergent solution. After 5–10 min of prefixation, the slide was carefully removed from the high salt-detergent-ethanol mixture so that the released chromatin remained streaming down the slide. The slide was held vertically for 20–30 s. A viscous consistency of the fluid dropping off the slide indicates optimum removal of nuclear proteins and straightening of

Figure 1. Immunofluorescent detection of short BrdU pulses on released chromatin by anti-BrdU antibody. (a) Nuclear halo of a fibroblast cell exposed to BrdU during the last 5 min before cell harvest. The halo was prepared by histone extraction with high salt and detergent for 2 min, stained with anti-BrdU antibody (red rhodamine fluorescence) and counterstained with DAPI (blue). Replicating DNA is retained in the residual nuclear matrix. Few replicated DNA segments appear as BrdU beads-on-a-string on the loop chromatin. Arrowhead indicates a replication unit of approximately 100 kb. (b) Nuclear halo prepared from a cell exposed to BrdU for 5 min and then grown in BrdU-free medium for 6 h. The halo is stained with anti-BrdU antibody (red) and hybridized with biotinylated chromosome 10-specific α -satellite DNA, detected with FITC-conjugated avidin (green). The bulk of newly replicated DNA is localized on the halo, whereas the nuclear matrix shows only weak, if any, BrdU incorporation. Note the hemizygous α -satellite DNA hybridization patterns. One α -satellite array is extended out onto the loop chromatin (long arrow), and the chromosomal homolog remains compact within the nuclear matrix (short arrow). (c) Straightened chromatin fibers after destruction of the nuclear matrix by detergent and high-salt lysis for 10 min, counterstained with DAPI. Open arrowhead indicates a single BrdU-labeled DNA fiber. (d and e) Chromatin fibers hybridized with biotinylated α -satellite DNA specific for chromosome 2 (d) and chromosome 10 (e), reacted with FITC-avidin (green). Multiple discrete dots of BrdU incorporation (red) suggest the presence of many independent replication sites in stretched α -satellite arrays (green). Arrowheads in Panel d indicate sites of BrdU incorporation in two independent α -satellite arrays. ($\times 500$ magnification)

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released chromatin fibers. Although an increasing loss of DNA occurred under prolonged extraction conditions, much of the protein-depleted chromatin remained attached to the slide. Finally, the released chromatin was fixed to the slide by incubating it in an ethanol series (70%, 80% and 100%) for 10 min each. The resulting slides can be stored in a desiccating box at 4°C for several weeks.

Fluorescence In Situ Hybridization

For FISH, standard protocols were followed (5,16). Briefly, the slides were treated with 100 µg/mL RNase A (Boehringer Mannheim, Mannheim, Germany) in 2× standard saline citrate (SSC), pH 7.0 at 37°C for 1 h, rinsed four times in 2× SSC and dehydrated in an ethanol series. Slides were denatured at 80°C in 70% formamide (Sigma Chemical), 2× SSC, pH 7.0 and again dehydrated in an alcohol series.

The hybridization mixture was composed of 50% formamide in 2× SSC, 10% dextran sulfate, 500 ng/µL salmon sperm DNA and 2 ng/µL biotinylated probe DNA. After 5-min denaturation at 70°C, 30 µL of hybridization mixture were applied to each slide and sealed under a coverslip. Hybridization was done overnight in a moist chamber at 37°C. The slides were then washed for 20 min in 50% formamide, 2× SSC at 42°C and twice for 5 min in 0.1× SSC, pH 7.0 at 60°C. Hybridization and washing conditions are dependent on the DNA probe and should be optimized for each sequence.

Anti-BrdU Immunofluorescence Staining

Following post-hybridization washings, the slides were incubated for 30–60 min at 37°C in a Coplin jar with a blocking solution of 3% bovine serum albumin (BSA; fraction V pow-

der, inexpensive grade), 0.1% Tween® 20 (both from Sigma Chemical), 4× SSC, pH 7.3. Two hundred microliters of anti-BrdU antibody solution were then added to each slide, covered with a large cover glass and placed in a moist chamber for 30 min at 37°C. Mouse monoclonal anti-BrdU antibody (Boehringer Mannheim) was diluted 1:50 in PBS. Since BrdU incorporated into released chromatin is only recognized if the DNA is in the single-stranded form, denaturation of the slides before in situ hybridization is a prerequisite for the immunodetection of BrdU. Following incubation with anti-BrdU antibody, the slides were washed three times for 5 min in PBS.

Hybridization of biotinylated probes was detected with fluorescein isothiocyanate (FITC)-conjugated avidin (DCS grade; Vector Laboratories, Burlingame, CA, USA). Digoxigenated probes can be immunolocalized by

means of fluorescent-labeled anti-digoxigenin antibody (Boehringer Mannheim). Anti-BrdU antibody bound to BrdU-substituted DNA was localized by indirect immunofluorescence with anti-mouse IgG (Dianova, Hamburg, Germany). FITC-avidin (diluted to 5 $\mu\text{g/mL}$) or FITC-conjugated anti-digoxigenin antibody (diluted 1:50) and rhodamine-conjugated anti-mouse IgG (diluted 1:200) were combined in detection buffer (1% BSA, 0.1% Tween 20, 4 $\times$ SSC, pH 7.3). Two hundred microliters of detection solution were added to each slide and incubated for 30 min at 37°C. The slides were washed again three times for 5 min in 0.1% Tween 20, 4 $\times$ SSC, and cell nuclei were counterstained with 1 $\mu\text{g/mL}$ 4',6-diamidino-2-phenylindole (DAPI) in 2 $\times$ SSC for 1–5 min. Finally, the slides were mounted in 90% glycerol, 0.1 M Tris-HCl, pH 8.0 and 2.3% 1,4-diazobicyclo-2,2,2-octane (DABCO).

Digital Imaging

Images were taken with an Axioskop[®] 20 Epifluorescence Microscope (Carl Zeiss Jena, Göttingen, Germany) equipped with a cooled charge-coupled device (CCD) PM512 camera (Photometrics, Tucson, AZ, USA), which was controlled by a Power Macintosh[®] 6100/66 computer (Apple Computer, Cupertino, CA, USA). Gray-scale source images were captured separately with filter sets for FITC, rhodamine and DAPI using the CCD Image Capture software (available through the Office of Cooperative Research, Yale University, New Haven, CT, USA). Merging and pseudocoloring were accomplished using Gene Join (Yale University). Although a CCD imaging system was used, it is worth emphasizing that both the BrdU and in situ hybridization signals described here were clearly visible by eye through the microscope.

RESULTS

Nuclear halos were prepared from fibroblast cells that had been grown in the presence of BrdU for 5 min. After washing the BrdU, cells were harvested immediately. After incubation of the

slides with high salt and detergent for 2 min, released DNA loops were arranged around a histone-depleted nuclear matrix. Almost all pulse-incorporated BrdU representing replicating DNA was retained in the residual nuclear matrix (Figure 1a, red), as reported previously (3). Linear strings of BrdU label were visible in the loop chromatin. These beads-on-a-string may represent individual replication units that have completed DNA synthesis during the short BrdU pulse and, therefore, are no longer attached to the matrix. By comparison with YAC hybridization signals on similar halo preparations (data not shown), the length of the BrdU strings was estimated 50–300 kb.

When cells were exposed to BrdU for 5 min and then chased for 6 h in thymidine-rich medium, the BrdU label was almost exclusively found out on the halos (Figure 1b, red). The nuclear matrix was virtually cleared of BrdU staining. This is consistent with a loop-chromatin organization of newly replicated DNA (3). Released chromatin provides a suitable target for FISH. Nuclear halos after a 6-h chase time were hybridized with the chromosome 10-specific α -satellite DNA clone p α -10RP8 (1). The α -satellite DNA family is located at the centromeric regions of all human chromosomes and provides the structural specificity for at least some aspect(s) of centromere function (4,5). It is based on 171-bp monomeric repeat units, which are organized in tandem arrays of lengths approximately 250 kb to >5000 kb (6,18). Two distinct in situ hybridization patterns were observed on nuclear halos (Figure 1b, green): (i) beads-on-a-string on the loop chromatin for a fully replicated α -satellite array and (ii) compact hybridization signals within the nuclear matrix for α -satellite DNA during or before its replication. Interestingly, some 20% of the analyzed halo preparations showed hemizygous hybridization patterns: one α -satellite array was fully extended on the loop chromatin, whereas the chromosomal homolog remained as a compact spot with the nuclear matrix. This supports the conclusion that replication of α -satellite arrays on homologous chromosomes is highly asynchronous (5).

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Nuclear extraction for at least 10 min produced long straight DNA fibers due to complete deattachment of the DNA loops from the nuclear matrix (Figure 1c). DNA halos (loop chromatin) and straightened DNA fibers can be found on the same slide. Chromatin fibers released from BrdU-substituted cells allow the fine mapping of DNA replication sites in specific DNA segments that are delineated by FISH. Figure 1, d and e gives representative examples of linearized chromosome 2 α -satellites hybridized with p α 2-11 (6) and chromosome 10 α -satellites hybridized with p α 10RP8 (1). Two to twenty discrete domains of BrdU incorporation (Figure 1, d and e, red) were detected on individual α -satellite arrays (Figure 1, d and e, green) that extend over approximately two Mb. This implies that DNA synthesis initiates at multiple discrete sites. Since the linearized arrays did not show a reproducible pattern of BrdU signals, there does not appear to be a specific replication order within a chromosome-specific α -satellite DNA subset.

DISCUSSION

Mammalian requirements for initiation of DNA replication are not well understood (7,8,10). Virtually all human DNA fragments larger than 10 kb can replicate in Epstein-Barr virus-derived vectors that promote nuclear retention in mammalian cells (8). This is consistent with the idea that replication may initiate at randomly chosen sites. Since genetic assays for mammalian autonomously replicating sequence (ARS) elements are not reliable, it is important to develop alternative techniques to map replication sites in chromosomal DNA. To this end, BrdU was pulse-incorporated into replicating DNA for 1–10 min. Based on the rate of replication fork movement (7,10), BrdU incorporation into replicating DNA proceeds at approximately 3 kb/min. Even very short BrdU pulses can be visualized on chromatin fiber preparations of BrdU-substituted cells by the use of anti-BrdU antibody. BrdU-substituted chromatin fibers serve as target DNA molecules onto which specific probes can be mapped

by FISH. Both the binding of anti-BrdU antibody and probe hybridization require denaturation of the chromosomal DNA *in situ* in order to expose the incorporated BrdU to antibody and the target DNA sequences to probe DNA. In this light, the application of fiber FISH to replication studies is straightforward. High-resolution fluorescent replication assays provide direct visual information on the spatiotemporal distribution of replication sites in specific DNA segments of up to several Mb. The method was tested by visualizing short BrdU pulses in replicating α -satellite DNA. It may be of major use in the identification of mammalian DNA replication origins and in the generation of high-resolution replication maps of the human genome. In addition, it may prove useful for studying the cell biology of DNA replication. Pulse-chase labeling of nuclear halos revealed dynamic changes in the higher-order organization of BrdU-substituted chromatin, suggesting that nuclear matrix attachment sites may be gained or lost during or after DNA replication (3).

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