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A NEW AND RAPID METHOD FOR VISUALISING DNA REPLICATION IN SPREAD DNA BY IMMUNOFLUORESCENCE DETECTION OF INCORPORATED 5-IODODEOXYURIDINE

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SUMMARY We have developed a new and rapid immunofluorescent method for visualisation of replicated regions on fixed DNA fibers. Using this method we have found in 5-fluorodeoxyuridine (FrdU)-blocked human cells distinct replication units covering about 60 kb of DNA and corresponding in size to single replicons or chromatin loops. Our results also suggest that nonadjacent replicons within a replicon cluster may be activated after FrdU arrest and that the method may be adapted for localization on fibers of specific DNA sequences.

Fiber radioautography of ^{3}H-thymidine-labeled DNA has been widely used to analyse DNA replication in mammalian cells (1-4). This method has the disadvantage of requiring long autoradiographic exposure times (up to several months; 3, 4). The localization of DNA replication in cell nuclei has been studied by immunofluorescence methods (5-7) but these have not been applied to DNA fibers. We demonstrate here that newly-replicated regions in spread DNA can be visualised using immunofluorescence microscopy to detect anti-5-iododeoxyuridine (IdU) antibodies bound to incorporated IdU.

MATERIALS AND METHODS

Cells, chromosomes and fixation procedures. Human cell line K562 was maintained in EMEM medium containing 5% fetal calf serum (FCS). For

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immunostaining the cells were incubated for 16 h in the growth medium containing 0.01 mM 5-fluorodeoxyuridine (FrdU) and then 5-iododeoxyuridine (IrdU, 0.001 mM) was added for 90 min. Labeled cells were then either fixed with methanol-acetic acid (3:1) and spread on slide, or lysed with 1% sodium dodecylsulfate-50 mM EDTA, pH 8, and lysate smeared across the slide, dried, treated for 10 min. at RT with 80% ethanol and dried again. Metaphase chromosomes were prepared (11) from human lymphoblastoid cell line BOLD grown in RPMI medium with 10% of FCS and then fractionated using ATC3000 sorter (Bruker). Fixed fibers from non-labeled chromosomes 3 for Alu-primer extension were prepared in the same way as from K562 cells except lysates were treated for 1 h at 60°C with 0.1 mg per ml of proteinase K.

Indirect immunofluorescence. For detection of incorporated IrdU fixed cells or DNA fibers were sequentially treated with: 45% acetic acid for 30 min. at RT and 0.2 N HCl for 30 min. at RT. Staining at 37°C with mouse monoclonal antibodies against 5-bromodeoxyuridine,BrdU (which also react with IrdU, clone Iu4 , 1:100 in PBS, a kind gift of Dr.L.Stanker) was for 60 min. and with secondary antibodies (FITC-tagged goat anti-mouse IgG, 1:100 in PBS) - for 30 min.

In situ extension of an Alu primer (MC oligonucleotide, upper strand, see ref.8) on fixed denatured DNA fibers was performed for 20 min. at 65°C with *Tth* DNA polymerase (a gift of Dr.O.Kaboev) in a mix containing 0.05 mM biotin-dUTP and 0.1 mM (each) dATP, dCTP and dGTP. After washing with PBS the fibers were stained 3 times with FITC-avidin DCS followed by biotinylated anti-avidin D antibodies (Vector Laboratories).

Immunofluorescence was analysed in a Zeiss epifluorescence microscope, or in an Opton inverted microscope, or in a Nikon inverted microscope coupled with Bio-Rad MRC-600 series laser scanning system.

RESULTS AND DISCUSSION

Since antibodies against 5-bromodeoxyuridine interact only with denatured DNA (5-7) first we have examined the completeness of DNA denaturation under our conditions (30 min. at RT in 0.2 N HCl) by staining of fixed fibers with Hoechst 33258, specific for double-stranded DNA, before and after the HCl treatment. The fibers were prepared from K562 cells not treated with FrdU and grown for 5 h in the presence of 0.001 mM IrdU. It was found that the acid treatment supresses Hoechst staining but left long (more than 100 μ) continuous denatured regions available for anti-BrdU-anibodies (data not shown).

To study whether fiber immunostaining method is able to detect short IrdU-substituted regions we have used long (16 h) treatment of cells with

FrdU which activates multiple normally silent replication origins spaced at 4 μ apart (3-4). As expected, short IrdU-substituted regions were found upon conventional (Fig.1A) or confocal (Fig.1B) fluorescent microscopy and an average center-to-center distance between the regions was estimated

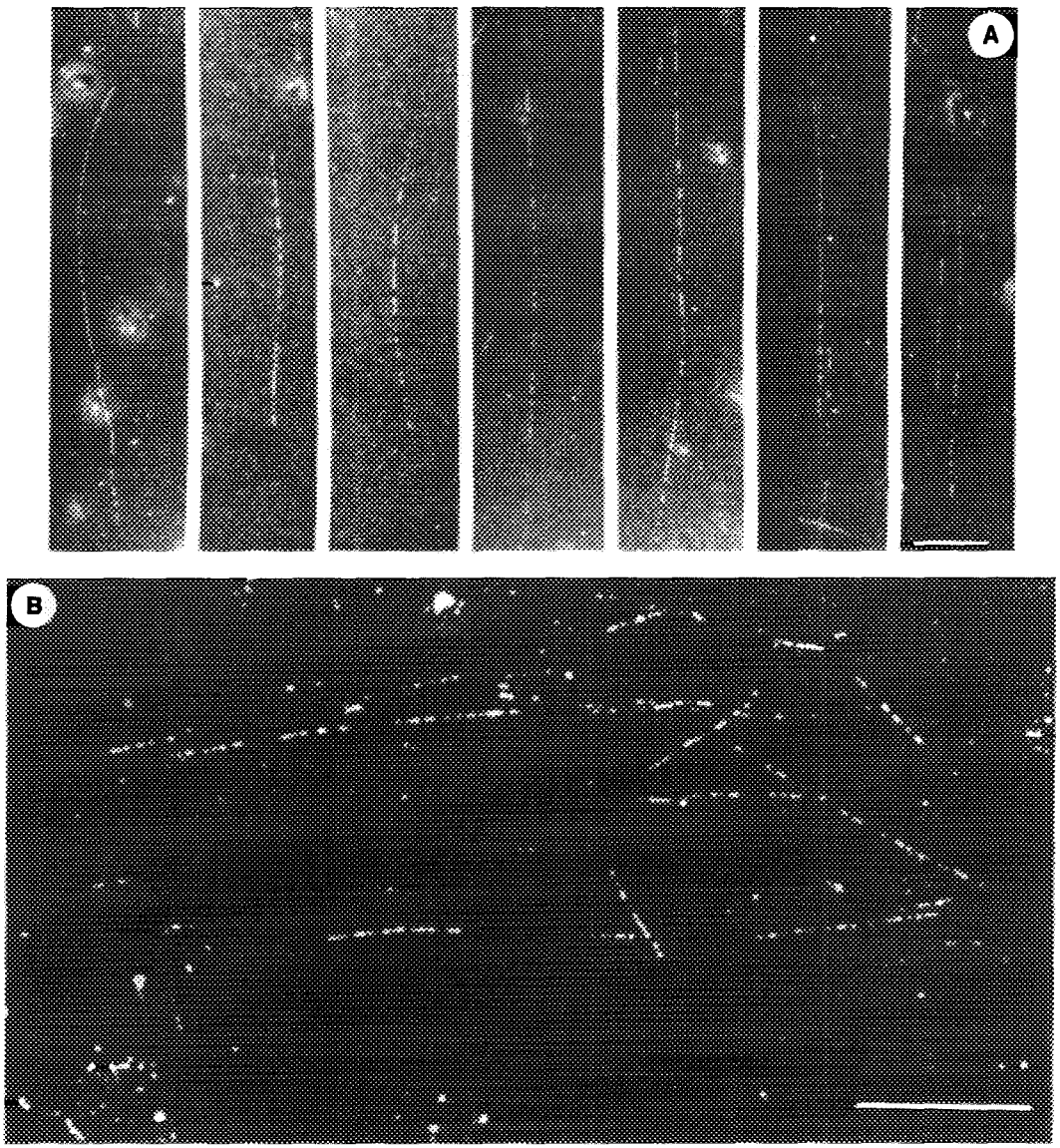

FIGURE 1. Immunofluorescent detection of the *in vivo* incorporated 5-iododeoxyuridine on DNA fibers from K562 cells treated for 16 h with 5-fluorodeoxyuridine. (A) - conventional fluorescent microscopy, bar is 10 μm.(B) – confocal laser scanning microscopy, bar is 25 μm.

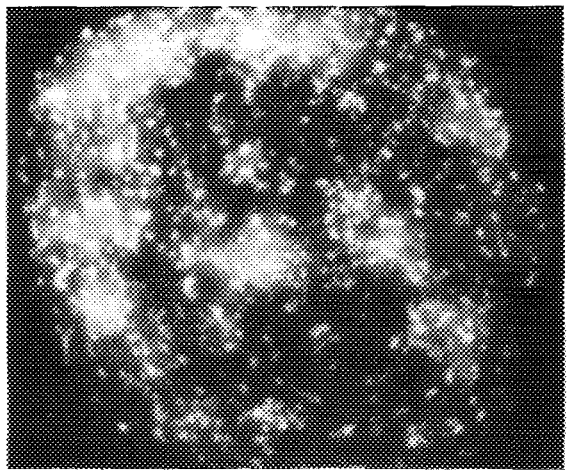

FIGURE 2. Replication centers in an FrdU-treated K562 cell. The scheme of FrdU treatment and labeling was exactly the same as in Fig. 1.

to be about 5 μ. Surprisingly, the short IrdU-substituted regions were found to be clustered into groups (Fig.1B) and an average size of the groups was 20 +/- 1 μ (M +/- SE, n=100). We also measured an average distance between pairs of the groups apparently located on the same fiber which was 34 +/- 3 μ (n=50), and visualised replication centers (5-6) in nuclei of the same cells from which the fibers were prepared (Fig. 2). Very faint (diameter 0.35 +/- 0.03 μ, n=400) replication centers (RCs) in nuclei were seen (Fig.2) much smaller than was observed earlier (5-6) for normally proliferating (non-treated with FrdU) mammalian cells after *in vivo* labeling (0.5 to several μ in diameter). Interestingly, recently published calibration allowing calculation of genomic distances from linear distances in interphase nuclei (9) suggests that 0.35 μ structures in interphase nuclei (the diameter of RCs in FrdU-treated cells) should accomodate about 70 kb of DNA which is close to the size of groups of short IrdU-labeled regions detected upon fiber immunostaining (20 μ or 60 kb) and to an average size of mammalian replicons (1-2) and of chromatin loops (10).

In normally proliferating cells each RC was estimated to contain 20-50 replication forks corresponding to replicating regions of 10-25 replicons

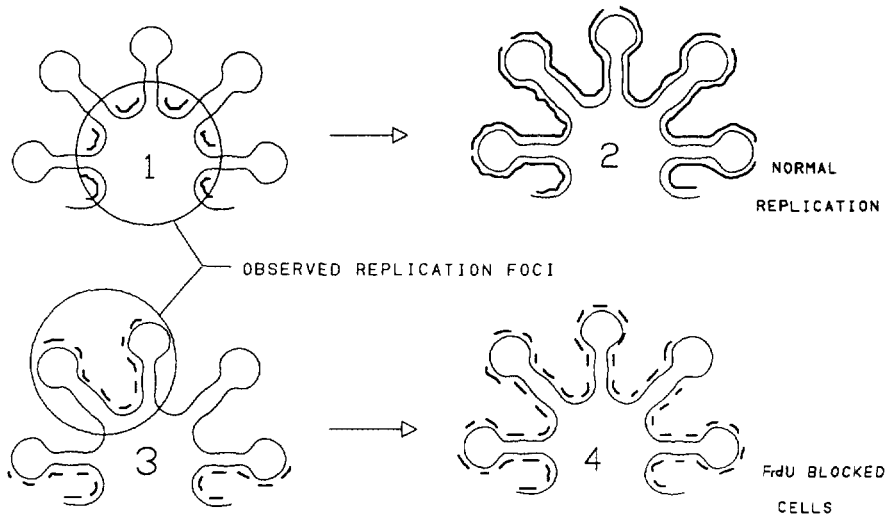

FIGURE 3. The scheme illustrating differences in the pattern of DNA replication in normally proliferating and FrdU-treated cells.

which are activated synchronously during specific interval of S-phase (1-2, 5-6). If the 60 kb clusters of IrdU-substituted regions corresponding in size to the amount of DNA accomodated by 0.35 μ RCs actually represent single replicons of normally proliferating cells (as shows Fig.3), our results indicate that in FrdU-treated cells not all replicons within a single domain are activated synchronously and, therefore, are functionally heterogeneous. The results also suggest that fiber immunostaining method for detection of IrdU or BrdU may be applied to the analysis of DNA replication.

In the search of other applications of the fiber immunostaining method described here we have attempted to localize Alu repeats on denatured DNA fibers prepared from the cell sorter-isolated (11) human chromosomes 3. Biotin-dUTP label was introduced into DNA by *in situ* extension of an Alu oligonucleotide (MC upper strand, see ref.8) by *Tth* DNA polymerase and visualised using FITC-avidin DCS and anti-avidin D. Fig.4 shows one of the fibers found on which unique pattern of fluorescent dots (presumably Alu repeats) is seen. The length of DNA products after primer extension by DNA polymerases rarely exceed 1 kb (12) which correspond to about 0.3 μ on fibers. It seems, therefore, that fiber

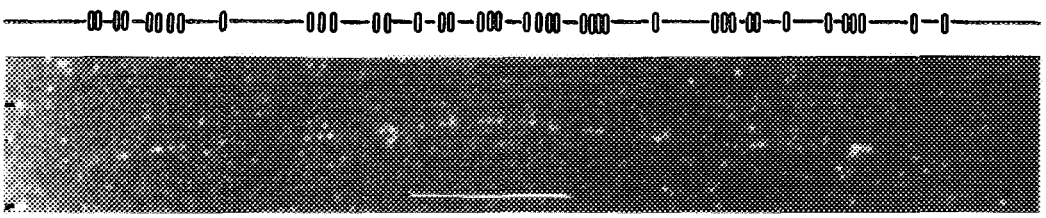

FIGURE 4. *In situ* extension of an Alu-repeat primer on fixed denatured DNA fiber from human chromosome 3 by *Tth* DNA polymerase in the presence of biotinylated dUTP. Bar is 25 μm.

immunostaining may be used for mapping of specific sequences on DNA and for fast determination of genomic distances which seems important for human genome analysis.

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REFERENCES

1. Edenberg,H.J. and Huberman,J.A. (1975) Ann. Rev. Genet. 9,245-284.
2. Hand,R. (1978) Cell 15, 317-325.
3. Taylor,J.H. (1977) Chromosoma 62, 301-325.
4. Taylor,J.H. and Hozier,J.C. (1976) Chromosoma 57, 341-350.
5. Nakamura,H., Morita,T. and Sato,C. (1986) Exp. Cell Res. 165, 291-297.
6. Nakayasu,H. and Berezney,R. (1989) J. Cell Biol. 108, 1-11.
7. O'Keefe,R.T., Henderson,S.C. and Spector,D.L. (1992) J. Cell. Biol. 116, 1095-1110.
8. Tomilin,N.V., Bozhkov,V.M., Bradbury E.M. and Schmid,C.W.(1992) Nucleic Acids Res. 20, 2941-2945.
9. van der Engh,G., Sachs,R. and Trask,B.J. (1992) Science 257, 1410-1412.
10. Hancock,R. and Boulikas,T. (1982) Int. Rev. Cytol. 79, 165-214.
11. Sillar,R. and Joung,B.D.(1981) J. Histochem. Cytochem. 29, 74-78.
12. Braman,J. (1989) Strategies in Molecular Biology 2, 56-64.