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Permanent attachment of replication origins to the nuclear matrix in BHK-cells

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ABSTRACT

The position of replication origins and replication forks relative to the nuclear matrix was analysed by autoradiography. Analysis of 2M NaCl-extracted BHK-nuclei, prepared on coverslips, showed that after brief pulses grains were exclusively found over the central core of the residual nuclei, which corresponds to positions in the nuclear matrix. In asynchronuous cells these grains were found to migrate into the DNA-halo surrounding the matrix during a subsequent chase. When the pulse had been administered to synchronuous cells at the onset of S-phase, it was observed, however, that in the majority of the structures no such migration had occurred. From this, and from the fact that label incorporated later in S-phase could be chased into the halo, we conclude that, contrary to DNA in replication forks, DNA containing replication origins is permanently attached to the nuclear matrix.

INTRODUCTION

Current models of DNA-replication in eukaryotes ascribe an important function to the nuclear matrix (1,2,3), to which DNA is bound in arrays of supercoiled loops (4,5) throughout the cell-cycle (3,6). In S-phase, DNA is reeled through matrix-bound "replisomes", which are the actual sites of DNA-replication (7,8). It has been postulated, on the basis of the observed relation between loop-size and replicon-size, that the bases of the DNA-loops behave as replication origins (7,9,10). By our laboratory evidence has been presented confirming this idea (11,12). In this study the position of replication origins relative to the nuclear matrix was studied in more detail by means of autoradiographic analysis of so-called matrix-halo structures (5). We have observed that, in synchronized BHK-cells, label incorporated at the beginning of S-phase remained matrix-associated, whereas label incorporated later in S-phase generally migrated into the DNA-halo eventually. Active replication origins, therefore, remain bound to the nuclear matrix after DNA-synthesis has been initiated.

MATERIALS AND METHODS

Cell culture, synchronization and labeling procedures. BHK A_3-cells (13) kindly provided by Dr. J.W.I.M. Simons of the Department of Radiation Genetics and Chemical Mutagenesis at Leiden University, were maintained in monolayer cultures in Minimal Essential Medium (MEM; Flow Labs), supplemented with 8% (v/v) foetal calf serum (Gibco).

For synchronization, cultures of asynchronuous cells in log-phase were treated for 4 hours with 0.5 µg/ml Nocodazole (14). Cells arrested in mitosis, generally approximately 30% of the total cell number, were selected by gentle shaking, pelleted by centrifugation and washed twice for 10 minutes with fresh, complete medium. The mitotic cells were subsequently seeded in fresh medium in petridishes containing coverslips.

Prior to labeling, to the medium 2.5 µg/ml Aphidicolin or 2 mM Fluorodeoxyuridine (FdUrd) and 40 µM Uridine (Urd) was added. After 30 minutes of incubation (^{3}H)thymidine was added (30-50 µCi/ml; specific activity 25 Ci/mmol) for different periods of time. In case a pulse was followed by a chase, coverslips were rinsed with fresh medium and transferred to label-free medium containing 100 µM thymidine.

Preparation of matrix-halo structures and autoradiography. The procedure of Vogelstein et al (5) was followed with minor variations. After the pulse, or after the pulse-chase, cells on coverslips were rinsed with ice-cold isotonic buffer (0.9% NaCl; 5 mM $MgCl_2$; 50 mM Tris-HCl pH 8.0) and permeabilized for approximately 1 minute in 0.5% Nonidet P-40 in 5 mM $MgCl_2$ and 50 mM Tris HCl pH 8.0. The permeabilized cells were then extracted sequentially with 0.0, 0.4, 0.8, 1.2, 1.6 and 2.0 M NaCl in 5 mM $MgCl_2$ and 50 mM Tris-HCl pH 8.0. To relax the supercoiled loops the 2.0 M NaCl buffer was supplemented with 4 ug/ml Ethidium bromide. NaCl was removed by repeating the extraction procedure in the opposite direction with the exception that the 0.0 M buffer contained 0.01% Triton X-100 to facilitate subsequent air-drying at room temperature. In each separate experiment, fluorescence microscopy was used to check whether the procedure had resulted in regular matrix-halo structures.

Dried coverslips were coated (0.5% gelatin; 0.1% $KCr(SO_4)_2$; 1.0% phenol) and autoradiographed with stripping film (Kodak Stripping Plate AR-10) with exposures of 2 to 3 weeks. The film was developed (Kodak D-19) for 10 minutes and fixed. Finally, matrix-halo structures were stained for 1.5 hours in 10% Giemsa in 0.15 M phosphate buffer pH 7.0. Micrographs were taken with a Zeiss Photomikroskop III using Agfapan-25 films. The diameter of the area covered by silver grains was determined using an ocular micrometer. For each histogram, 50 structures were analysed which had been selected at random and of which the halo did not appear to be disturbed.

RESULTS

To establish the position of origins of replication relative to the nuclear matrix, an experimental protocol was developed in which matrix-halo structures were prepared from cells specifically labeled either at the onset of S-phase or in middle/late S-phase. In some instances pulses were followed by a chase to analyse the fate of the label incorporated. The protocol consisted of synchronizing cells by mitotic selection using the reversible mitotic inhibitor Nocodazole (14). Cells were then seeded into medium containing (^{3}H)thymidine and one of the inhibitors of DNA-synthesis Aphidicolin

or Fluorodeoxyuridine (15,16). The inhibitors were present at concentrations which allow replication to be initiated. However, as elongation is severely inhibited under these circumstances, the initiated replicons accumulate leading to an increase of the yield of origins, labeled during the pulse.

We also observed Nocodazole to affect BHK-cells reversibly. The majority of cells obtained by the shake-off procedure reattached to coverslips in 1 to 2 hours and elongated subsequently. Approximately at the same time (^{3}H)thymidine incorporation was observed to start, reaching maximal rates 5 to 6 hours after release of the cells from the Nocodazole block (data not shown).

Compared to controls, in cells labeled in the presence of 10 uM Aphidicolin or 2 mM FdUrd, incorporation was depressed by greater than 95% and 75% respectively. In case of FdUrd this is an underestimation of the inhibition of the rate of chain elongation as FdUrd, interfering with thymidylate synthetase, induces cells to incorporate exogeneously provided thymidine to a greater extent. For this reason, in experiments with Aphidicolin, FdUrd was also added, albeit at a concentration of 10 uM, which, on its own, did not inhibit.

Inhibition induced either by Aphidicolin or FdUrd was found to be readily reversible since immediately after removal of the inhibitors from the medium (^{3}H)thymidine incorporation into DNA recovered to attain rates equal, or even superior, to those in control cells (data not shown).

Next, the location relative to the nuclear matrix of label incorporated into DNA during a pulse was analysed using matrix-halo structures obtained from asynchronuous cells. To this purpose, log-phase cells, grown on coverslips were labeled with (^{3}H)thymidine for intervals up to 60 minutes in the presence of 2 mM FdUrd. Half of each culture was subsequently allowed to grow on in inhibitor-free medium. Matrix-halo structures were then prepared as described before (5), except that routinely 4 ug/ml Ethidium Bromide was used in order to avoid UV-irradiation. In the fluorescence microscope matrices could be observed, which were surrounded by a halo consisting of relaxed DNA-loops as judged by the radius estimated (mean radius approximately 13 um in asynchronuous as well as synchronized cultures).

As a typical example, figure 1 A shows the distribution of grains over matrix-halo structures obtained from cells labeled in the presence of FdUrd for 40 minutes. Grains were found over the matrix only in at least 95% of the structures scored (fig. 1B). Consequently, only short stretches of DNA have been synthesized during the pulse and FdUrd can therefore be used to accumulate cells, released from the Nocodazole block, in early S-phase.

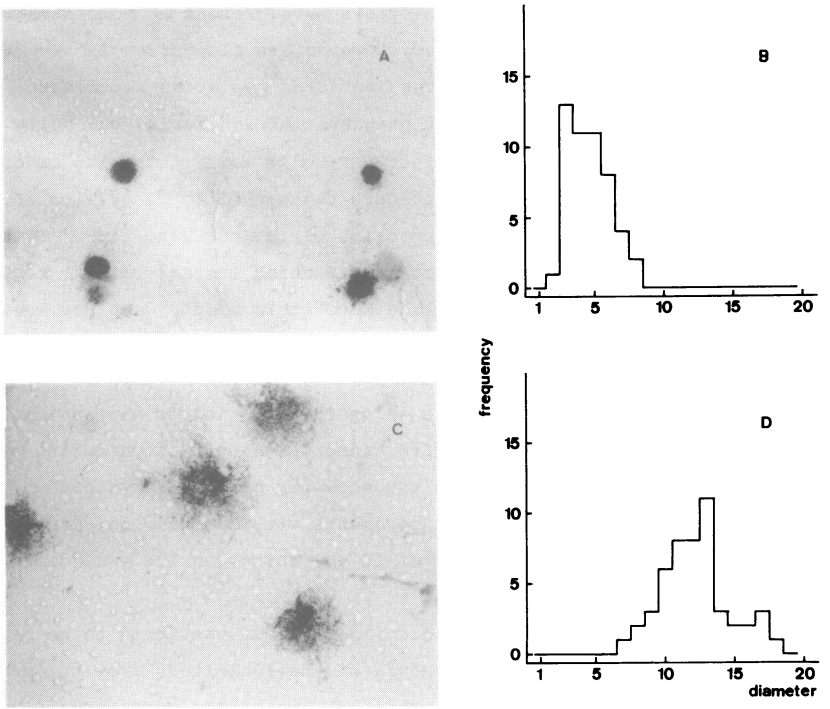

Figure 1. Autoradiographic analysis of matrix-halo structures from asynchronuous cells, pulse-labeled in the presence of FdUrd.
Panel A: autoradiogram showing the label distribution over matrix-halo structures after a 40 min. (^{3}H)thymidine pulse in the presence of 2 mM FdUrd.
Panel B: frequency distribution of the diameter of the labeled area (arbitrary units) in structures as shown in panel A.
Panel C: as under A, the pulse being followed by a 60 min. chase.
Panel D: frequency distribution of the diameter of the labeled area in structures as shown in panel C.

When the pulse had been followed by a chase of 1 hour in fresh medium containing 100 uM thymidine to bypass the FdUrd-block, label was found to have migrated into the surrounding DNA-halo in almost all structures that had incorporated (^{3}H)thymidine (figs. 1C and 1D). From this we again conclude that inhibition by FdUrd is readily reversible.

Similar results were obtained when instead of FdUrd, Aphidicolin had been used. It can therefore be concluded that both inhibitors tested are suited for the study intended. Our observations further confirm data presented by other authors, showing that replication takes place at the nuclear matrix and that newly replicated DNA is displaced into the halo eventually.

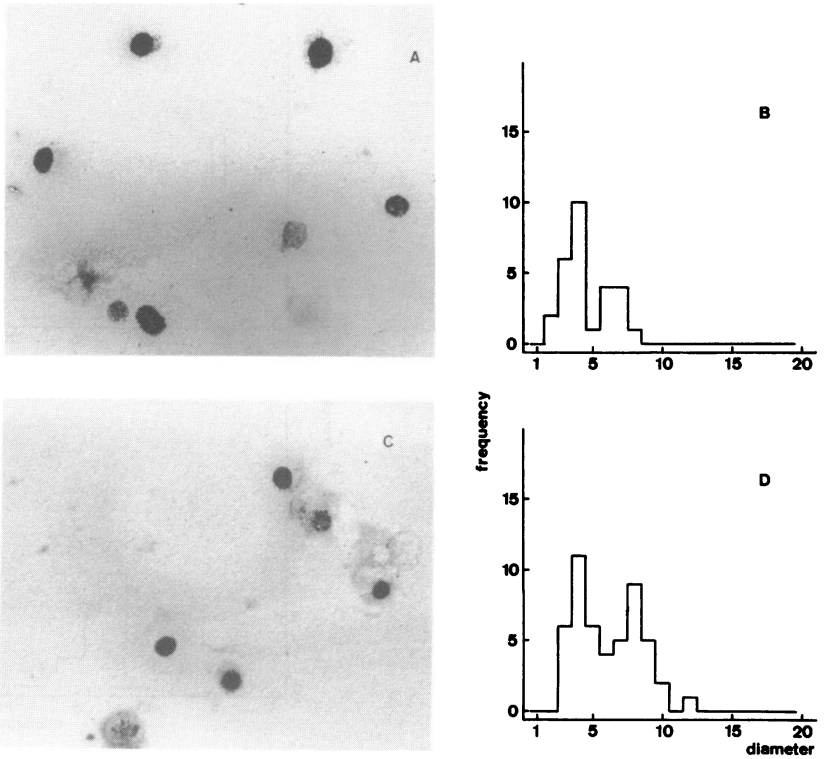

Figure 2. Autoradiographic analysis of matrix-halo structures from synchronuous cells labeled in early S-phase in the presence of FdUrd.
Panel A: autoradiogram showing the label distribution after a 2.5 hour (^{3}H)thymidine pulse administered in early S-phase to preferentially label replication origins.
Panel B: frequency distribution of the diameter of the labeled area (arbitrary units) in structures as shown in panel A.
Panel C: as under A, the pulse being followed by a 2 hour chase.
Panel D: frequency distribution of the diameter of the labeled area in structures as shown in panel C.

Next, for specific labeling of replication origins, a synchronuous cell-population was prepared by mitotic selection. After plating on coverslips, these cells were labeled for 2.5 hours with (^{3}H)thymidine in the presence of FdUrd, starting 1.5 hours after release of the cells from the Nocodazole block. After removal of the label, part of the culture was allowed to grow on in inhibitor-free medium for another 2 hours. Autoradiographic analysis revealed that in most of the matrix-halo structures, obtained from cells which had been pulse-labeled only, grains were exclusively found over the matrix (figs. 2A and 2B).

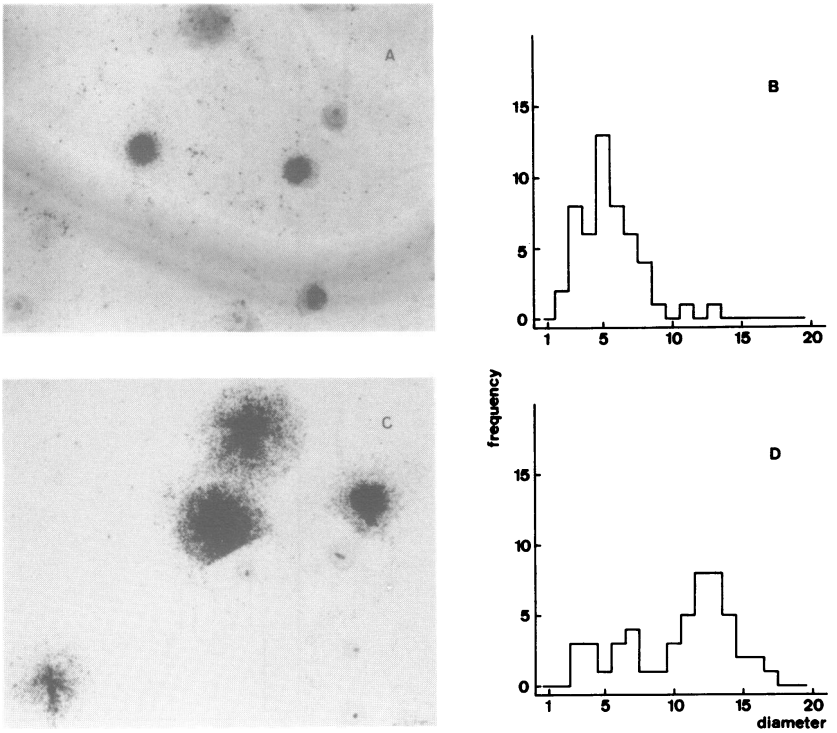

Figure 3. Autoradiographic analysis of matrix-halo structures from synchronuous cells labeled in middle/late S-phase in the presence of FdUrd.
Panel A: autoradiogram showing the label distribution after a 2.5 hour (^{3}H)thymidine pulse administered in middle/late S-phase to label DNA regions depleted of replication origins.
Panel B: frequency distribution of the diameter of the labeled area (arbitrary units) of structures as shown in panel A.
Panel C: as under A, the pulse being followed by a 2 hour chase.
Panel D: frequency distribution of the diameter of the labeled area of structures as shown in panel C.

This distribution was not affected by a subsequent chase in about 50% of the structures containing label, while in the other half the labeled region extended into the halo for a limited distance only. In a very minor fraction grains were found over the entire matrix plus halo (figs. 2C and 2D). The latter fraction most probably consists of cells which had initiated replication before addition of the inhibitor. Comparable data were obtained from experiments in which 10 uM Aphidicolin had been used instead of FdUrd (data not shown).

Experiments as outlined above were also performed with cells later in

S-phase. During the 2.5 hour pulse, administered from 5.5 hours after release of the cells from the Nocodazole-block onwards, the number of initiations occurring should be reduced to a considerable extent compared to early S-phase. Label incorporated during the pulse in DNA-loops which are subsequently allowed to be replicated to completion, should then be recovered at random positions in these loops. Consequently, after the chase, grains are expected to be found over the whole of the matrix plus halo.

Analysis of matrix-halo structures obtained from cells labeled in middle and late S-phase showed that immediately after the pulse, grains were found over the matrix only in over 70% of the structures that had incorporated (^{3}H)thymidine into their DNA (figs. 3A and 3B). After a chase of 2 hours, however, in at least 60% of the labeled structures, grains were found over the entire area covered by these structures. On the other hand, the fraction showing grains at matrix positions only had dropped to about 20% (figs. 3C and 3D). This fraction might have originated from cells which hade been slow in recovering from the mitotic block.

Summarizing, our data strongly suggest that non-origin regions of the DNA are not matrix-associated in a permanent fashion.

DISCUSSION

Approaching the concept of the nuclear matrix functionally has proven to be extremely rewarding in recent years. It has become clear that the proteinaceous nuclear skeleton is involved in a score of nuclear processes. With regard to replication it has been shown that the actual site of DNA-synthesis is located on the matrix (5,7,8). This strongly suggests that the nuclear matrix is associated with the multienzyme replicative complex (17,18) though, as yet, this still has to be proved in spite of the demonstrated association of DNA-polymeraseα with the matrix (19).

Our results, obtained by autoradiographic analysis of so-called matrix-halo structures, also show that DNA-synthesis takes place in the matrix compartment of the cell nucleus. Both after short pulses (data not shown) and after longer pulses in the presence of inhibitors of chain-elongation, grains were almost exclusively found over the nuclear matrix. As expected, the label could be chased into the halo, i.e. the DNA-loops emanating from the central matrix.

With synchronized cells similar results were arrived at if label was administered in middle/late S-phase. However, label incorporated into the chromosomal DNA at the onset of S-phase, which is therefore located in, or

close to, origins of replication, cannot be chased into the DNA-loops. This indicates that the loop-bases contain replication origins.

Our observations support the conclusions drawn from previous studies performed in our laboratory (11,12). In these studies it was shown that DNA-regions labeled in early S-phase resist detachment from the nuclear matrix by nucleases efficiently, whereas DNA labeled later in S-phase is removed preferentially.

Both autoradiographic and nuclease digestion studies support the concept that the bases of the matrix-attached DNA-loops contain replication origins and that these sites of the genome, therefore, are attached to the nuclear matrix permanently. In a recent study ars-sequences were mapped at positions in the DNA-loops distant from the nuclear matrix (20). Our findings clearly question the view that these sequences actually are origins of replication. Nucleotide sequence, therefore, might not be the sole parameter defining an eukaryotic origin of replication. For a sequence to be functional in this respect, it might be crucial to exist in a specific conformation, which could be induced by binding to the nuclear matrix proteins. Alternatively, and as suggested recently for regulatory elements involved in transcription (21), it might be required for any DNA-sequence to behave as an origin to be located in the matrix compartment of the nucleus, which is considered to be the appropriate micro-environment for the process of DNA-replication.

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