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Dynamics of three-dimensional replication patterns during the S-phase, analysed by double labelling of DNA and confocal microscopy

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Summary

The temporal and spatial progression of DNA replication in interphase nuclei of eukaryotic cells has been investigated. Application of a recently developed technique for the immunofluorescence double staining of cell nuclei labelled first with iododeoxyuridine (IdUrd) and subsequently with chlorodeoxyuridine (CldUrd) allows the visualization of two replication patterns in the same nucleus originating from two different periods of the S-phase. We have analysed changes in the three-dimensional replication patterns during the S-phase. To record dual colour three-dimensional images of doubly stained nuclei, a confocal microscope is used. This CSLM is equipped with a specific laser/filter combination to collect both fluorescence signals (FITC and Texas Red) in a single scan, thus precluding pixel shift between the images. A method for the quantitative evaluation of the degree of overlap between DNA regions replicated in two different periods of the S-phase is applied. The results confirm the generally accepted theory that DNA is replicated coordinately in a specific temporal order during the S-phase. The replication time of a DNA domain (i.e. the time between initiation and termination of DNA replication within a domain) at the very beginning of the S-phase was known to be one hour (Nakamura et al., 1986). Our observations show that in the rest of the S-phase, the replication time of a DNA region is also about one hour. We conclude that replicon clusters located in the same region are replicated in the same relatively short period of time. After this period there is no unreplicated DNA left in this region.

Key words: DNA replication, chlorodeoxyuridine, iododeoxyuridine, confocal microscopy, image analysis.

Introduction

Early studies have shown that DNA synthesis at the molecular level is coordinated in groups of adjacent replication units, named replicon clusters (Edenberg and Huberman, 1975; Hand, 1978). Within a period of one hour replication of DNA in one cluster is nearly complete (Huberman and Riggs, 1968; Housman and Huberman, 1975). How these replication clusters are organized within the nuclear structure is still largely unknown.

From later studies we know that eukaryotic DNA synthesis is concentrated in discrete foci in the interphase nucleus (Jackson, 1990). Note that a replication focus is defined as the site where replication takes place. As the replicating nucleus in the S-phase of the cell cycle is a dynamic structure, replicated DNA may be moved away from the replication foci. In pulse-chase experiments, the position of labelled DNA may not coincide with the position of the replication foci.

The spatial distribution of replication foci can be visualized by various labelling and detection techniques. Replicating DNA can be labelled with [^{3}H]thymidine (Huberman et al., 1973; Fakan and Hancock, 1974; Yanishevski and Prescott, 1978; Smith et al., 1984), biotin-11-dUTP (Mills et al., 1989; Banfalvi et al., 1990; Nakayasu and Berezney, 1989) or thymidine analogues like 5-bromodeoxyuridine (Gratzner, 1982; Nakamura et al., 1986, Dierendonck et al., 1989; Mazzotti et al., 1990). Using confocal fluorescence microscopy the three-dimensional position of nascent DNA, visible as fluorescent spots, in a nucleus can be determined (Mills et al., 1989; Banfalvi et al., 1990; Fox et al., 1991). The spatial distribution of these spots changes during the S-phase. Replication in the early S-phase is located in numerous (100-300) granules distributed throughout the nucleus, with the exception of condensed, heterochromatic regions. In the late S-phase the spots are larger and brighter and they are situated in the heterochromatic regions and in the nuclear periphery (Fox et al., 1991). These data show that pattern and timing of DNA replication are related to the structure and the

functional properties of chromatin in the interphase nucleus.

At the start of the S-phase, replication begins at discrete foci, which show replication for a period of about one hour (Nakamura et al., 1986; Mills et al., 1989), after which other active foci appear. How the positions of these newly appearing foci are related to the positions of foci that have already terminated activity has not yet been investigated. Even less is known about the positional relationship, in the late S-phase, between regions that terminated replication and regions that started replication.

The studies mentioned earlier illustrate the importance of comparing replication patterns originating from different periods in the S-phase. However, the strong variations in the orientation and position of chromosomes in interphase cells (Van Dekken et al., 1990) renders a detailed comparison of replication patterns in different nuclei very complicated. These cell-to-cell variations can be eliminated only by comparing different replication patterns in the same nucleus. This can be achieved by incorporating two independent DNA replication labels in the same nucleus. In that way the temporal development of replication patterns can be studied effectively. Recent developments in immunofluorescence double-staining methods (Shibui et al., 1989; Bakker et al., 1991; Aten et al., 1992) allows the separate detection of two different halogenated nucleotides that have been incorporated in DNA.

In the study presented here, Chinese hamster cell cultures were pulse-labelled with iododeoxyuridine and subsequently with chlorodeoxyuridine. The time interval between these labels ranged from one hour to five hours. The fluorescence signals were recorded by means of dual-colour confocal microscopy. The combination of double labelling and confocal microscopy allowed us not only to visualize early and late replicating DNA in the same nucleus, but also to detect changes in the three-dimensional replication patterns as a function of time. We quantified these changes by using a technique for the analysis of two-colour three-dimensional images.

Materials and methods

DNA labelling and staining

Cultures of asynchronously growing V79 Chinese hamster cells were labelled according to various labelling schedules (Aten et al., 1992). The cycle time of these cells is 11 h and the duration of the S-phase is 6 h. Cells were grown in medium supplemented with iododeoxyuridine (IdUrd, 10 μM) for 15 min, after which the medium was removed. The cells were then washed 3 times with normal, prewarmed medium and cultured for 5, 3 or 1 h in normal medium. Subsequently, the cells were cultured for 15 min in a chlorodeoxyuridine (CldUrd, 10 μM)-containing medium. Parallel cultures were used for control experiments. The cells in these cultures were grown for 15 min in the presence of IdUrd or CldUrd, or both halogenated nucleotides at the same time. At the end of the labelling procedure the cells were washed and harvested (Aten et al., 1992). Data shown in this paper were derived from two independent series of experiments. As an extra control experiment we carried out a double labelling with a variable duration of the first pulse (5, 15 and 60 min) and a time interval between the labels of 1 h. We used the data from these experiments to investigate the morphology of the domains containing newly synthesized DNA.

To detect the incorporated halogenated deoxyuridines an indirect immunofluorescence staining method was used (Aten et al., 1992). The IdUrd-labelled DNA and the CldUrd-labelled DNA were stained with Texas Red and FITC (fluorescein isothiocyanate).

Microscopy

For visual inspection of the preparations we used a Leitz fluorescence microscope (Ortholux II) and a Leitz 40×/1.30 NPL Fluotar oil immersion objective.

Three-dimensional, two-colour images of doubly stained nuclei were recorded by confocal scanning laser microscopy (Brakenhoff et al., 1985, 1989). The setup was equipped with a krypton-ion laser tuned at 520.3 nm (30 mW) to excite FITC and Texas Red simultaneously. A 510 nm longpass dichroic mirror partially reflected the laser beam to the object and transmitted the FITC and Texas Red fluorescence light from the object. A 580 nm longpass dichroic beamsplitter separated red and green fluorescence. A 610 nm longpass filter and a 530-550 nm bandpass filter in front of two detectors were used to minimize optical cross-talk and to block scattered laser light. The fluorescence signals from both fluorochromes were recorded simultaneously in one scan. Data of digitized fluorescence signals were stored according to the Image Cytometry Standard format as 8 bits memory arrays (Dean et al., 1990). The dimensions of a voxel were 0.066 μm lateral and 0.26 μm axial. From the preparations with cells labelled with variable duration of the first label, only singly labelled nuclei which incorporated IdUrd were selected. From these nuclei the Texas Red signal was recorded using a Leica confocal microscope.

Image analysis

For image processing and analysis the software package SCIL-IMAGE (developed at the University of Amsterdam) was used on an Apollo workstation. This package has been extended with several 3D routines developed by the Netherlands Project Team for Computer Science Research (SPIN).

Preprocessing

All images underwent a transformation to correct for optical cross-talk and histochemical cross-reactivity. Such a transformation is necessary because the intensities of red and green light as observed in a dual-channel image are not directly related to the quantity of incorporation of the labels IdUrd and CldUrd. The intensity of the fluorescence signals (red and green) is proportional to a linear combination of the label intensities, i.e. the quantity of incorporated label, as expressed by:

$$\text{Red} = A\,(\text{IdUrd} + \alpha\,\text{CldUrd}) \quad (1)$$

$$\text{Green} = B\,(\text{CldUrd} + \beta\,\text{IdUrd}), \quad (2)$$

where red and green are the intensities of the fluorescence signals, α and β are the cross-talk factors, A and B are gain constants and IdUrd and CldUrd are the relative quantities of the incorporated labels. We calculated the cross-talk factors by using cultures of cells labelled with either IdUrd or CldUrd (Fig. 1A-C). From each culture preparations were doubly stained and dual-channel images were recorded of several nuclei. For each image the intensities of both colours of the voxels were plotted in a bivariate fluorescence intensity histogram (Fig. 1D and E). The number of voxels was represented as a function of their red and green fluorescence. These histograms have an elongated shape. The part close to the origin of the histogram represents the low-intensity background voxels. Object voxels are represented by the tails further away from the origin. From the angles between the tails and the axes the cross-talk factors α and β were calculated.

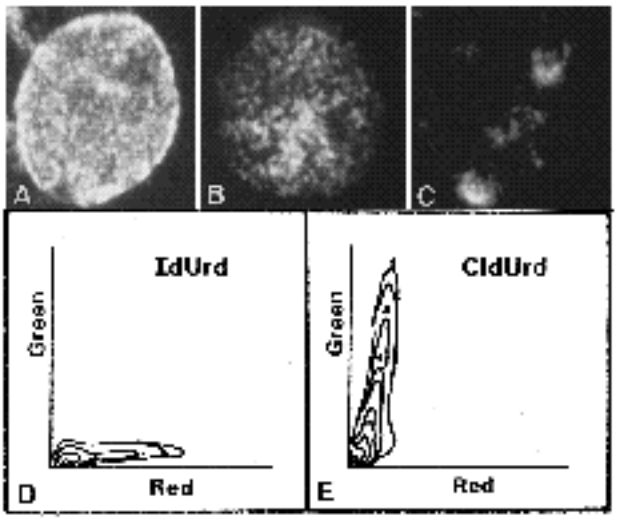

Fig. 1. Replication patterns typical for three different periods of the S-phase. The pattern with many small granules (A) is typical of the early S-phase. A few clusters of bright replication foci (C) were found in nuclei labelled in the late S-phase; (B) shows an example of the third, intermediate type. From the images of nuclei singly labelled with IdUrd or CldUrd and doubly stained with FITC and Texas Red, bivariate fluorescence intensity histograms were made (D and E). In these contour plots the number of voxels is plotted as a function of the local red and green fluorescence.

Once the cross-talk factors were determined, the relative amounts of CldUrd and IdUrd incorporation were derived from equations (1) and (2). A transformation using this solution was applied to all dual-channel images of doubly labelled nuclei. The transformed images, in contrast to the uncorrected images, are biologically relevant because local intensities are directly related to the amount of locally incorporated label.

Subsequently, local background, caused by residual out-of-focus fluorescence and non-specific staining, was removed. Background was determined using a 51×51×13 uniform filter (lowpass) on the three-dimensional images. The resulting background images were subtracted from the original images and negative voxels of the new images were set to zero.

Correlation

We have analysed the changes in the replication patterns in time by estimating the degree of overlap of the red and green spots. For this we used the Pearson correlation coefficient (r) between the red and the green component of each dual-channel image:

$$r = \frac{\sum_i (R_i - R_{av}) \cdot (G_i - G_{av})}{\{\sum_i (R_i - R_{av})^2 \cdot \sum_i (G_i - G_{av})^2\}^{\frac{1}{2}}}, \quad (3)$$

where R_i and G_i are the red and green intensities of voxel i, respectively, and R_{av} and G_{av} the average values of R_i and G_i, respectively.

Image representation

The colour photographs (Fig. 2A-F) show optical sections through the middle of six nuclei. These photographs were made directly from a screen. After cross-talk correction and background removal, the images were displayed on the screen using a colour lookup table (LUT) that was designed to visualize two fluorescence signals in a single image. Texas Red intensities ranged from black to red; FITC from black to green and combinations of both signals ranged from black to yellow.

Results

Replication patterns

Cells of singly labelled control cultures showed nuclei with incorporated halogenated deoxyuridine (56%) and nuclei without incorporated label (44%). In the population of labelled nuclei we distinguished three types of replication patterns. Of the labelled nuclei 65% showed a large number (hundreds) of small granules (Fig. 1A). This pattern is typical of early and mid S-phase (Nakayasu and Berezney, 1989). For simplicity we will call this kind of pattern the early type pattern. Eighteen per cent of the cells showed only a few large, bright replication foci (Fig. 1C), typical of late S-phase (Fox et al., 1991). Fig. 1B shows a third kind of pattern, an intermediate form with small granules in combination with a few bright spots (16% of the labelled nuclei). From these data we estimated that during the first 4 h of the S-phase replication foci are distributed throughout the nuclear interior (the early pattern), while during the last hour the typical late pattern is visible. The intermediate pattern is visible during the 5th hour of the S-phase.

In preparations of doubly labelled cell cultures we distinguished unlabelled, singly labelled and doubly labelled nuclei. The cells with doubly labelled nuclei were in S-phase during both labelling periods (15%, 35% and 51% of the total population of cells of cultures labelled with 5 h, 3 h and 1 h between the labels, respectively, and 56% of the simultaneously labelled cultures). In each doubly labelled nucleus we observed two replication patterns; a red pattern from the first label (IdUrd) and a green one from the second label (CldUrd).

When cells were scored for early, intermediate or late replication patterns, the doubly labelled nuclei observed in the 5-h experiment showed either two similar (10%) or two different (90%) types of replication patterns. Most of latter population (70%) showed a typical early pattern in red (first label) in combination with a late pattern in green (second label), in agreement with an S-phase of about 6 h and a time interval between the labels of 5 h.

In the experiments with a 3-h interval between the labels, 48% of the doubly labelled cells showed two similar patterns and 52% showed two different patterns. Some combinations of patterns, e.g. a late type pattern in red (first label) and an early or intermediate type pattern in green (second label), were never observed in nuclei of these experiments. This observation underlines the strict sequence of replication patterns during the S-phase.

In the population of doubly labelled nuclei in the experiments with the 1-h interval we very often found (82%) two very similar replication patterns (for example, an early type pattern in red and another (not identical) early pattern in green). The other 18% of the nuclei showed combinations of two different pattern types (early-intermediate (7%) or intermediate-late (11%)).

All doubly labelled nuclei of the simultaneously labelled cell culture, as expected, showed two identical replication patterns (for example, an early pattern in red and the same early pattern in green).

Spatial distribution of replication foci

We have studied the three-dimensional localization of regions containing DNA labelled at different periods of the S-phase, by dual-colour confocal scanning laser microscopy. Fig. 2 shows optical sections through the centre of six nuclei, representing typical results obtained with the double-labelling protocols used in these experiments. The red spots in the images represent DNA regions that were replicated only in the presence of IdUrd. The green spots are replication foci labelled by CldUrd. The yellow regions represent sites where both labels were incorporated.

Fig. 2A shows an optical section through a nucleus of a cell labelled with IdUrd and CldUrd at the same time. Here virtually all replication foci are yellow. All doubly labelled nuclei in this preparation showed an essentially complete overlap of replication patterns.

The time interval between IdUrd and CldUrd pulse labels in the nuclei in Fig. 2B and C was 1 h. In both images the green pattern of replication foci is similar to the red pattern of the chased IdUrd pulse (in Fig. 2B both are patterns of the early type and in Fig. 2C the patterns are both typical of late S-phase). Most of the fluorescent spots are either red or green. The overlap between the first and second label (the yellow regions) is much less than that in Fig. 2A. Fig. 2D and 2E shows nuclei labelled with a 3-h interval. Fig. 2D shows two similar but not identical early patterns and Fig. 2E shows two different pattern types (early pattern in red and a late pattern in green). When the time interval between the labels was increased to 5 h (Fig. 2F) we observed that most of the doubly labelled nuclei (70%) had a large number of small granules in the early pattern (red) and a few larger spots in the late pattern (green). A striking observation in this study was the almost complete absence of overlapping replication foci in nuclei of cells which were labelled with a time interval between the labels of 3 h or more. Even with a time interval between the labels of only 1 h the overlap was relatively small.

Image analysis

To perform a statistical analysis of the confocal images we quantified the degree of overlap between replication patterns in doubly labelled nuclei. First the images were preprocessed. Fig. 3 shows the result of preprocessing an image of a red and a green labelled nucleus. In this figure we used a square-root lookup table (LUT). This LUT accentuates the weak background fluorescence that was present in the images before preprocessing. To assess quantitatively the changes in replication patterns, we measured the extent of overlap of 69 preprocessed images of nuclei double labelled with a time interval of 1, 3 and 5 h between the labels and of nuclei incubated with both labels simultaneously. The measure used in these experiments was the correlation coefficient between the intensities of the two labels of the individual image voxels. In Fig. 4 the average values for this correlation coefficient are presented for the different pulse intervals. These data clearly demonstrate that the correlation coefficient decreases rapidly with increasing time interval between the labels. Nuclei that were labelled simultaneously had two almost identical replication patterns

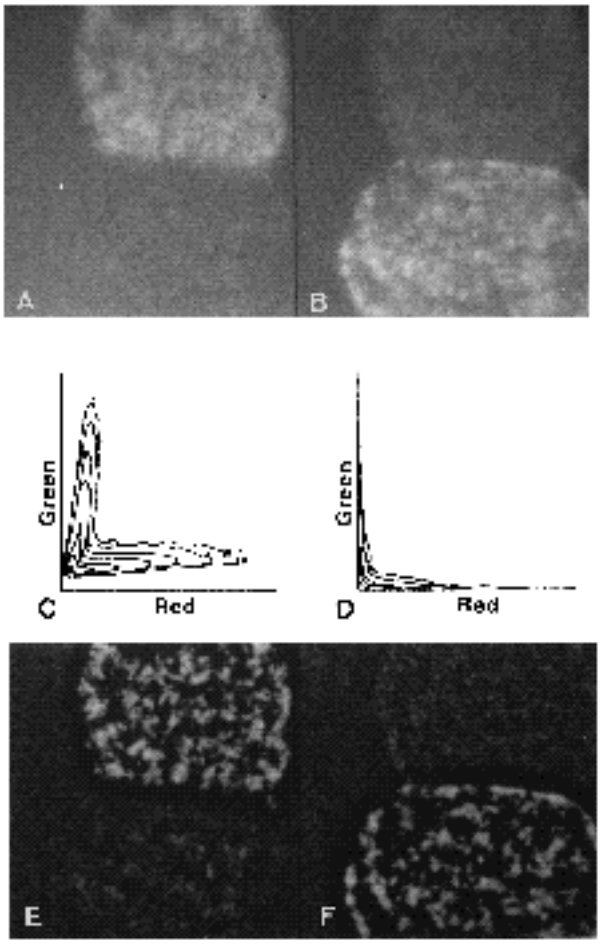

Fig. 3. A demonstration of the preprocessing procedure applied to two adjacent singly labelled nuclei recorded with the confocal microscope. The dual-colour image of these nuclei was split into two parts, a red component (A) and a green component (B). (C) Shows the bivariate histogram of the original image. After cross-talk and background correction local grey values of the corrected images reflect amounts of locally incorporated label. The red component of this corrected image is shown in (E) and the green component in (F). The bivariate histogram of the corrected image is shown in (D). To accentuate local background and fluorescence caused by cross-talk a square-root lookup table was used.

(high correlation coefficient). Early and late replication patterns did not overlap at all (low correlation at 5 h). With a time interval of 3 h between the labels little or no overlapping fluorescent spots were observed. Nuclei labelled with a time interval of 1 h showed a correlation coefficient of 0.24 (±0.9).

Morphology

Fig. 5A-C shows the Texas Red signal of optical sections through the middle of three nuclei labelled first for 5, 15 and 60 min with IdUrd, respectively, and then with CldUrd. The time interval between the labels was 1 h. By selecting singly (IdUrd) labelled nuclei we had confidence that replication patterns of the late S-phase were recorded. The regions of replicated DNA shown in Fig. 5C are obviously larger and more intense than those in Fig. 5A, which

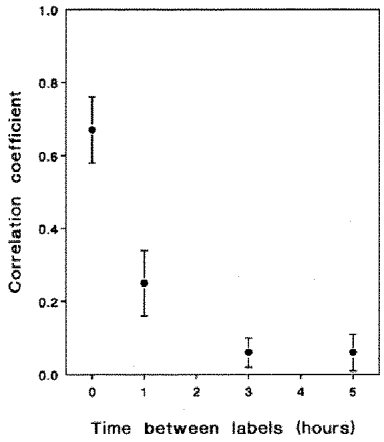

Fig. 4. The correlation coefficients between the label intensities of the red and the green components of images of doubly labelled cells reflect the amount of overlap between the labelled regions. When nuclei are labelled at the same time (0 h) the label intensities are highly correlated (almost complete overlap). At 3-h or 5-h intervals between the labels, almost no overlap is observed (correlation coefficient close to zero). Bars represent standard deviations.

suggests that the DNA domains spread away from the sites of synthesis.

Discussion

We have investigated the temporal and spatial behaviour of DNA replication in interphase nuclei of V79 Chinese hamster cells. This study was performed using a combination of three techniques: fluorescence double labelling of DNA replication, dual-colour confocal microscopy, and quantitative three-dimensional image analysis. Double labelling of replicating DNA with IdUrd and CldUrd allowed us to study the time dependence of the replication process in detail. DNA replicated at two different periods of the S-phase was visualized in the same nucleus. Dual-colour confocal microscopy was used for the analysis of the three-dimensional positions of fluorescent spots. To preclude pixel shift between the two colours we recorded fluorescence signals from both fluorochromes simultaneously. In our experiments we used a stage-scan confocal microscope. This CSLM requires on-axis imaging only. Thus, chromatic aberration is negligible for each point of the image. The presence of chromatic aberration would in fact disturb the measurement of the overlap between two patterns. Quantitative analysis of dual-colour three-dimensional images, using a correlation technique, allowed a statistical evaluation of the extent of overlap of regions of DNA replicated at two different periods of the S-phase. Only after preprocessing of the images, did the correlation coefficient provide reliable information about the extent of overlap.

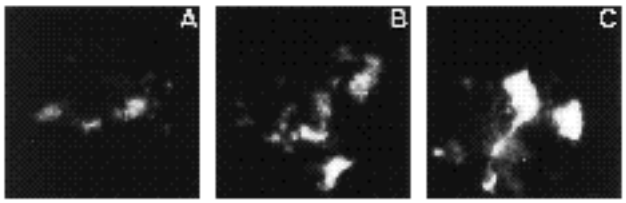

Fig. 5. Cells labelled for 5, 15 and 60 min with IdUrd in the late S-phase. Obviously the labelled regions grow, which indicates that DNA domains spread away from the sites of synthesis.

We distinguished three different types of replication patterns in the cell nuclei. This observation is consistent with that of Nakayasu and Berezney (1989), who described three distinct patterns of replication foci: type I (early to mid S), type II (mid to late S) and type III (late S). Nascent DNA in a nucleus with a type I pattern is located in numerous granules distributed throughout the nuclear interior. Patterns of type III show a few bright regions that often are composed of clusters of granules. Type II is a form intermediate between types I and III. We prefer to call these three pattern types the early, mid and late types. This nomenclature suggests a division of the S-phase into three periods, each with its own specific type of replication pattern. In the early S-phase, the first 4 h of the S-phase (total length 6 h) of the V79 cells, we observed the early pattern and during the last hour (late S-phase) cells displayed typical late patterns.

Based on DNA single labelling data and on biochemical studies (Laskey et al., 1989; Villarreal, 1991), progress has been made in understanding the organization of DNA replication in eukaryotic cells. In this study we investigated the organization of DNA replication using a double-labelling (pulse-chase-pulse) procedure. We visualized the spatial distribution of regions containing DNA labelled at two different periods of the S-phase. In our experiments, these regions are visible as fluorescent spots. It must be noticed that the red fluorescent spots (first label) represent DNA replicated one or more hours before fixation and do not necessarily reflect the sites in the nucleus where it was replicated. Nascent DNA may be moved through the nuclear interior, forced by the replication process, as suggested by other investigators (Nakamura et al., 1986; Mills et al., 1989) and our experiments shown in Fig.5. Cells were fixed immediately after the second pulse label. The green spots, therefore, do with certainty reflect the sites of replication, i.e. the replication foci.

Quantitative analysis (Fig. 4) showed that the degree of overlap between regions labelled with IdUrd and regions labelled with CldUrd 1 h later is small. It can therefore be concluded that the average replication time of a chromatin region, i.e. the time interval between initiation and termination of DNA replication in that region, is about 1 h. This conclusion is consistent with the observation of Nakamura et al. (1986) that cells labelled at the beginning of the S-phase showed replication at discrete foci. They observed that these fluorescent spots grew during 1 h, after which the replication machinery started at other sites. We have observed that the replication time of a region is about 1 h, not only in the early S-phase (Fig. 2B) but also in the late S-phase (Fig. 2C).

The DNA regions replicated in the early S-phase are small (Fig. 2B and D) and do not overlap when they are labelled at different periods of the early S-phase. This confirms the idea posed by several investigators (Mills et al.,

1989; Nakamura et al., 1986; Nakayasu and Berezney, 1989) that each replication focus in early S phase consists of a single cluster containing adjacent replication units, called replicon clusters. In late S-phase the fluorescent regions are larger and more intense than those in early S. Consequently, in the late S-phase there must be numerous replicon clusters situated in each fluorescent region. Fig. 2C shows a nucleus labelled in the late S-phase with 1 h between the labels. All fluorescent regions in nuclei labelled in this way are either red or green (besides some yellow voxels on red-green borders), each containing many replicon clusters. From this observation we conclude that during the late S-phase replicon clusters situated in the same region are replicated synchronously. Because the absence of yellow spots in the red regions we conclude that after a period of replication there are no unreplicated clusters left in this region. These observations indicate that clusters are replicated in a strictly sequential order.

In the nucleus displayed in Fig. 2C two green regions (arrowheads) can be observed, both with two adjacent red regions. This type of double-labelling pattern was observed frequently in late S-phase nuclei. The green spots, reflecting DNA that was labelled just before fixation, should colocalize with the site where the replication process takes place. If we assume that replication occurs at fixed sites in the nucleus (Berezney and Coffey, 1975; Padroll et al., 1980), it is likely that the red-labelled DNA regions were replicated at the sites of the green spots 1 h before fixation. After replication, this chromatin may have been moved away from the fixed dedicated polymerization sites, as postulated by Jackson (1990) and Cook (1991), forced by the replication process.

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Fig. 2. Optical sections (CSLM) through the middle of nuclei doubly labelled with two different halogenated nucleotides. The nucleus in (A) was labelled with IdUrd and CldUrd at the same time. The time interval between the incorporation of the labels in the other nuclei was 1 h (B and C), 3 h (D and E) and 5 h (F). IdUrd-labelled DNA (first label) is depicted in red and CldUrd-labelled DNA is green. (F) Shows early and late replicated DNA in the same nucleus.