

Dynamic Organization of DNA Replication in Mammalian Cell Nuclei: Spatially and Temporally Defined Replication of Chromosome-specific α -Satellite DNA Sequences

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Abstract. Five distinct patterns of DNA replication have been identified during S-phase in asynchronous and synchronous cultures of mammalian cells by conventional fluorescence microscopy, confocal laser scanning microscopy, and immunoelectron microscopy. During early S-phase, replicating DNA (as identified by 5-bromodeoxyuridine incorporation) appears to be distributed at sites throughout the nucleoplasm, excluding the nucleolus. In CHO cells, this pattern of replication peaks at 30 min into S-phase and is consistent with the localization of euchromatin. As S-phase continues, replication of euchromatin decreases and the peripheral regions of heterochromatin begin to replicate. This pattern of replication peaks at 2 h into S-phase. At 5 h, perinucleolar chromatin as well as peripheral areas of heterochromatin peak in replication. 7 h into S-phase interconnecting patches of

electron-dense chromatin replicate. At the end of S-phase (9 h), replication occurs at a few large regions of electron-dense chromatin. Similar or identical patterns have been identified in a variety of mammalian cell types. The replication of specific chromosomal regions within the context of the BrdU-labeling patterns has been examined on an hourly basis in synchronized HeLa cells. Double labeling of DNA replication sites and chromosome-specific α -satellite DNA sequences indicates that the α -satellite DNA replicates during mid S-phase (characterized by the third pattern of replication) in a variety of human cell types. Our data demonstrates that specific DNA sequences replicate at spatially and temporally defined points during the cell cycle and supports a spatially dynamic model of DNA replication.

DURING S-phase of the mammalian cell cycle, the cell must copy the millions of nucleotides which form its genome with precise fidelity within a complex three-dimensional network to maintain genetic integrity (Jackson, 1990). DNA replication initiates at origins of replication that occur in clusters or replication units (Cairns, 1966; Huberman and Riggs, 1968; Hand, 1978) which are activated at different times throughout S-phase (Taylor, 1960; Hand, 1978). The timing of replication of some multicopy genes (Stambook, 1974; D'Andrea et al., 1983) (reviewed by Balazs et al., 1974) and some single copy genes (Epner et al., 1981; Furst et al., 1981; Braunstein et al., 1982; Calza et al., 1984; Goldman et al., 1984; Brown et al., 1987; Hatton et al., 1988a,b; Dhar et al., 1989; Taljanidisz et al., 1989) during S-phase has been demonstrated at the biochemical level. Several factors have been shown to influence the timing of replication of different genes. The transcriptional activity of a gene appears to be important in the timing of its replication (Goldman et al., 1984; Hatton et al., 1988a,b; Dhar et al., 1989; Taljanidisz et al., 1989). In general, actively transcribed genes seem to replicate early in S-phase. Many inactive genes have been shown to replicate late, although, some

have been shown to replicate at other times during S-phase. The location of a gene on a chromosome has also been shown to affect its timing of replication (Calza et al., 1984; Hatton et al., 1988b). For example, when a late-replicating gene is brought into an early-replicating region of a chromosome by immunoglobulin gene rearrangement, the late-replicating gene replicates early (Calza et al., 1984). Chromatin structure is another factor that has been found to be involved in the replication timing of different genes. When the inactive, late-replicating β -globin gene from fibroblasts is transcriptionally activated by fusion with mouse erythroleukemia hybrid cells, the transcriptionally activated gene becomes early replicating and more sensitive to nucleases, indicating that there is a change in chromatin structure (Dhar et al., 1989).

The coordination of a specific temporal order of replication of DNA sequences found at different sites throughout the genome suggests the need for spatial order for efficient nuclear function. A number of recent studies indicate that the nucleus is organized. There is evidence that specific chromosomes or chromosomal domains occupy specific locations in the nucleus and these locations are consistent between cells of the same cell type. For example, the chromo-

somes of *Drosophila* cells have been shown to be organized within the nucleus by folding in a specific manner and by contacting the nuclear surface at specific sites (Agard and Sedat, 1983; Mathog et al., 1984; Hochstrasser et al., 1986; Hochstrasser and Sedat, 1987a,b). Often, the disposition of the nucleolus is characteristic of the cell type. In epithelial cells of insects, the nucleolar patterns of adjacent cells are mirror images of each other (Locke and Leung, 1985). In the interphase nuclei of both human and mouse central nervous system cells, specific chromosome domains (centromeric alphoid repeats) have been shown to be organized in a reproducible manner (Manuelidis and Borden, 1988). It has been hypothesized that the three-dimensional structure of the genome is maintained or altered by the nuclear pore complex, the peripheral nuclear lamina, and components of the nuclear core (Blobel, 1985). This hypothesis suggests that genes are "gated" to specific nuclear pores that are nonrandomly distributed on the nuclear surface reflecting the underlying organization of the genome. Nuclear regions enriched in small nuclear ribonucleoprotein particles (snRNPs)¹ and another non-snRNP pre-mRNA splicing factor (SC-35) have also been shown to be organized within the interphase nucleus in the form of a reticular network that extends between the nucleolar surface and the nuclear envelope (Spector, 1990; Spector et al., 1991).

In previous studies, the sites of DNA replication have been localized by the incorporation of [³H]thymidine and it has been determined that regions of euchromatin replicate early in S-phase and that regions of heterochromatin replicate later in S-phase (Hay and Revel, 1966; Milner, 1969; Williams and Ockey, 1970; Huberman et al., 1973; Fakan and Hancock, 1974; Fakan, 1978; Smith et al., 1984; Spector, 1990). More recently, the sites of DNA replication have been visualized by immunofluorescence microscopy of incorporated biotin labeled dUTP (Nakayasu and Berezney, 1989) or 5-bromodeoxyuridine (BrdU) (Nakamura et al., 1986; van Dierendonck et al., 1989; Fox et al., 1991). These studies have identified different patterns of dUTP or BrdU localization that occur in a specific order and at different times during S-phase (Nakayasu and Berezney, 1989; van Dierendonck et al., 1989) as well as within defined regions of the three-dimensional space of the nucleus (Fox et al., 1991). In addition, initial EM studies of immunogold-labeled incorporated BrdU in mammalian nuclei during S-phase have identified immunogold labeling patterns similar to those visualized by fluorescence microscopy (Mazzotti et al., 1990). Whereas these studies have identified the distribution of DNA replication sites in the mammalian cell nucleus, there has not yet been a precise study directed at visualizing which DNA sequences are replicating within these regionally defined sites of replication.

The availability of high resolution methods for visualizing the sites of DNA replication within a single cell allows us to determine when and where specific DNA sequences replicate. In an attempt to address this question, we have synchronized CHO and HeLa cells to determine the exact progression of the arrangement of DNA replication sites within the nucleus during S-phase. Short pulses of BrdU at

specific time points during S-phase following cell synchronization allows for the precise temporal localization of DNA replication sites. In this study five different patterns of DNA replication have been identified and their timing during S-phase has been determined. In addition, the three-dimensional organization of these five patterns of DNA replication and the types of chromatin that are replicating in each of these patterns has been established. By combining BrdU labeling with in situ hybridization of DNA sequences and examining by confocal microscopy, we can determine when these sequences replicate within a cell in relation to the replication patterns and assess the differences and/or similarities in replication between individual cells within a population. This has enabled the spatial and temporal replication of specific DNA domains to be determined. We have found that α -satellite DNA sequences, which localize to either the nucleolar surface and/or the nuclear periphery, replicate during mid S-phase.

Materials and Methods

Cell Culture

CHO cells (CCL61; American Type Culture Collection, Rockville, MD) were cultured in Ham's F12 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS, 1% penicillin, and 1% streptomycin. HeLa cells (human cervical carcinoma; ATCC CCL 2) and MRC5 cells (male human lung diploid fibroblast; ATCC CCL 171) were cultured in DME (Gibco Laboratories) supplemented with 10% FBS and 1% penicillin/streptomycin. WI-38 cells (female human lung diploid fibroblast; ATCC CCL 35), Detroit 551 cells (female human skin diploid fibroblast; ATCC CCL 110), and MG-63 cells (male human osteosarcoma; ATCC CRL 1427) were cultured in MEM (Gibco Laboratories) supplemented with 10% FBS and 1% penicillin/streptomycin. SW-620 cells (male human colon adenocarcinoma; ATCC CCL 227) were cultured in Leibovitz's L-15 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS and 1% penicillin/streptomycin. Human umbilical vein endothelial (HUVE) cells, collected from a single female umbilical vein, were cultured in CS-C medium (Cell Systems, Kirkland, WA). All cell types were plated and grown on glass coverslips in 35-mm culture dishes.

Cell Synchronization

CHO cells were synchronized by a modification of the method described by Ashihara and Baserga (1979). At 10% confluency, CHO cells on coverslips were washed three times with serum-free F12 media at 37°C and then cultured in this medium for 66 h. This arrests the cells in G₀ of the cell cycle. G₀ arrest was determined by observing the number of cells in mitosis at different time points following the addition of serum-free F12 medium. The time point of 66 h was the first during which no mitotic cells were observed. F12 with 10% FBS was then added to the growth-arrested cells for 4 h to allow the cells to enter G₁. Addition of hydroxyurea (Sigma Chemical Co.) to a final concentration of 1.5 mM subsequently blocked the cells at the G₁/S border. 14 h after addition of hydroxyurea, the cells were washed three times with PBS, pH 7.4, at 37°C to release the cells from the G₁/S block. F12 with 10% FBS was then added to the cells to allow the cells to progress synchronously through S-phase. At specific time points following removal of the hydroxyurea the cells were fixed and processed as described below.

HeLa cells were cultured in DME with 10% FBS for 2 d before the addition of hydroxyurea to a final concentration of 1.5 mM to block cells at the G₁/S border. Cells were then incubated in hydroxyurea-containing medium at 37°C for an additional 14 h. Cells were released from hydroxyurea block by washing three times with PBS, pH 7.4, at 37°C and incubating in fresh DME containing 10% FBS at 37°C which allowed them to progress synchronously through S-phase.

BrdU Labeling

BrdU (Sigma Chemical Co.) was added to both synchronous and asynchro-

1. Abbreviations used in this paper: BrdU, 5-bromodeoxyuridine; HUVE, human umbilical vein endothelial; snRNPs, small nuclear ribonucleoprotein particles.

nous cultures to a final concentration of 10 μ M for 10 min at 37°C. Cells were sampled from synchronized cultures on an hourly basis beginning immediately after removal of the hydroxyurea block. BrdU was added 10 min before the collection of each coverslip.

Fixation

After incorporation of BrdU, the cells were washed three times with PBS, pH 7.4, at 37°C and fixed in 2% formaldehyde in PBS, pH 7.4, for 15 min at room temperature. Following fixation, the cells were washed three times with PBS, pH 7.4, permeabilized with 0.2% Triton X-100 in PBS for 5 min at 4°C and then washed in PBS. For studies which combined BrdU labeling with *in situ* hybridization, the fixative consisted of 2% paraformaldehyde plus 5 mM MgCl₂ in PBS, pH 7.4. Postfixation washes were in 0.3 M glycine in PBS, pH 7.4. The cells were then permeabilized and washed as outlined above.

To preserve nuclear morphology, hypotonic swelling, ethanol dehydration, and air drying (steps commonly employed in *in situ* hybridization protocols) were not used.

Denaturation

For conventional immunofluorescence studies, the cellular DNA was denatured with 4N HCl for 30 min at room temperature. For *in situ* hybridization combined with BrdU labeling, the cellular DNA was denatured using a modification of the method described by Humbert et al. (1990). Cells were treated with 0.7% Triton X-100 in 0.1 M HCl for 10 min on ice. The cells were next washed twice with 2 \times SSC and then were incubated in 50% deionized formamide in 2 \times SSC at 80°C for 30 min by floating the petri dishes containing the coverslips in an 80°C waterbath. The cells were then washed twice with ice-cold 0.5% Tween-20 in PBS, pH 7.4 followed by two washes in 2 \times SSC. Both denaturation protocols gave identical BrdU-labeling patterns. The temporal order and distribution of BrdU labeling were unaffected by either strong acid at room temperature or weak acid/formamide at 80°C in all cell types studied. Furthermore, electron microscopic examination of BrdU incorporation (see below), in which the BrdU was made accessible by sectioning and involved no DNA denaturation, gave similar labeling patterns.

Probes

Biotinylated DNA probes to α -satellite DNA of human chromosomes 2, 3, 8, 12, 17, and X were purchased from Oncor (Gaithersburg, MD). DNA probes to α -satellite DNA of human chromosome 1 (pE25.a/ATCC 61304/0.349-kb insert; Carine et al., 1989), chromosome 8 (pJM128/ATCC 59904 2.55-kb insert; Donlon, T., H. R. Wyman, J. Mulholland, D. Barker, G. Bruns, S. Latt, and D. Botstein. 1986. *Am. J. Hum. Gen.* 39:A196), chromosome 10 (p α 10RP8/ATCC 61396/1.35-kb insert; Devilee et al., 1988), chromosome 12 (p α 12H8/ATCC 61398/1.35-kb insert; Looijenga et al., 1990), chromosome 17 (p18H8/H. Willard/2.7-kb insert; Wayne and Willard, 1986), chromosome 18 (LI.84/ATCC 61394/0.68-kb insert; Devilee et al., 1986), and chromosome X (pBamX7/H. Willard/2.0-kb insert; Wayne and Willard, 1985) were labeled with biotin-11-dUTP by nick translation (Langer et al., 1981).

Antibody Labeling

For immunolabeling of replication alone, after DNA denaturation, the cells were washed with PBS, pH 7.4, and incubated with a fluorescein-conjugated mouse mAb to BrdU (Boehringer-Mannheim Biochemicals, Indianapolis, IN) at a dilution of 1:5 for 1 h at room temperature in the dark. The cells were then washed with PBS, pH 7.4, and coverslips were mounted in 9:1 glycerol/PBS containing 0.1% *p*-phenylenediamine buffered to pH 8.0 with 0.5 M carbonate/bicarbonate buffer (Johnson and Nogueira Araujo, 1981).

In situ Hybridization and Detection

50 ng of each probe plus 10 μ g of sheared salmon sperm DNA and 15 μ g of *Escherichia coli* tRNA was dried in a SpeedVac DNA 100; (Savant Instruments, Inc., Hicksville, NY) and then resuspended in 13 μ L of 100% deionized formamide. The probes were denatured by heating in an 80°C waterbath for 10 min and then were placed immediately on ice. 7 μ L of concentrated hybridization buffer was added to each probe to give a final concentration of: 65% formamide, 2 \times SSC, 2 \times Denhardt's solution, 10% dextran sulfate, and 50 mM Tris, pH 7.5. The probes in hybridization buffer (20 μ L total volume) were added to the coverslips, placed cell-side down

on a glass microscope slide, and sealed with rubber cement. After incubation in a 42°C incubator overnight, the coverslips were washed twice (30 min each) in 65% formamide in 2 \times SSC at 42°C and then washed (30 min each) in 2 \times , 1 \times , and 4 \times SSC at room temperature. The coverslips were then incubated in FITC-conjugated anti-BrdU (1:5) (Boehringer Mannheim Biochemicals), Texas red-conjugated avidin D (1:200) (Vector Laboratories Inc., Burlingame, CA), 1% BSA in 2 \times SSC in the dark for 60 min at room temperature. After labeling, the cells were washed with 2 \times and 4 \times SSC containing 0.1% Tween-20, and three washes of PBS, pH 7.4. Coverslips were then mounted as described above.

Light Microscopy

Cells prepared for immunofluorescence were photographed with Kodak T-Max 3200 film (Eastman Kodak Co., Rochester, NY) using a Nikon Microphot-FXA microscope (Nikon Inc., Garden City, NY) equipped with standard epifluorescence and differential interference contrast optics.

A Zeiss Confocal Laser Scanning microscope (Carl Zeiss, Thornwood, NY), equipped with a 63 \times /1.4 N.A. oil immersion lens, an Argon Ion Laser (λ = 488 nm) to excite FITC fluorescence and a Helium/Neon laser (λ = 543 nm) to excite Texas red fluorescence, was used for optical sectioning. Data sets of optical sections were collected at 250-nm intervals through each nucleus. Single optical sections, left/right stereo pairs of labeled nuclei reconstructed from serial optical sections, and two-color double-labeled images were generated using the Zeiss Confocal Laser Scanning software Version 2.03 (Carl Zeiss). For double-label studies, the focal plane for hybridization of α -satellite DNA (Texas red fluorescence) was determined and an optical section was taken, protected (i.e., stored in memory) and assigned the color red. Immediately, a second section in the same focal plane, of the BrdU labeling, was taken in the other fluorescence channel (i.e., FITC fluorescence), assigned the color green, and superimposed on the first section. Areas of coincidence appeared as yellow by this method. Images were recorded on a Sony (Montvale, NJ) color video printer (Mavigraph) and on Kodak T-Max 100 film or Kodak Ektachrome 400 film using a Matrix MultiColor image recording system.

Immunoelectron Microscopy

The technique of freeze substitution employed in this study, offers the advantages of improved ultrastructural preservation with no loss of antigenicity of the sample. CHO cells were grown on 3 \times 5 mm Thermoform coverslips (Miles Scientific; Naperville, Illinois) in 35-mm culture dishes and synchronized as described above. After synchronization, BrdU was added to the cells for 1 h at 37°C at a concentration of 10 μ M. The cells on Thermoform coverslips were then washed in serum-free F12 medium at 37°C, rapid frozen with a CF100 cryofixation unit (Research and Manufacturing Company Inc., Tucson, AZ), and transferred to liquid nitrogen. The cells were then freeze substituted for 3 d at -80°C and 1 d at -20°C with 0.5% osmium tetroxide in acetone. After freeze substitution, the cells were slowly warmed to room temperature and then washed three times for 10 min with acetone before infiltration with 50% acetone and 50% Epon/Araldite overnight. Infiltration continued with three changes of 100% Epon/Araldite after which, the cells were embedded in Epon/Araldite and polymerized overnight at 60°C. Ultrathin (60–80 nm) sections were cut *en face* with a diamond knife on a Reichert Jung Ultracut E ultramicrotome and collected on gold grids. The sections were incubated with 1% BSA/1% normal goat serum for 1 h. The grids were washed with 1% Tween-20 in TBS, pH 7.6, for 15 min and then incubated with a mouse mAb to BrdU (Boehringer-Mannheim Biochemicals) at a dilution of 1:3 overnight at 4°C. The grids were washed for 15 min with TBS, pH 7.6, and then incubated with 30-nm gold-conjugated goat anti-mouse IgG (Amersham Corp., Arlington Heights, IL) diluted 1:15 for 1 h at room temperature. The grids were then washed with TBS, pH 7.6, for 15 min, washed with filtered, deionized water for 15 min, and air dried. The grids were stained with aqueous 2% uranyl acetate and lead citrate (Reynolds, 1963) and viewed with a Hitachi H-7000 transmission electron microscope (Hitachi Ltd., Rockville, MD) operated at 75 kV.

Results

Cell Synchronization and BrdU Labeling

Visualization of DNA replication sites by immunofluorescence microscopy of BrdU incorporation in an asynchronous

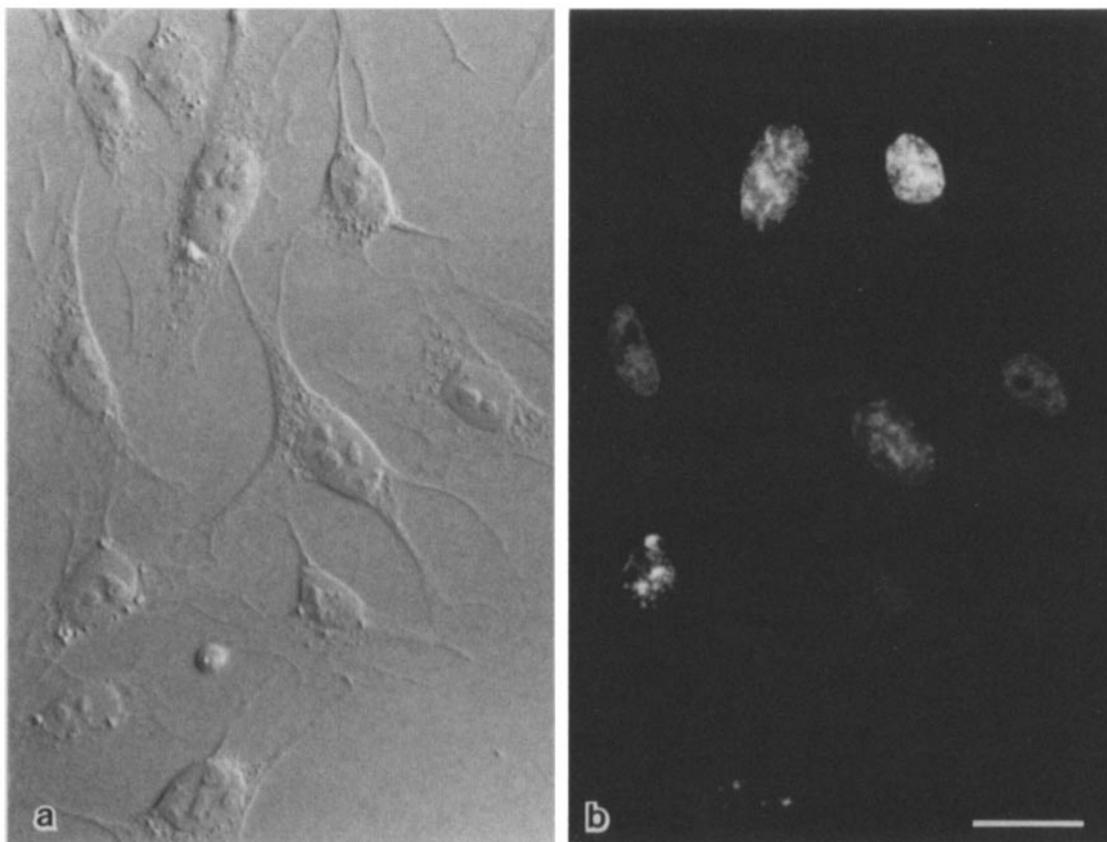


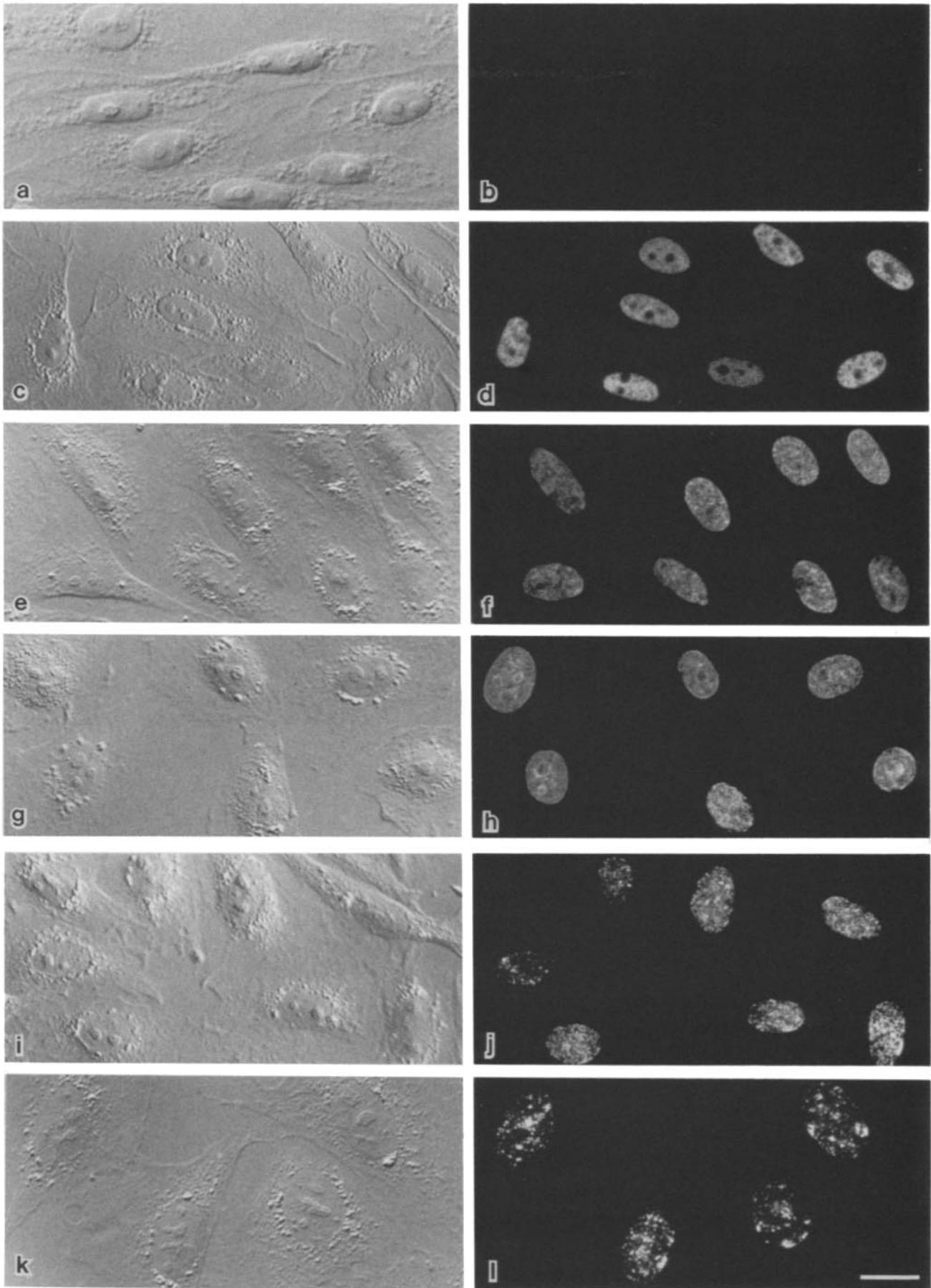
Figure 1. Different patterns of BrdU incorporation are found in an asynchronous population of CHO cells. CHO cells were labeled with BrdU followed by FITC-conjugated anti-BrdU antibodies. Differential interference contrast optics (*a*) and immunofluorescence (*b*) indicate that in a random cell population there are five different patterns of BrdU incorporation and that some cells do not incorporate BrdU. Bar, 20 μm .

population (Figs. 1, *a* and *b*) revealed that the labeled cells exhibited five different patterns of localization of DNA replication sites. To determine if these patterns of replication were associated with a temporal progression through S-phase of replicating cells, CHO cells were synchronized by serum deprivation and hydroxyurea block. Cells were then released from the hydroxyurea block and allowed to progress through S-phase synchronously. BrdU labeling of CHO cells, at specific time points after hydroxyurea release, made it possible to determine the temporal relationship between the different patterns of DNA replication. This relationship was observed by a number of different methods. First, cells were observed by standard immunofluorescence microscopy to determine if the patterns of replication observed in the asynchronous population were enriched at certain time points in

S-phase (Fig. 2). Immunofluorescence was also used to quantitate the occurrence and timing of the five patterns of DNA replication observed during S-phase (Fig. 3). Next, to investigate the three-dimensional organization of the five patterns of replication, we analyzed each pattern by confocal laser scanning microscopy (Figs. 4, 5, and 6). This three-dimensional analysis clearly revealed the differences in the organization of the five DNA replication patterns. Subsequently, in an attempt to determine the ultrastructural localization of the sites of DNA replication and to identify the types of chromatin associated with these sites, immunoelectron microscopy was performed (Figs. 7, 8, and 9).

CHO cells blocked in G₁ were labeled with BrdU and visualized by immunofluorescence microscopy. Cells blocked in G₁ did not display any fluorescent labeling indicating that

Figure 2. Five patterns of DNA replication are found at specific times in synchronized CHO cells following BrdU incorporation. CHO cells were observed by differential interference contrast microscopy (*a*, *c*, *e*, *g*, *i*, and *k*) and by immunofluorescence microscopy of an FITC-labeled antibody specific for incorporated BrdU (*b*, *d*, *f*, *h*, *j*, and *l*). CHO cells blocked in S-phase by the synchronization procedure do not display any BrdU labeling (*a* and *b*). At 0.5 h after release of synchronized CHO cells into S-phase there is an enrichment of a first pattern of replication (*c* and *d*). 2 h into S-phase a second pattern of replication predominates (*e* and *f*). As S-phase continues, a third pattern of replication peaks at 5 h (*g* and *h*). A fourth pattern of replication is enriched at 7 h into S-phase (*i* and *j*). At 9 h after release into S-phase, a fifth pattern of replication is the most common (*k* and *l*). Bar, 20 μm .



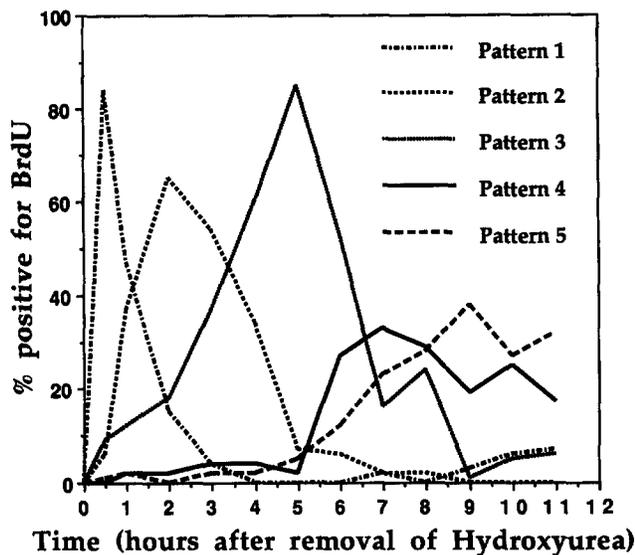
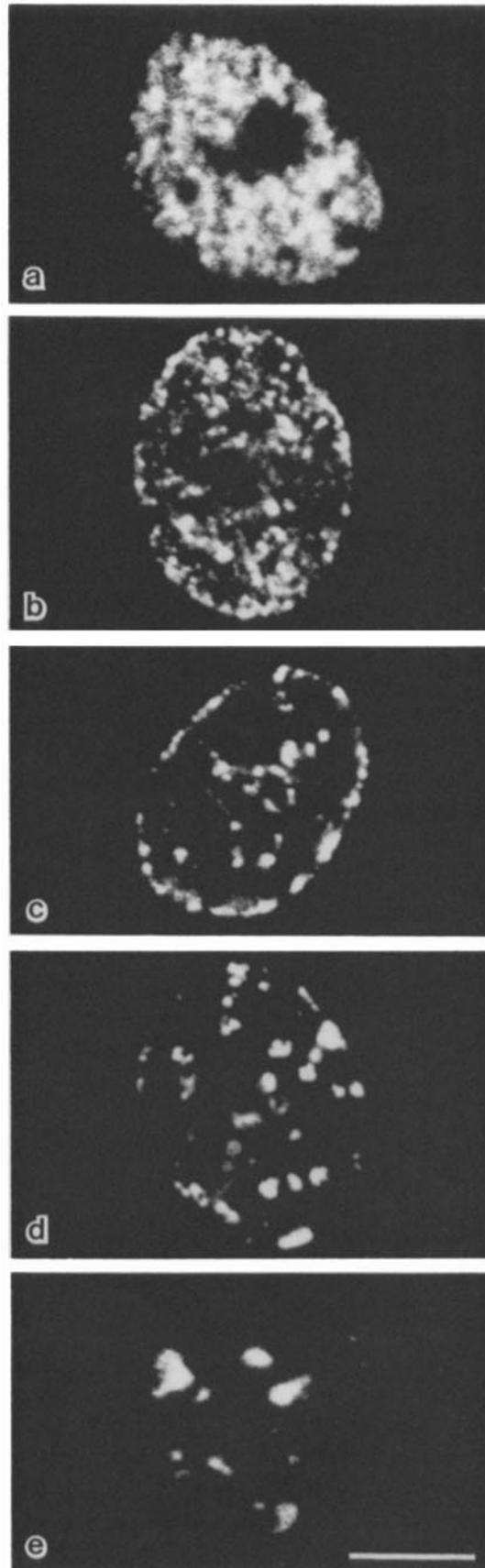


Figure 3. The five replication patterns in CHO cells as observed by immunofluorescence after cell synchronization were quantified at hourly intervals. CHO cells were synchronized as described in Materials and Methods and then released into S-phase. At hourly intervals, cells were labeled with 10 μ m BrdU which was subsequently detected by FITC-tagged antibodies against BrdU. At each time point 100 nuclei were counted and sorted by pattern. The graph indicates that there is an ordered sequence of the patterns, with each pattern reaching its maximal occurrence at different time points. The first pattern of replication reaches its peak at 0.5 h, the second at 2 h, the third at 5 h, the fourth at 7 h, and the fifth at 9 h.

no cells were in S-phase (Fig. 2, *a* and *b*). After release into S-phase, each of the five replication patterns were enriched at specific time points. The first pattern of replication peaked at 0.5 h after release into S-phase (Figs. 2, *c* and *d*, and Fig. 3). Optical sections were collected by confocal laser scanning microscopy at 250-nm intervals through the depth of the nucleus of each pattern and displayed as single slices (Fig. 4) and as reconstructed stacks of sections projected in stereo (Figs. 5 and 6). A single optical section through the nucleus representative of the first pattern is shown in Fig. 4 *a*. This pattern displays areas of replicating DNA that are distributed at sites throughout the nucleoplasm. The sites of replicating DNA appear to be concentrated in certain areas of the nucleoplasm. However, there is no apparent labeling of the nucleolus and some other nuclear regions. A stereo image representative of the first pattern optimally reveals the three-dimensional character of this pattern (Fig. 5 *a*). The sites of DNA replication extend throughout the depth of the nucleus. It is apparent from the stereo image that, aside from the nucleolus, there are areas at the periphery and in the interior of the nucleus that are not labeled. The ultrastructural localization of the sites of DNA replication in this first pattern was determined by immunogold electron microscopy (Fig. 7 *a*).

Figure 4. The five patterns of DNA replication identified in CHO cells by BrdU immunofluorescence are revealed by single section confocal laser scanning microscopy. Optical sections of labeled nuclei were collected at 250-nm intervals through the depth of the nucleus for each replication pattern and a single optical section representative of each pattern is shown. Pattern 1 (*a*), pattern 2 (*b*), pattern 3 (*c*), pattern 4 (*d*), and pattern 5 (*e*). Bar, 10 μ m.



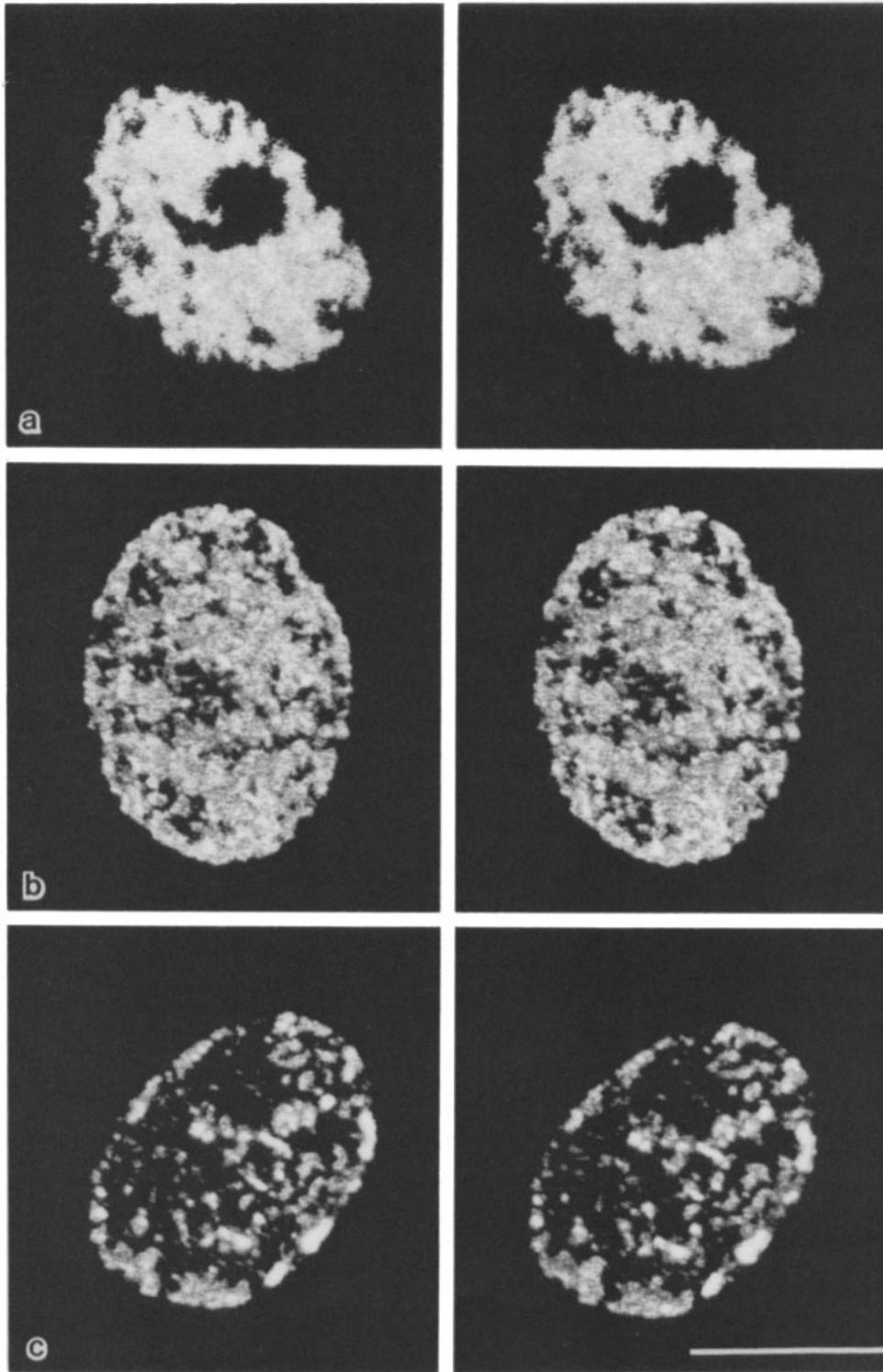


Figure 5. Stereo pairs of reconstructed optical sections collected through the depth of CHO nuclei optimally show the three-dimensional organization of the DNA replication sites for each of the five patterns of BrdU incorporation. The first pattern of replication (a) was reconstructed from half the optical sections collected to reveal the nature of the sites of DNA replication in the nuclear interior. The sites of replicating DNA are distributed throughout the nucleoplasm but appear to be concentrated in some areas more than others. However, there is no apparent labeling of the nucleolus and some areas of the nucleoplasm. The second pattern of replication (b) is a reconstruction of all of the optical sections revealing a decrease in internal and an increase in peripheral sites of replication. The third pattern of replication (c) is a reconstruction of half the optical sections in order to reveal the sites of replication associated with the periphery of the nucleus and nucleolus. Bar, 10 μ m.

Replication occurs throughout the nucleoplasm, specifically in regions of euchromatin. Furthermore, the peripheral heterochromatin and some regions of the internal nucleoplasm are devoid of replication sites.

As the CHO cells progressed further into S-phase, a second pattern of DNA replication was observed to peak at 2 h after hydroxyurea release (Figs. 2, e and f, and Fig. 3). The second pattern is characterized by sites of replication that are

larger and more discrete than those of the first pattern (Fig. 4 b). The sites of replication are now localized more towards the periphery of the nucleus with fewer interior sites. The stereo image of this second pattern reveals discrete sites of replication distributed in areas around the nuclear periphery as well as clusters in the nuclear interior (Fig. 5 b). Immunoelectron microscopy of this second pattern indicates that the sites of replication are now associated with the peripheral

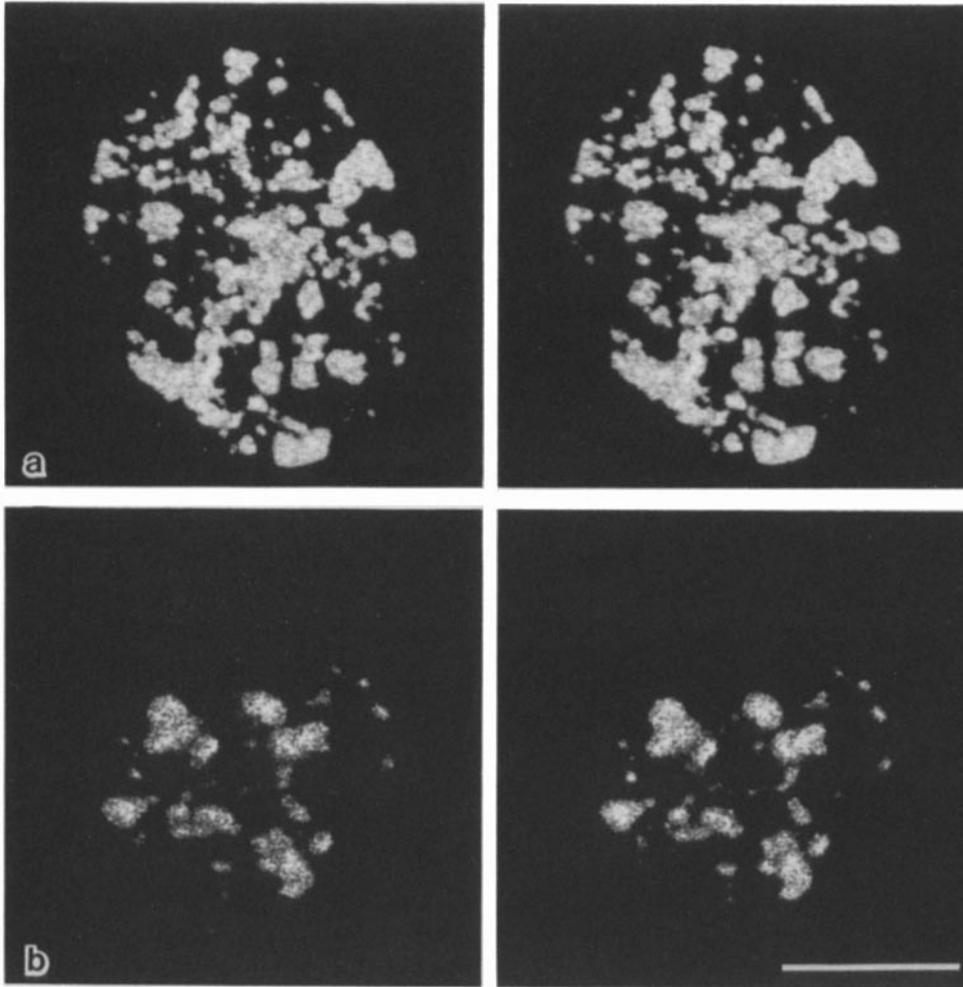


Figure 6. Collected optical sections were reconstructed as stereo pairs to reveal the three-dimensional character of the fourth pattern of replication (*a*). In this pattern the sites of replication are organized into chains, loops, and semi-circular structures. The fifth pattern of replication was reconstructed from all of the collected optical sections to show the replication sites through the depth of the nucleus (*b*). This pattern is characterized by large areas of replicating DNA that span portions of the interior as well as smaller areas of replication at the periphery of the nucleus. Bar, 10 μm .

heterochromatin (Fig. 7 *b*). Replication of euchromatin still occurs at this time but the sites of replication now seem to be more clustered than those of the first pattern.

As the CHO cells continued through S-phase, the third pattern of DNA replication observed by immunofluorescence (Figs. 2, *g* and *h*) peaked at 5 h (Fig. 3) after hydroxyurea release. This pattern (Fig. 4 *c*) is characterized by sites of replication restricted to the nuclear periphery and to the perinucleolar region. A stereo image of this pattern reveals that the sites of DNA replication occur in large patches, elongated areas, and smaller, discrete areas at the nuclear periphery as well as sites in the interior of the nucleus associated almost exclusively with the nucleolar surface (Fig. 5 *c*). The DNA replication sites occur primarily in regions of the peripheral heterochromatin and in association with the surface and portions of the interior of the nucleolus (Fig. 8 *a*). At this time point in S-phase the sites of replication are excluded from the euchromatin-containing regions of the nucleus.

A fourth pattern of DNA replication (Figs. 2, *i* and *j*) peaked at 7 h after release into S-phase (Fig. 3). The sites of DNA replication have now become larger in size and fewer in number (Fig. 4 *d*). This pattern, viewed in stereo, exhibits sites of replication that are distributed throughout the in-

terior and at a few discrete sites at the periphery of the nucleus (Fig. 6 *a*). At this time, however, the sites of replication are often connected in a chain-like manner or they take the form of loops or semi-circular structures. EM reveals that replication occurs at a few areas of the peripheral heterochromatin as well as internal electron-dense regions which are presumably interior regions of heterochromatin (Fig. 8 *b*). Little to no replication of euchromatin occurs at this time point.

Towards the end of S-phase, the last pattern of replication (Figs. 2, *k* and *l*) peaked at 9 h after hydroxyurea release (Fig. 3). This pattern is characterized by fewer, larger areas of replicating DNA in the interior of the nucleus as well as some smaller sites of replication in the interior and periphery of the nucleus (Fig. 4 *e*). The chain, loop, and semi-circular structures of pattern four are not apparent in this fifth pattern of replication. Stereo imaging of this pattern indicates that the larger areas of replicating DNA mostly span portions of the interior of the nucleus (Fig. 6 *b*). The smaller sites of DNA replication are associated with the peripheral heterochromatin and areas in the interior of the nucleus (Fig. 9 *a*). The larger areas of DNA replication are associated with internal electron-dense regions of heterochromatin. As was the

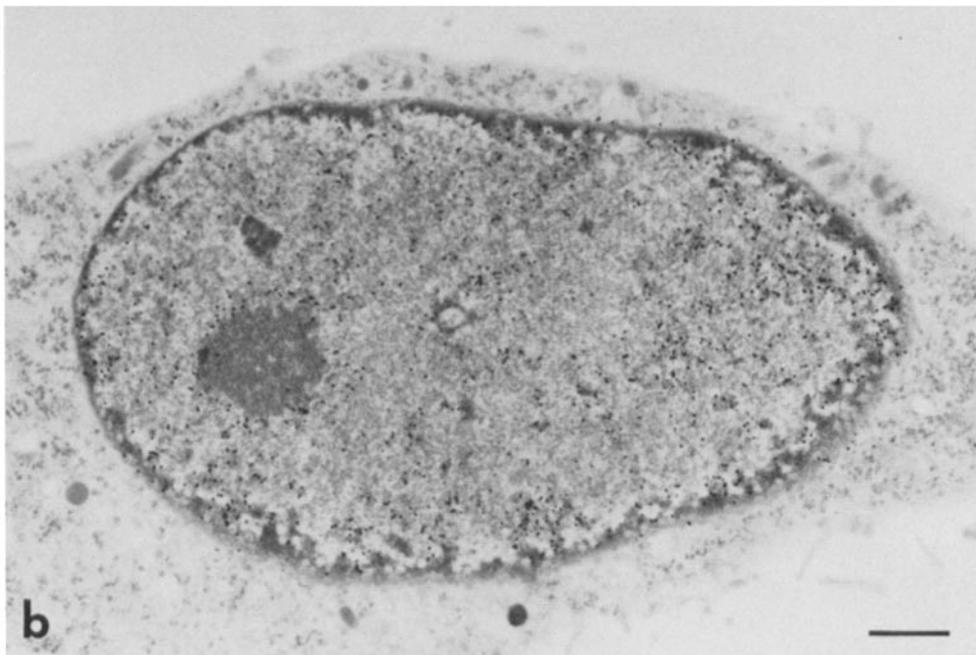
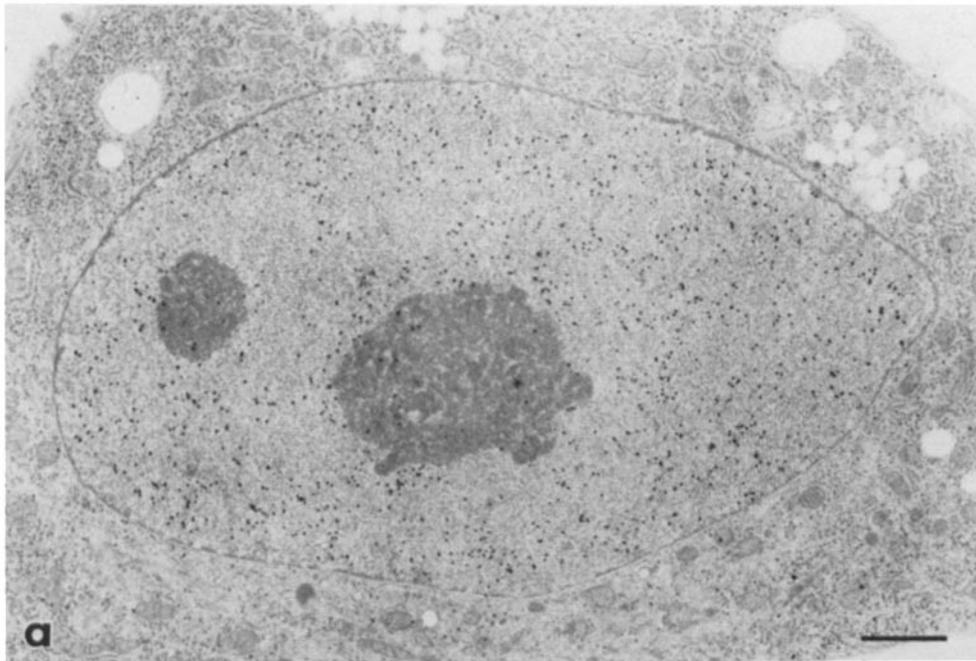


Figure 7. The first pattern of replication seen at 1 h into S-phase (a) is characterized by sites of replication throughout the nuclear interior (euchromatin) but is excluded from the nucleoli and the peripheral heterochromatin. Immunoelectron microscopy of the sites of DNA replication was performed by freeze substitution as described in Materials and Methods. At 2 h into S-phase, the second pattern of replication (b) is characterized by sites of replication more towards the nuclear periphery (peripheral heterochromatin) with less labeling of the nuclear interior (euchromatin). Bars, 1 μ m.

case for the fourth BrdU-labeling pattern, there does not appear to be much replication of euchromatin exhibited at this time point.

DNA Replication and In Situ Hybridization of Chromosome-Specific DNA Sequences in Human Cells

To determine when specific DNA sequences replicate within the context of these five replication patterns, we have combined BrdU labeling with in situ hybridization in human cells. To establish whether human cells had similar patterns of replication to those of CHO cells, immunofluorescence labeling was performed on asynchronous and synchronous

BrdU-labeled HeLa cells. Fig. 10 shows that HeLa cells displayed five patterns of DNA replication that were similar to those identified in CHO cells. Synchronization of HeLa cells revealed that the five patterns of replication identified in these cells occurred in the same order as in CHO cells. A number of human cell lines (MRC 5, WI-38, Detroit 551, HUVE, SW 620, MG-63) we have observed also display five patterns of replication similar to those of CHO and HeLa cells (data not shown).

Replication of α -Satellite DNA Occurs during Mid S-Phase

α -satellite DNA, which is associated with the chromosome

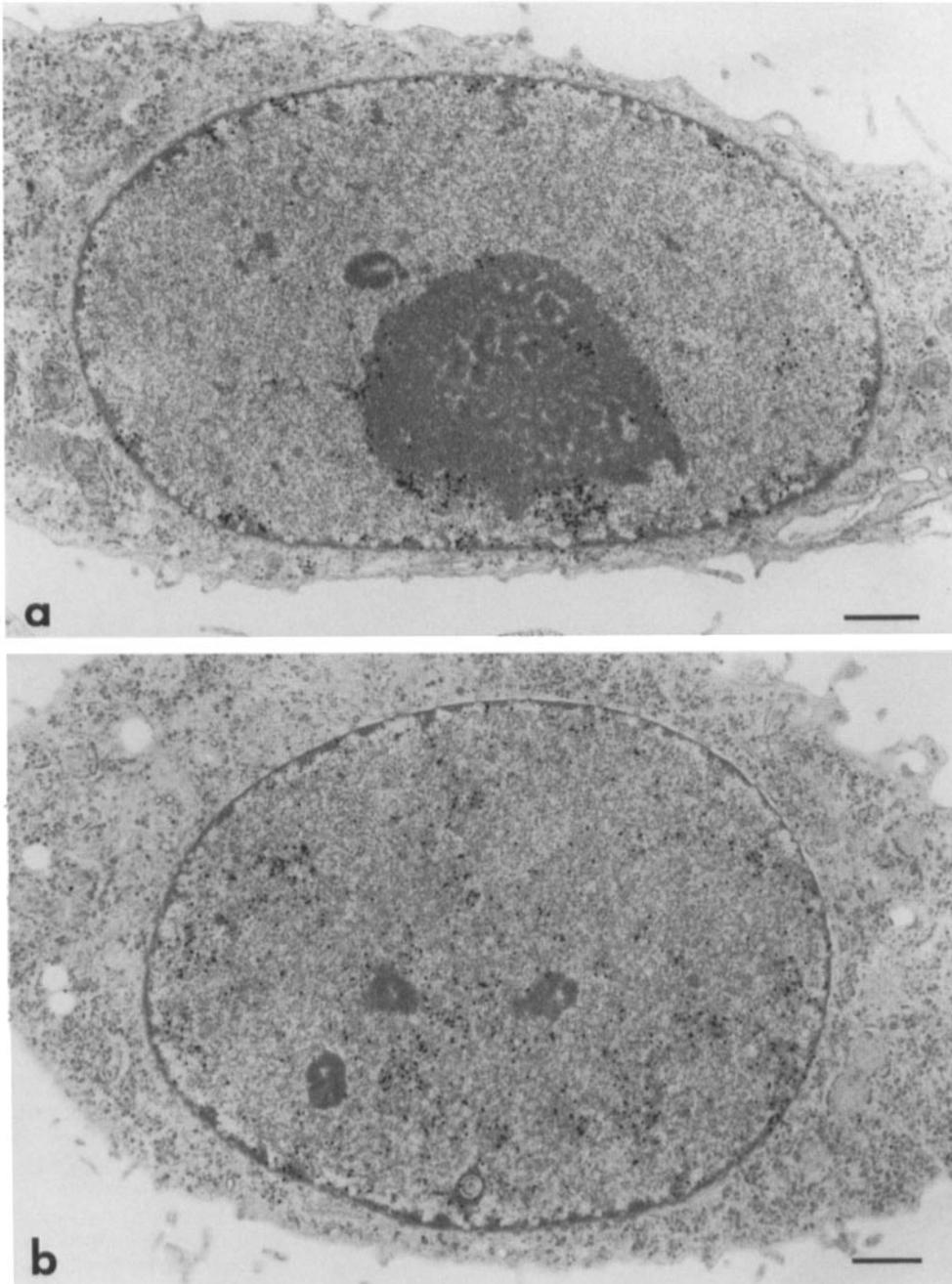


Figure 8. The third pattern of replication (a) is characterized by sites of replication that are exclusively associated with the peripheral heterochromatin and the surface of the nucleolus. The third pattern of replication occurs 5 h into S-phase. The fourth pattern of replication (b), at 7 h into S-phase, is characterized by the sites of replication which appear to connect and form chains of replication clusters associated with electron-dense regions of the nucleoplasm (heterochromatin) and a few regions of peripheral heterochromatin. Bars, 1 μ m.

centromere, consists of chromosome-specific “monomers” of ~ 171 bp which are tandemly repeated for several kilobases (for reviews see Willard and Wayne, 1987; Brinkley, 1990; Willard, 1990). As an example, in the X chromosome α -satellite DNA, 12 monomers form a higher-order repeat of 2.0 kb which is present in $\sim 5,000$ copies (10,000 kb) in tandem array per chromosome (Willard and Wayne, 1987; Willard, 1990).

Hybridization of the probe to the X chromosome α -satellite DNA occurred in all cells regardless of the state of DNA replication in any individual cell (i.e., whether or not a cell incorporated BrdU). The localization of the X chromosome α -satellite DNA was visualized relative to each of the BrdU-labeling patterns in both synchronous and asynchronous

populations of cells by superimposition of optical sections (sampled in each channel of fluorescence) generated by confocal laser scanning microscopy. Because of the disposition of centromeres within the three-dimensional space of the nucleus, usually α -satellite DNA from only one centromere is detected in any one optical section. Occasionally, two centromeres lie within the same focal plane (Figs. 11, d, h, i, and m). At times of DNA synthesis characterized by the first (Fig. 11 a), second (Fig. 11 b), fourth (Fig. 11 d), and fifth (Fig. 11 e) patterns of DNA replication, the X chromosome α -satellite DNA is visualized as red spots which do not overlap with the green labeling of BrdU in all cells examined. Only at the time characterized by the third pattern of DNA replication (Fig. 11 c), which occurs during mid S-phase,

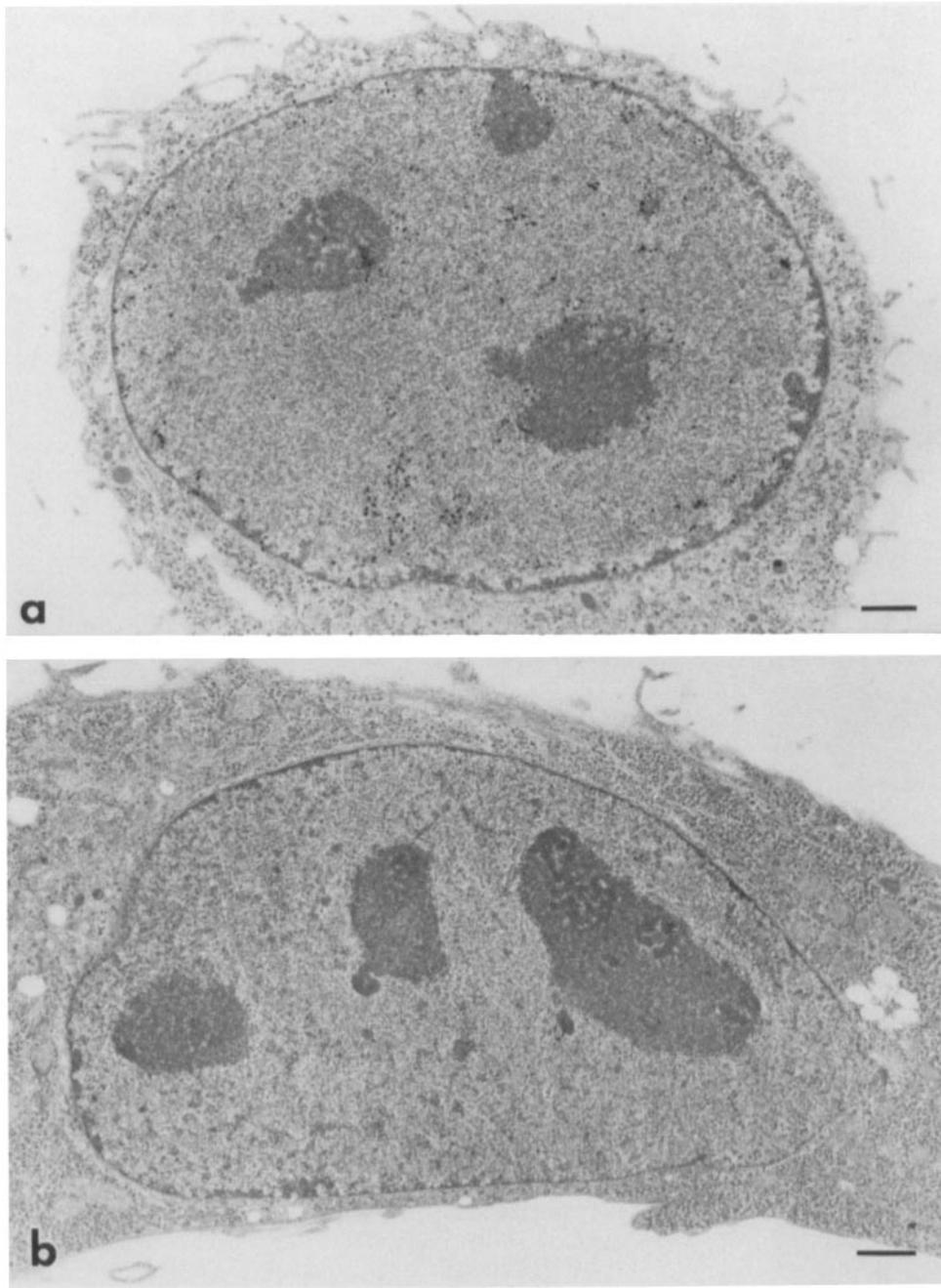


Figure 9. At 9 h into S-phase, the fifth pattern of replication (a) is characterized by fewer and larger sites of replication over electron-dense regions of chromatin as well as smaller areas of replication in the peripheral heterochromatin. A control cell (b), incubated without primary antibody but with the secondary gold-conjugated antibody, shows no specific labeling. Bars, 1 μ m.

does the hybridization to the X chromosome α -satellite DNA colocalize with the BrdU labeling (i.e., the superimposition of red and green images produces a yellow spot in optical sections). Therefore, the replication of the X chromosome α -satellite DNA appears to occur during mid S-phase which is characterized by the third pattern of DNA replication. The replication of α -satellite DNA during mid S-phase appears to be true for other chromosomes as well. Probes to α -satellite DNA from chromosomes 1 (Fig. 11 f), 3 (Fig. 11 g), 10 (Fig. 11 h), 17 (Fig. 11 i), and 18 (Fig. 11 j) as well as chromosomes 2, 8, and 12 (data not shown) colocalize with the third pattern of DNA replication. Thus, in general, α -satellite DNA replicates during mid S-phase. α -satellite DNA from homologous chromosomes does not

necessarily replicate at precisely the same time although α -satellite DNA from both centromeres replicates within the same pattern. Often, the replication of α -satellite DNA from one member of a chromosome pair precedes the other (e.g., Fig. 11 i), yet both replicate during the third pattern of DNA replication (i.e., mid S-phase) since replication of α -satellite DNA is not detected in other replication patterns (i.e., early or late S-phase) in all cells examined. The replication of α -satellite DNA during mid S-phase appears to be independent of cell type, nuclear size or shape, or ploidy. In a variety of human cell types: HUVE (Fig. 11 k), Detroit 551 (Fig. 11 l), WI-38 (Fig. 11 m), SW-620 (Fig. 11 n), and MG-63 (Fig. 11 o), X chromosome α -satellite DNA replicates during the third pattern of BrdU labeling (mid S-phase). This pat-

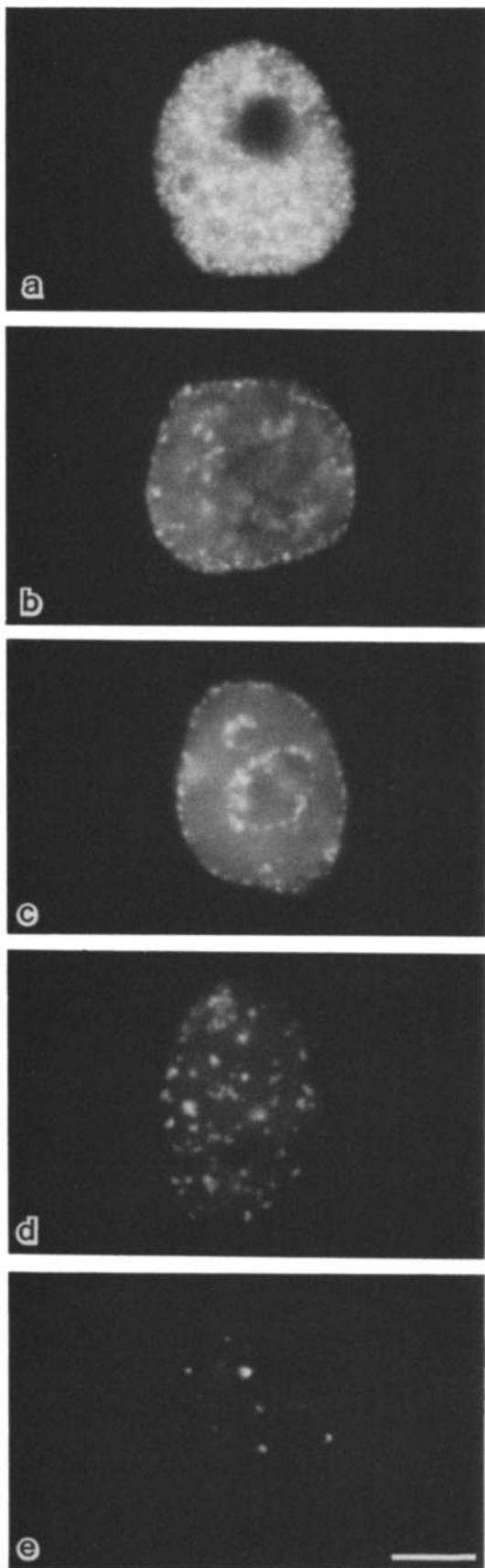


Figure 10. HeLa cells display five patterns of DNA replication that are similar to those of CHO cells. HeLa cells were pulse labeled with BrdU which was detected by immunofluorescence using the

tern differs between some cell types in that the extent of perinucleolar BrdU incorporation is less in some cell types (i.e., fibroblasts vs. epithelial cells). However, in all cases, regardless of chromosomal location or cell type, the α -satellite DNA is found associated with either the surface of the nucleolus or the nuclear periphery (Fig. 11).

Discussion

In this study we have characterized the spatial and temporal nuclear organization of DNA replication sites in mammalian cell nuclei. We have found that the spatial patterns and the temporal order of these patterns are similar in all of the mammalian cell types that we have studied thus far. Short pulses of BrdU incorporation have allowed us to resolve five patterns of DNA replication in both CHO and HeLa cells. Identical patterns occur in both synchronized and asynchronous populations of cells. Visualization of chromosome specific DNA sequences (α -satellite DNA) within the context of the five patterns of replication identified has enabled us to determine that these sequences replicate in specific spatial and temporal positions during S-phase.

Our work is in agreement with previous studies of others who have found different patterns of DNA replication during S-phase (Nakayasu and Berezney, 1989; van Dierendonck et al., 1989; Mazzotti et al., 1990; Fox et al., 1991). However, other studies find less distinct patterns of BrdU (or dUTP) incorporation and that regions of replicating DNA appear to vary only in size (and not pattern) of replication clusters during S-phase (Nakamura et al., 1986; Banfalvi et al., 1990). In vitro, the sites of replication of *Xenopus* sperm nuclei, chicken erythrocyte nuclei, and bacteriophage λ DNA induced by a *Xenopus* cell-free extract occur as discrete foci which are maintained during replication (Mills et al., 1989; Cox and Laskey, 1991; Leno and Laskey, 1991). These differences raise the question of whether replication sites are dynamic (and move from one region of the nucleus to another) or whether they are fixed, with the DNA "spooling" through them (Pardoll et al., 1980). Several studies have shown that DNA replication factors which colocalize with BrdU-labeling (e.g., PCNA) display similar temporal changes in distribution as those which we have found (Bravo and Macdonald-Bravo, 1985; Celis and Celis, 1985; Madsen and Celis, 1985; Bravo, 1986; Bravo and Macdonald-Bravo, 1987; Raska et al., 1989). Immunolabeling of DNA cruciforms (regions at or near replication origins) also shows a similar change in distribution during S-phase (Ward et al., 1990; 1991). These results suggest that DNA replication factors move throughout the nucleus (to the DNA being replicated) during S-phase. However, this does not exclude the possibility that the sites of replication are "fixed". It has been proposed that replication occurs at discrete sites (Mills et al., 1989; Cox and Laskey, 1991), the positions of which are maintained via an attachment to an underlying nuclear skeleton (or nuclear matrix) (Pardoll et al., 1980; Berezney

same procedure used for CHO cells (see Materials and Methods). The patterns of replication in HeLa cells are spatially and temporally organized similar to those of CHO cells; Pattern 1 (a), pattern 2 (b), pattern 3 (c), pattern 4 (d), and pattern 5 (e). Bar, 10 μ m.

et al., 1982; Dijkwel et al., 1986; Jackson and Cook, 1986; Razin, 1987). Furthermore, DNA polymerases (Foster and Collins, 1985; Tubo and Berezney, 1987) and replication forks (Vaughn et al., 1990) have been found to be associated with the nuclear matrix. Replication patterns, similar to those which we have observed, have also been shown to be associated with the nuclear matrix (Nakayasu and Berezney, 1989). These results suggest a possible model in which replication factors, capable of moving throughout the nucleus, bind to specific DNA sequences at (or before) their initiation of replication. These complexes then temporarily associate with the nuclear matrix where DNA is replicated.

The spatial order of DNA replication is further revealed by three-dimensional reconstruction of BrdU-labeled nuclei via confocal laser scanning microscopy. Our findings of the three-dimensional distribution of replication patterns are in partial agreement with a recent report by Fox et al. (1991). Our results, however, indicate that the bulk of replication at the periphery of the nucleus occurs primarily during mid S-phase not at the end of S-phase as suggested by Fox et al. (1991). Our observations are therefore more consistent with those of other groups (Nakayasu and Berezney, 1989; van Dierendonck et al., 1989).

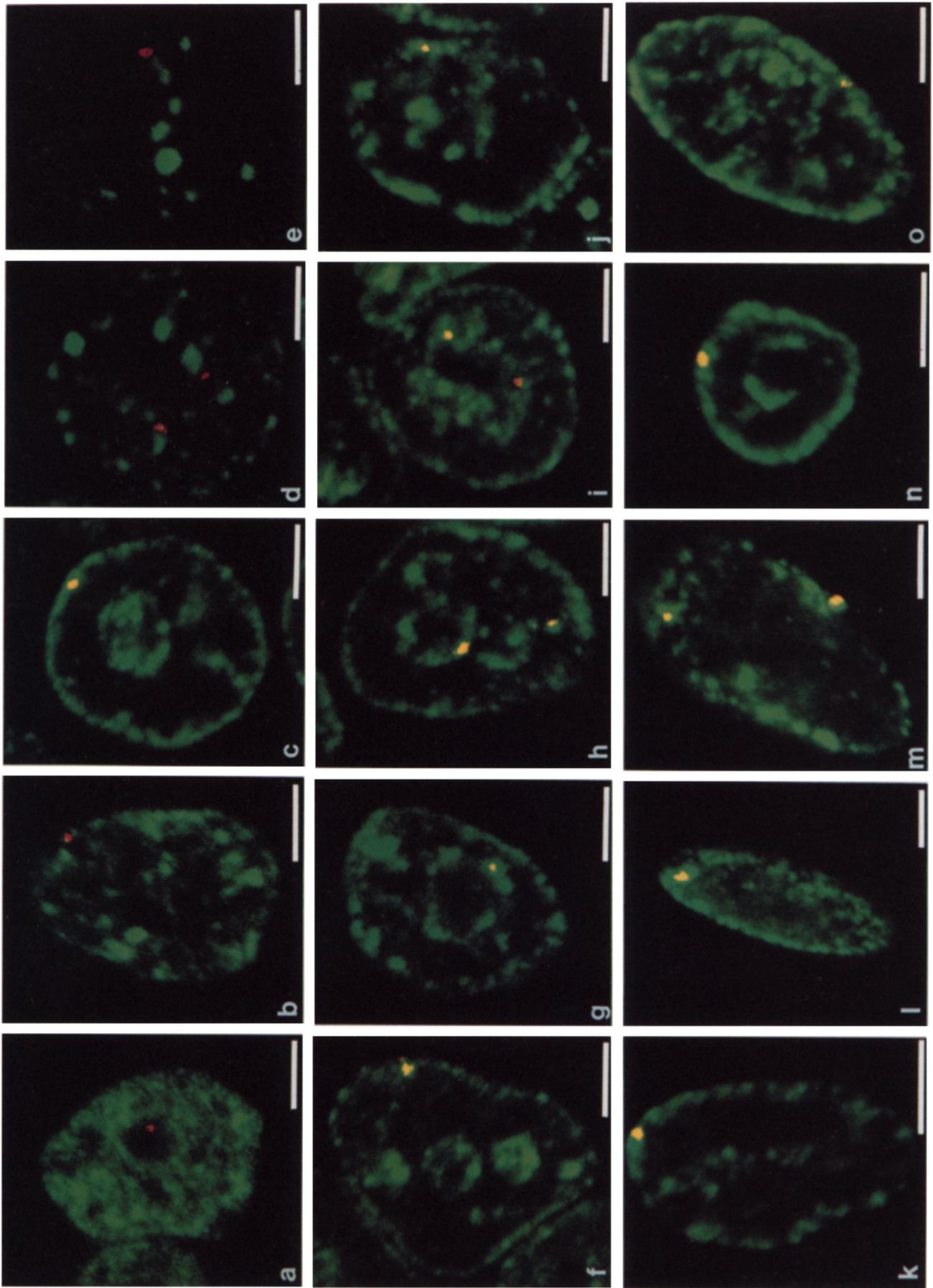
We have made an attempt to establish when different types of chromatin replicate by the use of EM of BrdU-labeled cells. We have found five patterns of BrdU incorporation which are similar to those characterized by light microscopy. Initially, only euchromatin is replicated. As S-phase progresses, the replication of euchromatin subsides as replication of peripheral heterochromatin increases. In mid S-phase, discrete regions of the perinucleolar chromatin and peripheral heterochromatin replicate. At the end of S-phase, regions of internal heterochromatin replicate as replication in regions of the peripheral heterochromatin decreases. These results are in agreement with the idea that "active" DNA is preferentially replicated early and "inactive" DNA is predominantly replicated late in S-phase (Lima-de-Faria and Jaworska, 1968; Goldman et al., 1984). At the ultrastructural level, Mazzotti et al. (1990) have examined BrdU incorporation in asynchronous murine erythroleukemia cells and have identified three types of immunogold labeling. They were able to identify labeling of either the diffuse chromatin alone (euchromatin), the border of the euchromatin/heterochromatin or the peripheral heterochromatin. The temporal sequence of these three different immunogold labeling patterns, however, can only be inferred from their study because only asynchronous cells were examined. In our study, the synchronization of cells has enabled us to determine the exact progression of DNA replication patterns observed by immunogold labeling and to determine what type of chromatin is replicated at specific time points.

In this study, we have also been able to visualize, within single cells, when a chromosome-specific sequence of DNA replicates via confocal microscopy of BrdU-labeled cells hybridized in situ with chromosome-specific probes. By superimposition of α -satellite DNA hybridization with BrdU labeling, we have found that several of the chromosome specific α -satellite DNA sequences replicate during mid S-phase (i.e., the third pattern of DNA replication). The replication of several of the α -satellite DNA sequences during mid S-phase is true for all of the human cell types studied

thus far. Our findings are in agreement with a recent study (Bartholdi, 1991) which followed centromeres labeled with antibodies throughout the cell cycle and found that the intensity of centromere fluorescence increased during mid S-phase, well before the appearance of double dots. It is noteworthy that the third pattern of DNA replication delineates the previously established positions of centromeres (i.e., perinucleolar and/or peripheral) (Manuelidis, 1984; Rappold et al., 1984; Bourgeois et al., 1985; Manuelidis, 1985; Manuelidis and Borden, 1988; Haaf and Schmid, 1989; Mukherjee and Parsa, 1990; Popp et al., 1990; van Dekken et al., 1990; Haaf and Schmid, 1991). Furthermore, our results are supported by previous biochemical studies which found that centromeres replicated in mid S-phase, but not in the first three hours (Dooley and Ozer, 1977) or last two hours (Bostock and Prescott, 1971) of S-phase. However, our results differ with previous studies which suggest that centromeric DNA replicates very late in (or at the end of) S-phase (Lima-de-Faria and Jaworska, 1968; Camargo and Cervenka, 1982; Goldman et al., 1984; Ten Hagen et al., 1990). In this study, we pulse-labeled synchronized cells with BrdU for brief (10 min) periods of time and sampled cells on an hourly basis. Thus, we were able to examine replication with a greater temporal resolution. The assessment of replication based upon absolute timing rather than accumulated DNA content avoids any variations in the rate of DNA replication during S-phase. Furthermore, by colocalization studies, we have been able to determine not only the time at which centromeric DNA begins to replicate, but also when it completes replication. Any variability in the timing of replication assessed from a pooled sample of a potentially heterogeneous population of cells has been eliminated by examining replication in synchronized cells on an individual cell basis. Thus, we believe that our studies allow for a more precise determination of α -satellite DNA replication within a cell.

The technique of combining BrdU labeling with in situ hybridization and examination by confocal microscopy allows for the determination of the timing of replication of specific genes. Furthermore, changes in the timing of replication can be determined based upon whether or not a cell expresses a gene, cell differentiation, or cell transformation. Additionally, adjacent DNA probes along the length of a chromosome can be hybridized in situ to BrdU labeled cells to map the differences in timing of contiguous DNA sequences (i.e., to determine if there is a smooth wave of replication within specific regions of a chromosome or whether there are abrupt starts and stops in replication of sequences along the chromosome).

In the present study we have identified five spatial and temporal patterns of DNA replication. Furthermore, we have shown that chromosome specific α -satellite DNA sequences replicate during mid S-phase. The identification of the five patterns of DNA replication and the fact that the chromosome specific α -satellite sequences replicate within only one of these replication patterns supports the idea that the position of DNA sequences in the interphase nucleus is important in determining the timing of replication. Thus, we conclude that the replication of DNA follows a dynamic order in the mammalian cell nucleus, with specific regions of DNA replicating at defined times and in defined locations of



the nucleus during S-phase. Our studies support the hypothesis that the nucleus is a highly organized system of defined structural and functional activities.

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Figure 11. Replication of centromeric (α -satellite) DNA occurs during mid S-phase of human cells. Human cells were labeled with BrdU and hybridized *in situ* with biotinylated DNA probes to chromosome specific α -satellite DNA. BrdU incorporation and hybridization of probes were visualized by labeling with FITC-conjugated anti-BrdU and Texas red-conjugated avidin, respectively. An optical section was taken by confocal laser scanning microscopy of the hybridized probe within the nucleus and pseudo-colored red. A second optical section, in the same focal plane, of the incorporated BrdU was pseudo-colored green and superimposed upon the first section (i.e., of α -satellite probe hybridization). Areas in which DNA replication (green) and α -satellite DNA localization (red) colocalize appear yellow in color. Optical sections of synchronized HeLa cells, labeled with BrdU, and hybridized with a probe to the X chromosome α -satellite DNA, show that the X chromosome centromere replicates during mid S-phase which is characterized by the third pattern of BrdU incorporation (c). No colocalization of the X centromere and DNA replication occurs in either the first (a), second (b), fourth (d), or fifth (e) patterns of BrdU incorporation. The replication of centromeric (α -satellite) DNA in HeLa cells during mid S-phase (characterized by the third pattern of BrdU incorporation) also occurs for α -satellite DNA of chromosomes 1 (f), 3 (g), 10 (h), 17 (i), and 18 (j). Furthermore, the replication of X chromosome α -satellite DNA occurs during mid S-phase (third pattern of BrdU incorporation) in a variety of human cell types including: human umbilical vein endothelial (HUVE) cells (k), Detroit 551 (fibroblast) cells (l), WI-38 (fibroblast) cells (m), SW-620 (adenosarcoma) cells (n), and MG-63 (osteosarcoma) cells (o). Bars, 5 μ m.

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