Mapping Replicational Sites in the Eucaryotic Cell Nucleus

Hiroshi Nakayasu and Ronald Berezney

Department of Biological Sciences, State University of New York at Buffalo, Buffalo, New York 14260

Abstract. We have used fluorescent microscopy to map DNA replication sites in the interphase cell nucleus after incorporation of biotinylated dUTP into permeabilized PtK-1 kangaroo kidney or 3T3 mouse fibroblast cells. Discrete replication granules were found distributed throughout the nuclear interior and along the periphery. Three distinct patterns of replication sites in relationship to chromatin domains in the cell nucleus and the period of S phase were detected and termed type I (early to mid S), type II (mid to late S) and type III (late S). Similar patterns were seen with in vivo replicated DNA using antibodies to 5-bromodeoxyuridine. Extraction of the permeabilized cells with DNase I and 0.2 M ammonium sulfate revealed a striking maintenance of these replication granules and their distinct intranuclear arrangements with the remaining nuclear matrix structures despite the removal of >90% of the total nuclear DNA. The in situ prepared nuclear matrix structures also incorporated biotinylated dUTP into replication granules that were indistinguishable from those detected within the intact nucleus.

DESPITE considerable progress in defining specific molecular components involved in eucaryotic DNA replication such as DNA polymerases α, δ, and DNA primase (Kornberg, 1988), our understanding of native replicational sites and their structural organization and associations in the cell nucleus has lagged behind. Most previous studies designed to localize the sites of DNA replication in eucaryotic cells have used autoradiographic microscopy. These techniques have been very useful in determining cells active in DNA replication and the general distribution of replication sites in the cell nucleus over peripheral versus internal sites and over condensed heterochromatin versus diffuse euchromatin (Hay and Revel, 1963; Milner, 1969; Huberman et al., 1973; Fakan and Hancock, 1974; Fakan, 1978; Smith et al., 1984). The level of resolution of these techniques, however, severely limits their potential usefulness for studying the structural organization of individual replicational sites in the cell nucleus.

This has prompted us to explore more sensitive and higher-resolution approaches to this problem. Langer et al. (1981), who first synthesized biotin-labeled nucleotides for use as nucleic acid–affinity probes also demonstrated that 5-([*N*-biotinamidocaproyl]-3-aminoallyl)-2′-deoxyuridine-5′-triphosphate (biotin-11-dUTP)[1] is effectively incorporated into DNA by a variety of DNA polymerases, including the mammalian replicative enzyme DNA polymerase α. Numerous studies have also indicated that permeabilized cell systems maintain many basic features of eucaryotic replication in vivo, including semiconservative replicative-like synthesis, which continues DNA synthesis at in vivo initiated replication sites (Berger et al., 1977; Reinhard et al., 1977; van der Velden et al., 1984), Okazaki fragment synthesis and ligation into DNA of replicon size (Berger et al., 1977; Gautschi et al., 1977; van der Velden et al., 1984*a*; Miller et al., 1985), and bidirectional replication at discrete replicon subunits that are of similar size as replicons active in vivo and arranged in tandemly repeated arrays or replicon clusters (Hand and Gautschi, 1979).

With this in mind, we developed a permeabilized cell system to study the incorporation of biotin-11-dUTP into newly replicated DNA. The sites of the biotinylated, newly synthesized DNA were then directly visualized by fluorescent microscopy after reaction with Texas red-streptavidin. Although our study is the first reported attempt with this technique for visualizing DNA replication sites in cells from multicellular organisms, Olins and Olins (1987) recently used biotin-11-dUTP in an in vitro nuclear system to visualize DNA synthesis in the macromolecular replication band of the ciliated protozoa *Euplotes eurystomus*. Their studies demonstrated that the biotin-11-dUTP incorporation system maintained certain characteristic structural features of DNA synthesis which were previously derived from in vivo studies. Our results demonstrate that DNA synthesis in the

Dr. Nakayasu's present address is Department of Medical Biochemistry, Shiga, University of Medical Sciences, Seta, Otsu, 52021, Japan. Address correspondence to Dr. Ronald Berezney, Department of Biological Sciences, State University of New York, Buffalo, NY 14260.

1. *Abbreviations used in this paper*: biotin-II-dUTP, 5-([*N*-biotinamidocaproyl]-3-aminoallyl)-2′-deoxyuridine-5′-triphosphate; BrdU, 5-bromodeoxyuridine; glycerol buffer, 20 mM Tris-HCl, pH 7.4, 25% glycerol, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM PMSF; TBS buffer, 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 5 mM $MgCl_2$; TBS-Tween buffer, 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.2 mM $MgCl_2$, 0.2% Tween 20.

cell nucleus occurs at several hundred discrete granular sites termed replication granules. The distribution of these replicational sites in relationship to chromatin domains in the cell nucleus was altered markedly as the cells traversed S phase. Three distinct S phase-dependent patterns were defined (types I, II, and III) and there was a remarkable maintenance of each of these three replication types in the nuclear matrices prepared from the corresponding permeabilized cells.

Materials and Methods

Cell Culture and Synchronization

Kangaroo kidney PtK-1 cells (CCL35; American Type Culture Collection [ATCC], Rockville, MD) and mouse Balb/3T3 fibroblasts (ATCC CCL163) were grown in DME (4.5 g glucose/liter; Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS, 20 μg/liter penicillin G (Gibco Laboratories) and 1 ml/liter gentamicin (Gibco Laboratories) in 6-well tissue culture dishes (Falcon Labware, Oxnard, CA). At 50–60% saturation, the cells were transferred onto cover slips (~1.5×10^4 cells/cm^2) and allowed to grow for 40–48 h. For synchronization, the culture medium on cover slips (~0.5 ml) was replaced by the same medium supplemented with 0.5% FCS instead of 10% and cultured for 72 h in this low serum medium. To count the cell number and mitotic index, the cells on cover slips were fixed in absolute methanol at −20°C for at least 10 min and stained with Hoechst 33258 dye (Sigma Chemical Co., St. Louis, MO) for easy observation of mitotic chromosomes. After this serum starvation, the number of mitotic cells decreased to 0%. The arrested cells were released from the G0 stage by replacement with 10% serum medium and incubated for 0–24 h in a CO_2 incubator.

In Situ Incorporation of Biotin-11-dUTP

PtK-1 or 3T3 cells on cover slips were washed twice with TBS buffer (10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 5 mM $MgCl_2$), then with glycerol buffer (20 mM Tris-HCl, pH 7.4, containing 25% glycerol, 5 mM $MgCl_2$, 0.5 mM EGTA and 0.5 mM PMSF), and then permeabilized with glycerol buffer containing 0.04% or 0.5% Triton X-100 at room temperature for 3 min and washed with glycerol buffer. The cover slips were then covered with 50 μl of 50 mM Tris-HCl, pH 7.4, containing 10 mM $MgCl_2$, 0.5 mM EGTA, 25% glycerol, 40 μM dATP, 40 μM dGTP, 40 μM dCTP, 16 μM biotin-11-dUTP (Bethesda Research Laboratories, Gaithersburg, MD) and 2 mM ATP (DNA synthesis medium), incubated at 37°C for 2–60 min, and washed with TBS buffer containing 0.5% Triton X-100. To minimize disruption of nuclei, the permeabilized cells were treated with TBS buffer containing 0.5 mM $CuSO_4$, 0.5 mM sodium tetrathionate, 0.5% Triton X-100, and 0.5 mM PMSF at room temperature for 10 min, or they were fixed in absolute methanol at −20°C for 10 min. Both treatments gave the same results. The addition of ATP in the DNA synthesis medium is not absolutely required for incorporation of biotin-11-dUTP. With ATP, however, the fluorescence increased severalfold in intensity.

Preparation of In Situ Nuclear Matrix

Preparation of in situ nuclear matrix was carried out before or after incorporation of biotin-dUTP. The samples (untreated or permeabilized cells after biotin-dUTP incorporation) were washed twice with TBS buffer, then treated with the same buffer containing 0.5% Triton X-100 and 0.5 mM PMSF at room temperature for 30 min. The chromatin was digested with 100 μl of DNase I (5 U/ml of TBS buffer, at room temperature for 10 min; U; Sigma Chemical Co.), then extracted with 200 μl of 20 mM Tris-HCl, pH 7.4, containing 0.2 M ammonium sulfate and 0.2 mM $MgCl_2$ at room temperature for 1 min. The samples were then washed twice with TBS-Tween buffer (10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 0.2 mM $MgCl_2$, and 0.2% Tween 20). For DNA synthesis on in situ nuclear matrix, the samples were washed twice with glycerol buffer and incubated with DNA synthesis medium at 37°C for 5 min as described above.

In Vivo Incorporation of 5-Bromodeoxymidine (BrdU)

Mouse 3T3 cells grown on cover slips were incubated at 37°C for 30–120 min. with DME containing 10 μM BrdU and 1 μM fluorodeoxyuridine. After four rinses in TBS, the cells were fixed with 4% paraformaldehyde freshly made in TBS buffer at 4°C for 15 min, washed with TBS six times, permeabilized in TBS containing 0.5 mM $CuSO_4$ and 0.5% Triton X-100 for 10 min at room temperature, and rinsed four times in TBS buffer.

Fluorescent Microscopy

To detect biotinylated DNA, the appropriately rinsed permeabilized cells or in situ nuclear matrix were washed twice with TBS-Tween buffer, and incubated with 60 μl of Texas red-conjugated streptavidin (5 μg/ml in TBS-Tween; Sigma Chemical Co.) at 37°C for 30–60 min. We found streptavidin was much better than avidin for our purposes. Because of its very basic pI (~10), fluorochrome-conjugated avidin stained nucleoli slightly with or without biotin-dUTP incorporation. Moreover, it has been reported that avidin nonspecifically binds to chromatin. These problems were eliminated with streptavidin.

To detect BrdU-substituted DNA, the rinsed permeabilized cells were incubated in 4 N HCl for 30 min at room temperature to denature the DNA, rinsed five times in TBS-Tween, incubated at 37°C for 1 h with an affinity-purified monoclonal mouse IgG antibody to BrdU in TBS-Tween, rinsed five times in TBS-Tween and reacted with a rhodamine-conjugated rabbit anti-mouse IgG (1:50 in TBS-Tween; Cooper Biomedicals, Malvern, PA).

All samples were then stained with Hoechst 33258 dye (0.5 μg/ml in TBS-Tween buffer) at room temperature for 10 min, washed four times with TBS-Tween buffer, mounted with 5 μl of PBS containing 50% glycerol, and observed immediately under a Zeiss Photomicroscope III equipped with a III RS vertical illuminator for epifluorescence, a 100-W mercury lamp, and Zeiss filter sets (nos. 48-77-02 and 44-7-14) for visualizing Hoechst and Texas red or rhodamine staining, respectively. Photographs were taken using Kodak technical pan film 2415 (ASA 400; Eastman Kodak Co., Rochester, NY). Exposure time was generally 2 min for newly synthesized DNA or 15 s for Hoechst 33258 staining.

Results

Incorporation of Biotin-11-dUTP in Permeabilized Cells

We have used a highly sensitive fluorescent microscopic technique to study the structural localization of newly synthesized DNA in permeabilized cells. Briefly, kangaroo kidney PtK-1 or mouse 3T3 fibroblast cells were grown on cover slips and permeabilized as described in Materials and Methods. DNA synthesis was performed with biotin-11-dUTP, an analogy of dTTP that is effectively incorporated into DNA by eucaryotic DNA polymerases (Langer et al., 1981). The structural localization of the newly synthesized DNA in the cells was then visualized under the fluorescent microscope after treatment with Texas red–conjugated streptavidin.

About 25% of the nuclei of exponentially growing PtK-1 cells were stained by this fluorescent probe. The other cells were completely devoid of stain and were likely non-S phase cells. The nascent DNA was located in numerous granules that were distributed throughout the nuclear interior, except for the nucleoli (Fig. 1, *a* and *d*). The replication granules were of similar size (ranging from 0.4 to 0.6 μm in diameter with most ~0.5 μm) and often appeared to be clustered into tandemly linked, chain-like arrays (Figs. 1, *a* and *c*, 2, *a*, *d*, *g*, and *j*). Whereas the nuclei of most cells that incorporated biotin-11-dUTP showed a similar granular pattern, some nuclei (~10%) had mainly peripheral staining (data not shown). From results to be described later (see Fig. 6), we believe that the peripheral staining represents DNA replication during the latter stages of S phase. Preincubation with inhibitors of the replicative enzyme DNA polymerase α (aphidicolin or *N*-ethylmaleimide), completely prevented the incorporation of biotin-11-dUTP into the nuclear DNA (Fig.

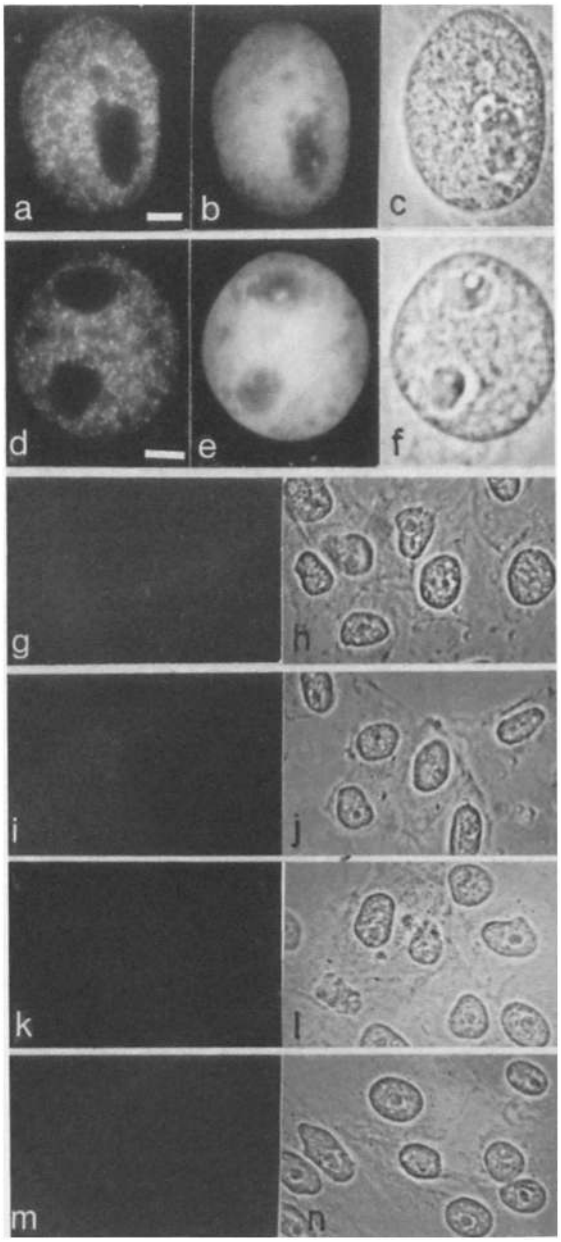

Figure 1. Incorporation of biotin-dUTP into PtK-1 nuclei. Kangaroo kidney PtK-1 cells on coverslips were permeabilized as described in Materials and Methods using 0.5% Triton X-100, and incubated with DNA synthesis medium at 37°C for 10 min. Complete DNA synthesis medium contained 50 mM Tris-HCl, 7.4, containing 10 mM $MgCl_2$, 0.5 mM EGTA, 25% glycerol, 40 μM dNTP, 16 μM biotin-11-dUTP, and 2 mM ATP. (*a* and *d*) Complete medium; (*g*) Preincubated with 10 μg/ml aphidicolin in glycerol buffer at 0°C for 30 min; (*i*) Preincubated with 10 mM *N*-ethylmaleimide as *g*; (*k*) Complete medium − dNTP + biotin-dUTP; (*m*) Complete medium − biotin-dUTP; (*b* and *e*) Corresponding Hoechst 33258 staining of *a* and *d*; (*c*, *f*, *h*, *j*, *l*, and *n*) Corresponding phase microscopy to *a*, *d*, *g*, *i*, *k*, and *m*, respectively. Visualization of newly synthesized biotinylated DNA and Hoechst 33258 staining are described in Materials and Methods. Bars, 4 μm.

1, *g–j*). There was also no fluorescence within nuclei in the absence of dNTP (Fig. 1 *k*) or biotin-11-dUTP (Fig. 1 *m*).

A time study of incorporation from 2 to 60 min is shown in Fig. 2. The granular intranuclear replication sites do not appreciably increase in size or number as DNA synthesis continues in the cells. Rather, there is a progressive increase in the intensity of individual replication granules with increasing time of incorporation. At any given incorporation time, the staining intensity of most of the granules appeared to be very similar. The total number of replication granules in each nucleus was estimated by direct counting of granules from prints of photographed structures. The values ranged from 180 to 300 granules per nucleus with an average of ~250. This represents a minimal estimation, because some of the granules in each nucleus were not visible in the particular focal plane that was photographed. These results support the notion that replication occurs at a multitude of discrete structural domains within the cell nucleus and further suggest that these numerous sites are carrying out DNA synthesis at comparable rates.

Newly Synthesized DNA on In Situ Nuclear Matrix

As an approach to identifying the structural associations of the replication granules within the cell nucleus, we extracted the cells after DNA synthesis with a series of treatments (e.g., DNase I and 0.2 $(NH_4)_2SO_4$, see Materials and Methods) designed to remove chromatin and soluble nuclear components while minimizing perturbation of the general size, shape, and nonchromatin structures of the cell nucleus. The remaining in situ nuclear matrix was surrounded by a cytoskeletal framework that likely adds to the stability of this nuclear substructure. A detailed study of these in situ prepared nuclear matrices will be published elsewhere (Nakayasu, H., and R. Berezney, manuscript in preparation). The preparations were very similar to those previously described (Capco et al., 1982; Staufenbiel and Deppert, 1984; Fey et al., 1986). They were devoid of histones, contained <10% of the total nuclear DNA and EM revealed a typical nuclear matrix structure consisting of a surrounding nuclear lamina, residual nucleoli, and an elaborate intranuclear fibrogranular network.

As shown in Fig. 3, there was a remarkable maintenance of the replication granules after preparation of DNA-depleted in situ nuclear matrices. Both the size of individual granules and their distribution throughout the in situ matrices (Fig. 3 *c*), including exclusion from residual nucleoli, were strikingly similar to the corresponding nuclei of permeabilized cells (Fig. 3 *a*). Because the intranuclear fibrogranular structure of the in situ prepared nuclear matrix is clearly visible under phase-contrast microscopy (Fig. 3 *d*), we were able to further observe that many of the replication granules are sequestered over this internal network. The approximate number of granules per matrix structure (150–300) and the fluorescence intensity of the individual granules were also comparable to that in nuclei, despite the removal of >90% of the total nuclear DNA (the effective removal of DNA from individual matrices was always monitored by Hoechst staining).

A number of investigations have demonstrated in vitro DNA synthesis in isolated DNA-depleted nuclear matrices that is mediated virtually exclusively by the replicative enzyme, DNA polymerase α (Berezney and Smith, 1980, 1982; Jones and Su, 1982; Mikhailov and Tsanev, 1983; Nishizawa et al., 1984; Smith et al., 1984; Foster and Collins, 1985; Wood and Collins, 1986; Tubo and Berezney, 1987*a*). We, therefore, investigated the structural organization of DNA synthesis in the in situ nuclear matrix prepara-

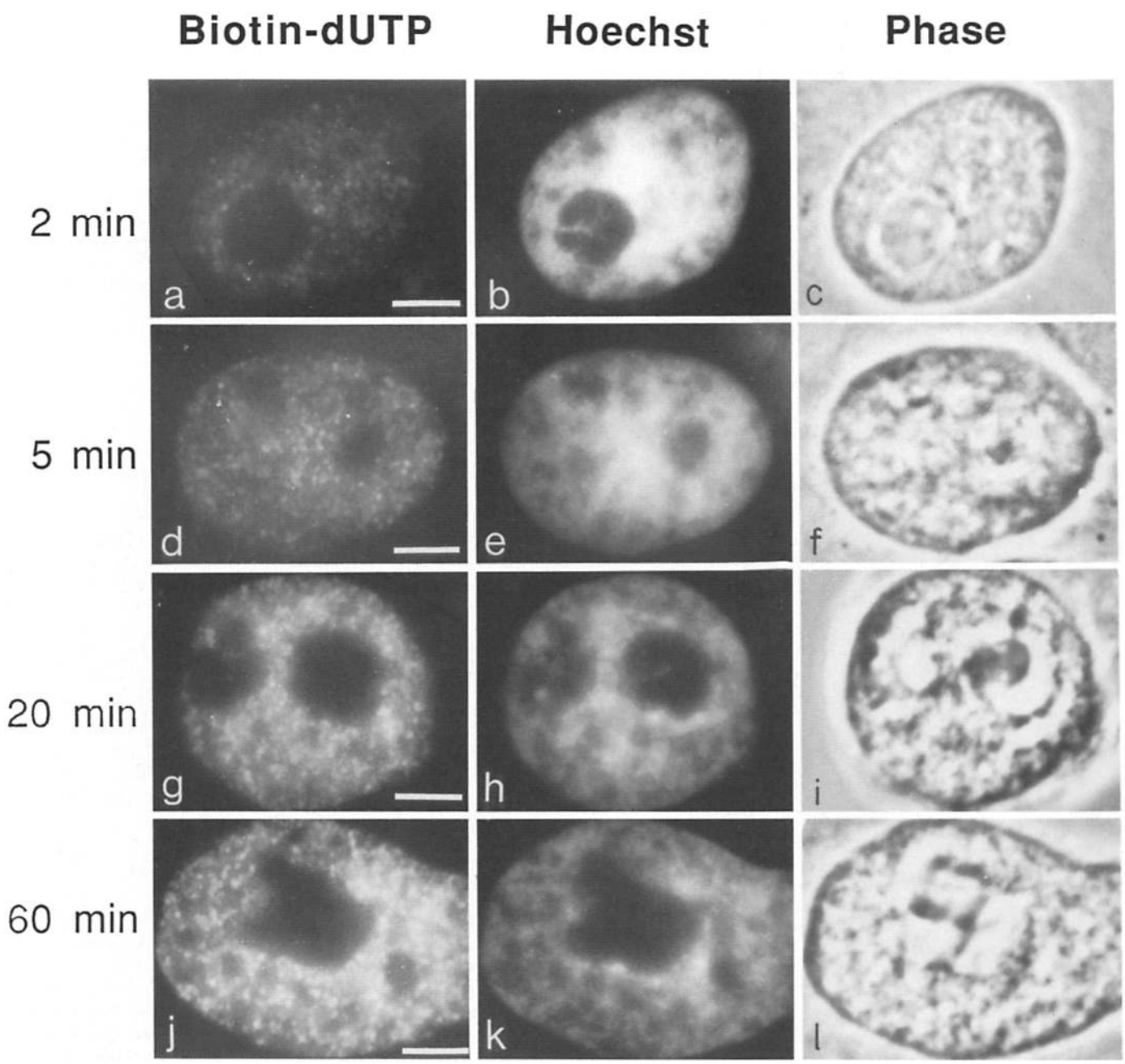

Figure 2. Time course of incorporation of biotin-dUTP into permeabilized PtK-1 cells. PtK-1 cells on coverslips were permeabilized with 0.04% Triton X-100 and then incubated with DNA synthesis medium containing biotin-11-dUTP at 37°C for 2, 5, 20, and 60 min. Visualization of newly synthesized DNA and staining with Hoechst 33258 dye were carried out as described in Materials and Methods. Bars, 4 μm.

tions after biotin-11-dUTP incorporation. The results demonstrated a granular pattern of localization strikingly similar to that of the in situ nucleus (Fig. 3 *e*). The matrix-bound replication granules were, again, preferentially distributed along the internal network of the nuclear matrix but not in residual nucleoli (Fig. 3, *e* and *f*) and were of similar size and found in similar numbers (150–300) as in permeabilized cells.

Relationship of DNA Replication Patterns to Chromatin Structural Domains in the Cell Nucleus

PtK-1 cells, due to their extraordinary flat shape (and consequently flat nuclei), are ideally suited for structural localization studies of the cell nucleus. Unfortunately, they have little visible condensed chromatin (heterochromatin). This made it virtually impossible to distinguish between incorporation over dense heterochromatic versus diffuse euchromatic regions in the nucleus. We, therefore, studied the structural localization of newly synthesized DNA in mouse 3T3 fibroblasts, which have well-defined perinuclear and intranuclear heterochromatic regions in their nuclei. This is evident in Fig. 4, where Hoechst staining of 3T3 cells showed both extremely intense staining regions (heterochromatin) and much fainter diffuse staining regions (euchromatin). Our observations, which are described below, lead us to propose three

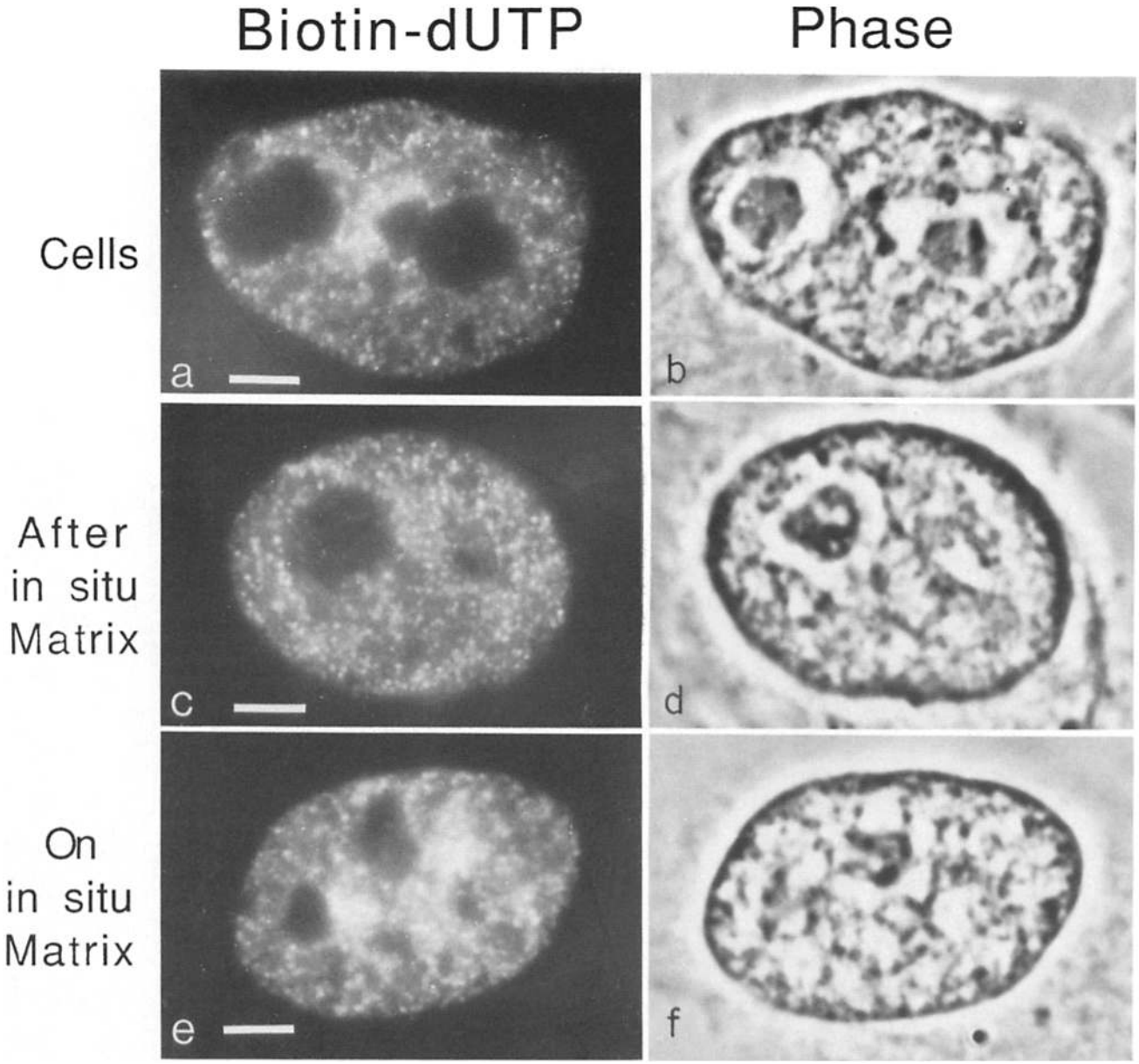

Figure 3. DNA synthesis in permeabilized cells and after extraction for in situ nuclear matrix. (*a* and *b*) PtK-1 cells on coverslips were permeabilized with 0.04% Triton X-100 and incubated with the DNA synthesis medium at 37°C for 5 min. In *c* and *d*, the cells were then treated with DNase I and 0.2 M ammonium sulfate to prepare in situ nuclear matrix structures (see Materials and Methods). In *e* and *f*, in situ nuclear matrices were first prepared followed by the incubation for DNA synthesis. Bars, 4 μm.

distinct patterns of replication sites in the cell nucleus termed types I, II, and III.

(a) Type I. Approximately 35% of the total 3T3 cells exhibited detectable DNA synthesis. Of these, about half of the nuclei revealed discrete intranuclear granules (Fig. 4, *a*, *b*, and *g*) that were very similar to those in PtK-1 cells. The replication granules were, again, completely excluded from nucleoli (visible as darker regions in phase microscopy and also as unstained regions in Hoechst staining), but were not excluded from heterochromatic regions (visible as slightly darker regions in phase microscopy and also as impressively bright regions after Hoechst staining). In cells of type I, therefore, DNA replication was proceeding at both euchromatic and heterochromatic regions.

(b) Type II. About 25% of nuclei synthesizing DNA showed a different replication pattern, termed type II. In these nuclei, significant fluorescence appeared over the perinuclear region including the perinuclear heterochromatin (Fig. 4, *c*, *d*, and *h*). Whereas there were also many intranuclear granules, these replication granules, unlike type I patterns, were preferentially associated with the intense Hoechst staining heterochromatic regions including the perinucleolar heterochromatin. The replication granules were also often arranged in small clusters.

(c) Type III. Another pattern (type III) was seen in the remaining 25% of nuclei synthesizing DNA. Extremely bright fluorescence was distributed over many of the intranuclear heterochromatic regions (Fig. 4, *e*, *f*, and *i*). These bright

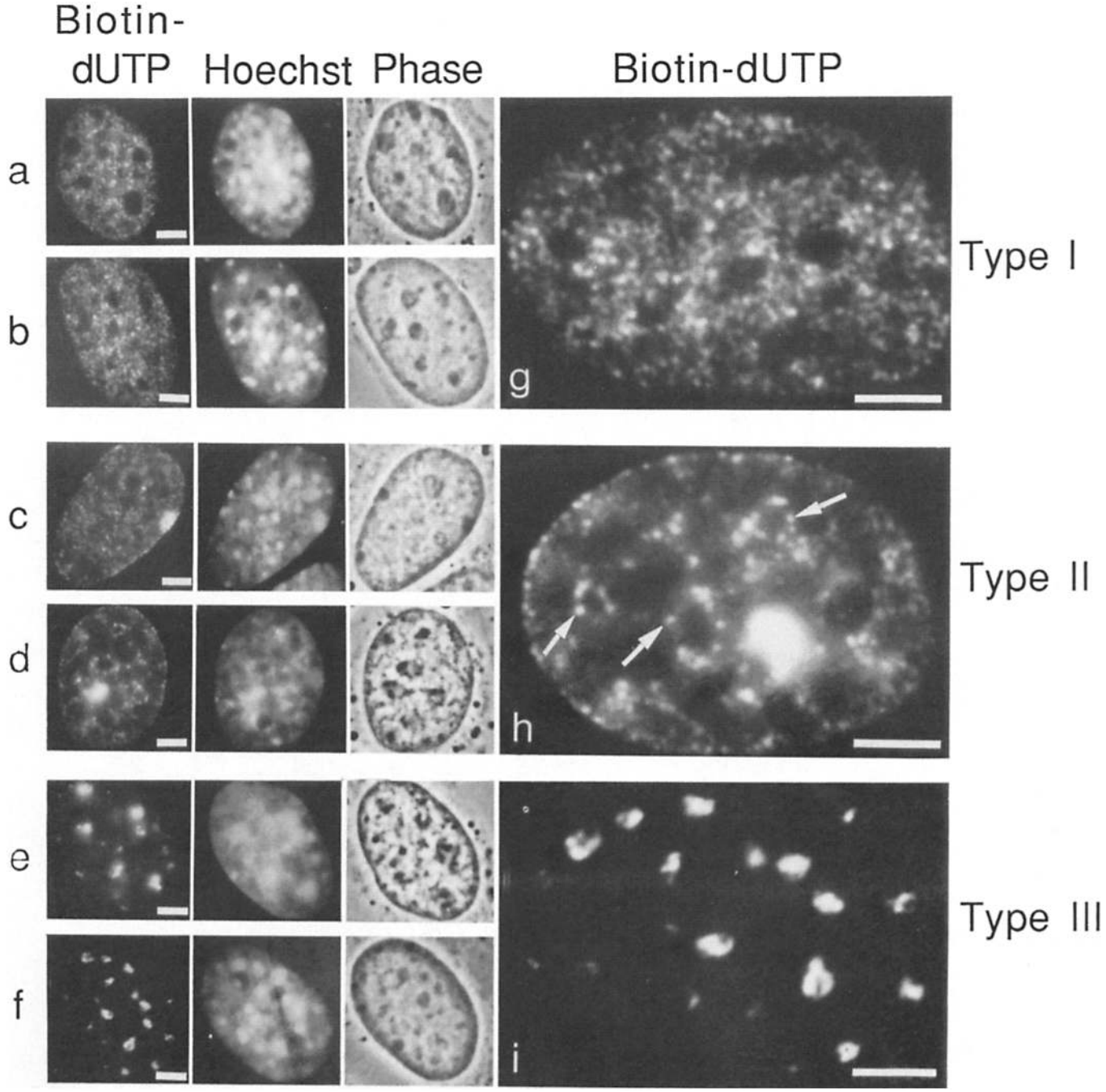

Figure 4. DNA synthesis on permeabilized 3T3 cells. Mouse 3T3 cells were permeabilized with 0.04% Triton X and incubated with DNA synthesis medium at 37°C for 10 min. (*a* and *b*) Type I DNA replication pattern; (*c* and *d*) type II DNA replication pattern; (*e* and *f*) type III DNA replication pattern; (*g*, *h*, and *i*) enlargements of types I, II, and III, respectively; *arrows* point to replication granules forming ring-like structures surrounding nucleoli in type II structures. Bars, 4 μm.

regions often appeared to be composed of clusters of granules with a size similar to the replication granules of types I and II. This was most obvious in the perinucleolar heterochromatic, in which the replication granules clustered into ring- or horseshoe-like arrays. Fluorescent were also observed at the perinuclear heterochromatic region. In contrast, the euchromatic regions were completely devoid of newly synthesized DNA. We conclude that DNA replication is proceeding virtually exclusively at heterochromatic regions in type III.

These three patterns were well maintained during preparation of in situ 3T3 nuclear matrix. Type I (Fig. 5 *a*), type II (Fig. 5 *c*), and type III (Fig. 5 *e*) distributions were easily recognizable and were found in similar proportions as in permeabilized cells.

DNA Replication Patterns in 3T3 Cells after Cell Synchronization

To determine the possible relationship of these three different patterns of DNA synthesis to the S phase, we synchronized

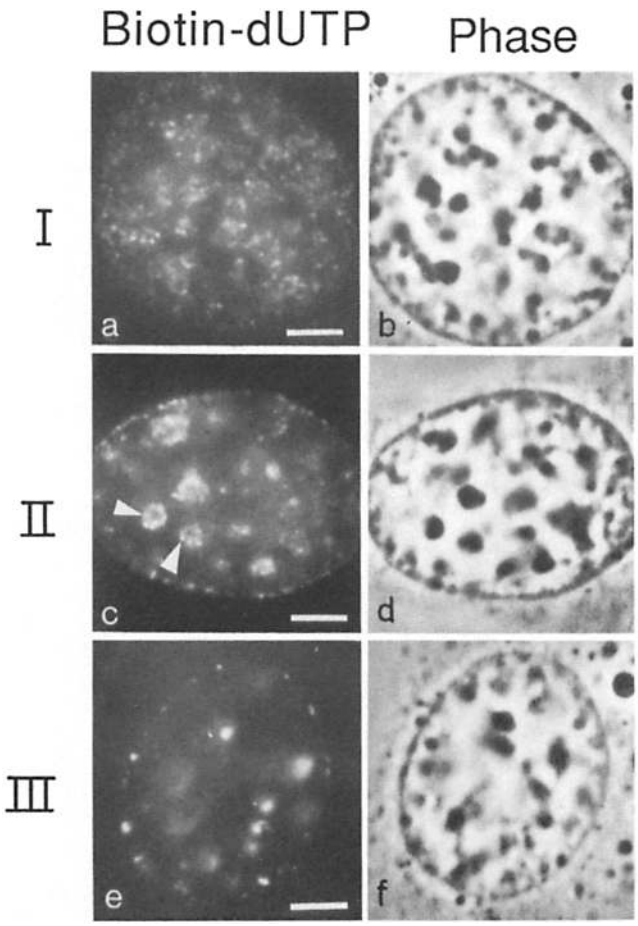

Figure 5. Maintenance of newly synthesized DNA on in situ 3T3 nuclear matrix. 3T3 cells on coverslips were permeabilized and DNA synthesis was performed as in the legend to Fig. 4. The cells were then treated with DNase I and 0.2 M ammonium sulfate to prepare in situ nuclear matrix structures (see Materials and Methods). (*a*) Type I structure on in situ nuclear matrix; (*c*) type II structure; (*e*) type III structure; (*b*, *d*, and *f*) corresponding phase microscopy of *a*, *c*, and *e*, respectively, arrowheads point to replication granules forming ring-like structures surrounding residual nucleoli in type II structures on matrix; Bars, 4 μm.

the 3T3 cells by serum deprivation (see Materials and Methods). 3T3 cells were arrested at the Go phase after incubation in medium containing 0.05% serum for 72 h. As shown in Fig. 6 *a*, DNA synthesis began between 10 and 12 h after addition of 10% serum and mitosis after 22–24 h. Fig. 6 *b* indicates the frequency of each replication type during S phase. At early S phase, the bulk of the nuclei synthesizing DNA (~80%) were type I. In contrast, most nuclei synthesizing DNA during late S phase (~70%) were type III. These results indicate that type I is specific for early stages of DNA replication and type III is typical of the later stages. Whereas type II nuclei were found throughout S phase, the ratio of type II increased significantly during mid S phase (Fig. 6 *b*). The type II pattern may, therefore, represent a transition stage from type I to type III.

Relationship to In Vivo Sites of Replication

Although considerable studies indicated that permeabilized cells are likely to be suitable in vitro model systems to study the structural organization of DNA replicational sites in the cell nucleus (see Introduction), it was important to attempt to test directly this assumption. Recently mAbs to BrdU have been effectively used to detect nuclei synthesizing DNA in vivo (Gratzner, 1982). We, therefore, pulsed 3T3 cells with BrdU and performed indirect immunofluorescent microscopy after incubation of the cells with an affinity-purified mAb to BrdU and an appropriate rhodamine-conjugated secondary antibody (see Materials and Methods). After incorporation times ranging from 30 to 120 min, the newly replicated DNA was distributed in spatial patterns that were remarkably similar to the types I, II, and III patterns identified in permeabilized cells after biotin-11-dUTP incorporation (Fig. 7). The replication sites in the type I-like patterns, however, displayed considerable size heterogeneity, with dimensions ranging from those of the replication granules detected with biotin-11-dUTP (~0.5 μm) to several microns. Moreover, many of these larger structures had ring-like or chain-like shapes (Fig. 7 *a*). Unfortunately, the fluorescent patterns of pulse periods <30 min were too weak to interpret accurately. We were, therefore, unable to determine whether the larger structures correspond to actual sites of replicating DNA or postreplicated DNA organized into higher-ordered structures. In any case, a considerable portion (30–50%) of the total nuclear sites that contained in vivo replicated DNA were strikingly similar in size and shape to the replicational granules detected with the biotin-11-dUTP system.

Discussion

Eucaryotic DNA is replicated in a series of tandemly repeated subunits along the continuous DNA molecule, termed replicons (Huberman and Riggs, 1968; Hand, 1978). Adjacent replicons are further organized into clusters that replicate as a unit at particular times in S phase (Huberman and Riggs, 1968; Hori and Lark, 1974; Hand and Tamm, 1974; Hand, 1975, 1978; Painter and Young, 1976; Painter, 1978; Lau and Arrighi, 1981). The numerous reports that specific DNA sequences are replicated at discrete times during S phase (Goldman et al., 1984; Pierron et al., 1984; Jalouzot et al., 1985; Gilbert, 1986), supports the conclusion that replicon cluster synthesis is temporally and spatially regulated along the chromosomal DNA (Hand, 1978). Our results further suggest that there are precise structural sites of replicon cluster synthesis in the cell nucleus. We find that DNA synthesis is sequestered within discrete sites distributed throughout the nuclear interior and termed replication granules. Each replication granule may correspond to a replicon cluster assembly, in which numerous tandemly arranged replicons are coordinately synthesizing DNA. Assuming $\sim 5 \times 10^4$ replicons per average diploid mammalian nucleus (Huberman and Riggs, 1968) and an average of ~25 replicons per replicon cluster (Painter and Young, 1976; Hand, 1978), the number of replication granules detected in type I nuclei (200–300) is consistent with the predicted number of replicon clusters presumed to be active at a given time in S phase. These discrete granular sites of replicon cluster synthesis are also maintained after extraction of the permeabilized cells to prepare DNA-depleted nuclear matrix (Figs. 4 and 5). Moreover, DNA synthesized on nuclear matrices was also arranged in similar replication granules (Fig. 4).

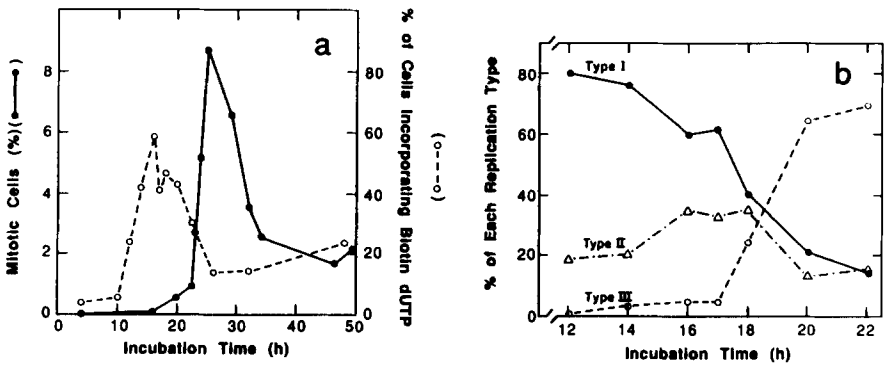

Figure 6. Synchronization of 3T3 cells. 3T3 cells was arrested by serum starvation (see Materials and Methods) then released from the G_0 stage by the addition of 10% serum. (*a*) Changes of DNA synthesis and mitotic index after the addition of 10% serum. (*b*) Percent of each structural pattern of newly synthesized DNA at several time points during S phase.

The structural maintenance of these in situ sites of DNA synthesis in nuclear matrix is consistent with numerous reports that conclude that the nuclear matrix contains the attachment sites for both replicon origins and replication forks (Berezney and Coffey, 1975; Dijkwel et al., 1979; McCready et al., 1980; Vogelstein et al., 1980; Berezney and Buchholtz, 1981; Aelen et al., 1983; Valenzuela et al., 1983; van der Velden et al., 1984*b*; Tubo and Berezney, 1985; Carri et al., 1986; Dijkwel et al., 1986; Jackson and Cook, 1986*a*; for a recent review see van der Velden and Wanka, 1987). DNA polymerase α, primase, and other putative replicational enzymes are associated with the nuclear matrix in a cell cycle and replicative dependent manner (Berezney and Smith, 1980, 1982; Jones and Su, 1982; Mikhailov and Tsanev, 1983; Nishizawa et al., 1984; Smith et al., 1984; Foster and Collins, 1985; Wood and Collins, 1986; Collins

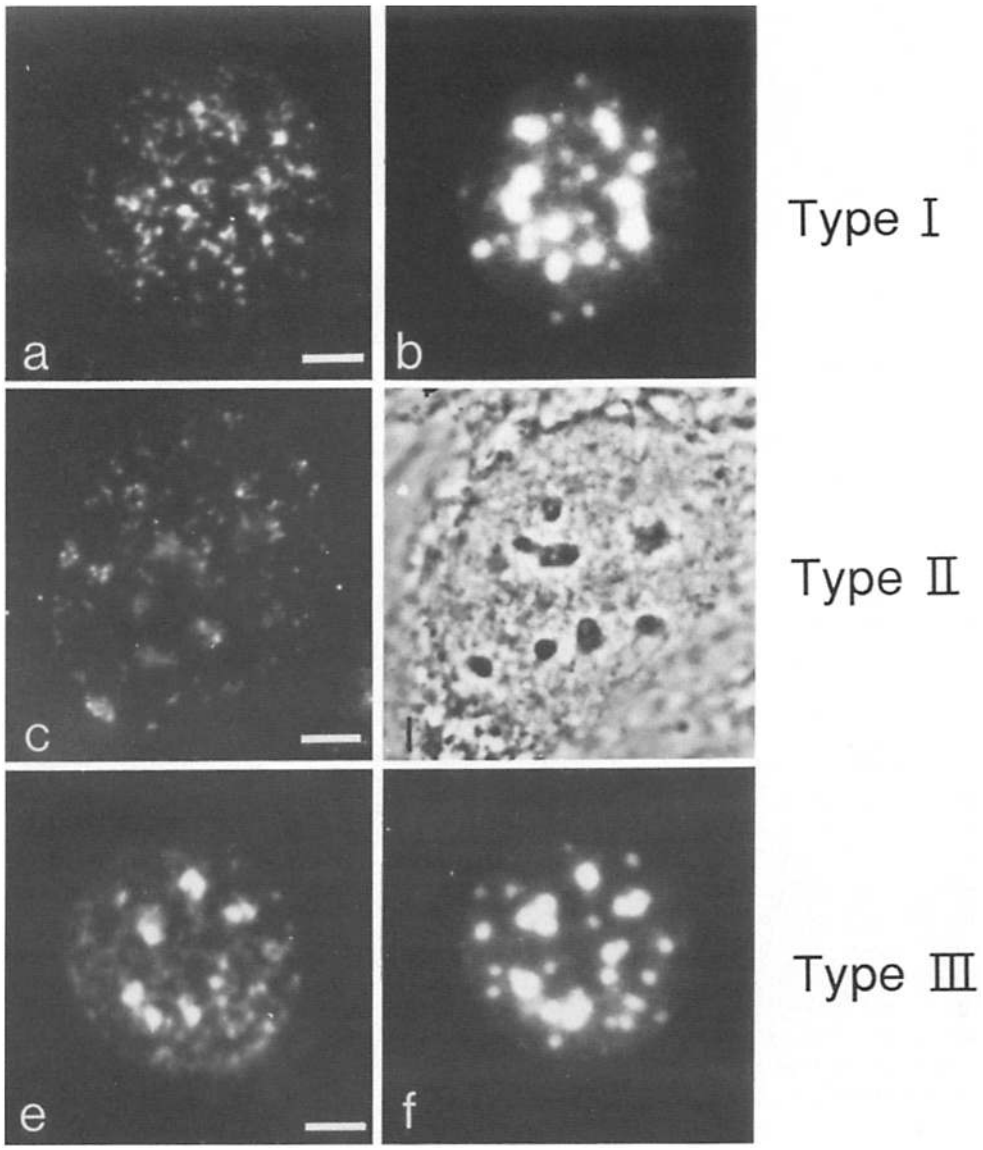

Figure 7. Visualization of in vivo DNA synthesis for 3T3 cells using antibodies to BrdU. Exponentially growing 3T3 cells were incubated at 37°C with 10 μM BrdU and 1 μM flurodeoxyuridine and processed for immunofluorescence staining with antibodies to BrdU as described in Materials and Methods. The results shown are after a 60-min BrdU pulse. No differences were seen in pulse periods ranging from 30 to 120 min. (*a*) Type I replication pattern; (*c*) type II replication pattern; (*e*) type III replication pattern; (*b* and *f*) corresponding Hoechst 33258 staining to *a* and *e*; (*d*) corresponding phase microscopy to *c* to better see the relationship of type II replication sites to the nucleoli. Bars, 4 μm.

and Chu, 1987; Jackson and Cook, 1986*b*; Tubo and Berezney, 1987*a*, *b*). Recently we demonstrated that the nuclear matrix-bound DNA polymerase and primase are organized into huge megacomplexes that sedimented at ~100–150 S on sucrose gradients (Tubo and Berezney, 1987*c*). The presence of only more typically sized 10 S complexes in nuclear matrices prepared just before the onset of S phase and the rapid in vitro conversion of the megacomplexes to 10 S complexes after release from the nuclear matrix led us to propose that the megacomplexes represent the corresponding clustering of the replicational assemblies which presumably underlie the replicon clusters (Tubo and Berezney, 1987*c*). We further speculated that the cell cycle–dependent and subsequent clustering of matrix-bound replicational complexes is a driving force for the formation of these higher ordered assemblies of DNA replication or "clustersomes" in the cell nucleus and may, thus, be an important factor in the regulation of DNA replication in the cell. The structural localization studies presented in this paper lend support to these previous biochemical studies and suggest that isolated nuclear matrix is a potentially important in vitro model system for studying the structural organization and regulation of higher ordered replicational assemblies in the cell nucleus.

In this regard, the correlation of sites of DNA replication with chromosome banding patterns have led several investigators to conclude that chromosome bands correspond to the basic units of replication or replication clusters in the interphase nucleus (Latt, 1975; Stubblefield, 1975; Kondra et al., 1978; Lau and Arrighi, 1981; Meer et al., 1981; Holmquest et al., 1982). Typically it is found that R bands (euchromatin) preferentially replicate in early S phase, G bands (noncentromeric heterochromatin) in late S phase, and C bands (centromeric heterochromatin) in very late S phase. (Kondra, 1978; Schempp and Vogel, 1978; Camargo and Cervenka, 1982; Holmquest et al., 1982).

The three types of structural patterns resolved in interphase cells by our study both confirm and extend these previous investigations. The type I granules of early S phase thus likely correspond to sites of organization at which the chromatin of R bands replicate. The type III patterns of late S phase over the intense Hoechst positive spots (see Fig. 4) correspond to the sites where the centromeric heterochromatin of C bands replicate (Pardue and Gall, 1970). The type II pattern particularly prominent in mid to late S phase likely represents a transition where both sites of R and G bands are replicating. Moreover, some G bands probably also replicate in type III. Such temporal overlap of R and G bands in mid S, and G and C band replication in late S was previously described (Schempp and Vogel, 1978; Camargo and Cervenka, 1982).

Replication units similar in dimensions to those of this study were also reported by Lau and Arrighi (1981) after premature chromosome condensation (PCC). In a scanning electron microscopic study, Mulinger and Johnson (1983) found that S phase PCC fragments were arranged in repeating arrays of granular aggregates of fibers with diameters ranging from 0.25–1.6 μm and a mean of 0.75 μm. Most of the replication granules observed in our study ranged from 0.4 to 0.6 μm in early S phase with much larger aggregates up to several microns observed in later stages. Significantly, Lau and Arrighi (1981) also observed a progressive increase in the size of replication units along PCC fragments from 0.4 to 0.6 μm in early S to several microns in late S. Our results, therefore, provide support for the previously stated view (Lau and Arrighi, 1981; Holmquest et al., 1982; Marcus, 1985) that replication structures corresponding to chromosome bands and containing replicon clusters are associated with the replicational machinery on the nuclear matrix.

Nakamura et al. (1986), using antibodies against BrdU, have also reported discrete structural sites of replicon cluster synthesis in the cell nucleus. Direct comparison with our results, however, is complicated by the relatively long cumulative labeling of cells with BrdU performed by these investigators. It may be significant, however, that replication granules of similar size were detected in these experiments after relatively short labeling periods. Similarly we found that a significant proportion (30–50%) of the total replicational sites seen after BrdU incorporation appear identical in size and shape to the replication granules revealed by biotin-11-dUTP incorporation into permeabilized cells (Fig. 7). Structures much larger than the replication granules are detected in the remaining replicational sites decorated by the anti-BrdU antibodies, which sometimes form ring- or horseshoe-like shapes. Nakamura et al. (1986) have proposed that these larger sites represent later stages in replicon cluster synthesis. This is conceivable, as one limitation of the permeabilized cell system is the apparent inability to ligate DNA of replicon size into larger units of replicated DNA (Berger et al., 1977). It is also possible, however, that these larger structures represent the close association of numerous individual replication granules over heterochromatic areas, as strikingly demonstrated by the type II and III patterns of our studies. In this regard we also found type II and type III patterns in our experiment with BrdU incorporation (Fig. 7). We are currently developing more sensitive immunofluorescent microscopic procedures to address this issue in BrdU labeled cells.

Our studies also predict that various replicational enzymes such as DNA polymerase α, and primase should colocalize within the discrete replication granules demonstrated in this study. Immunofluorescent microscopic studies of DNA polymerase α have indeed demonstrated a granular distribution of this enzyme in the nucleus (Nakamura et al., 1984; Yamamoto et al., 1984) and maintenance of this granular organization after preparation of nuclear matrix structures (Yamamoto et al., 1984). Moreover, Bravo and Macdonald-Bravo (1987) recently used immunofluorescent microscopy to demonstrate similar structural distributions of PCNA (proliferating cell nuclear antigen, also called cyclin), a presumptive subunit of DNA polymerase δ (Bravo et al., 1987; Prelich et al., 1987), with sites of in vivo replicated DNA. These latter findings confirm previous studies based on anti-PCNA immunofluorescence and autoradiographic analysis of newly replicated DNA (Bravo and Macdonald-Bravo, 1985; Celis and Celis, 1985; Madsen and Celis, 1985).

The experiments on BrdU incorporation were kindly performed by Dr. Alberto M. Martelli and Linda A. Buchholtz. We are extremely grateful to Dr. George L. Mayers and Dr. Richard B. Bankert from Roswell Park Memorial Institute for their kind gift of affinity-purified mAb to BrdU. We also thank Dawn Styres for typing this manuscript and Jim Stamos and Alan Siegel for their help with the photography and illustrations.

This work was supported by National Institutes of Health grant GM-23922. Dr. Nakayasu was supported in part by a State University of New York at Buffalo Presidential Post-doctoral Fellowship Award.

Received for publication 29 March 1988, and in revised form 16 September 1988.

References

Aelen, J. M. A., R. J. G. Opstelten, and F. Wanka. 1983. Organization of DNA replication in Physarum polycephalum. Attachment of origins of replicons and replication forks to the nuclear matrix. *Nucleic Acids Res.* 11:1181–1195.

Berezney, R., and L. A. Buchholtz. 1981. Dynamic association of replicating DNA fragments with the nuclear matrix of regenerating liver. *Exp. Cell Res.* 132:1–13.

Berezney, R., and D. S. Coffey. 1975. Nuclear protein matrix: Association with newly synthesized DNA. *Science (Wash. DC).* 189:291–293.

Berger, N. A., S. J. Petzold, and E. S. Johnson. 1977. High molecular weight DNA intermediates synthesized by permeabilized L cells. *Biochim. Biophys. Acta.* 478:44–58.

Bravo, R., R. Frank, P. A. Blundel, and H. Macdonald-Bravo. 1987. Cyclin/PCNA is the auxiliary protein of DNA polymerase-δ. *Nature (Lond.).* 326: 515–517.

Bravo, R., and H. Macdonald-Bravo. 1985. Changes in the nuclear distribution of cyclin (PCNA) but not its synthesis depend on DNA replication. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:655–661.

Bravo, R., and H. Macdonald-Bravo. 1987. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle. Association with DNA replication sites. *J. Cell Biol.* 105:1549–1554.

Camargo, M., and J. Cervenka. 1982. Patterns of DNA replication of human chromosomes. II. Replication map and replication model. *Am. J. Hum. Genet.* 34:757–780.

Capco, D. G., G. Krochmalnic, and S. Penman. 1982. The nuclear matrix: Three dimensional architecture and protein composition. *Cell.* 29:847–858.

Carri, M. T., G. Micheli, E. Graziano, T. Pace, and M. Buongiorno-Nardelli. 1986. The relationship between chromosomal origins of replication and the nuclear matrix during the cell cycle. *Exp. Cell Res.* 164:426–436.

Celis, J. E., and A. Celis. 1985. Cell cycle-dependent variations in the distribution of the nuclear protein cyclin in cultured cells: Subdivision of S phase. *Proc. Natl. Acad. Sci. USA.* 82:3262–3266.

Collins, J. M., and A. K. Chu. 1987. Binding of the DNA polymerase alpha-DNA primase complex to the nuclear matrix in HeLa cells. *Biochemistry.* 26:5600–5607.

Dijkwel, P. A., L. H.F. Mullenders, and F. Wanka. 1979. Analysis of the attachment of replicating DNA to a nuclear matrix in mammalian interphase nuclei. *Nucleic Acids Res.* 6:219–230.

Dijkwel, P. A., P. W. Wenink, and J. Poddighe. 1986. Permanent attachment of replication origins to the nuclear matrix in BHK-cells. *Nucleic Acids Res.* 14:3241–3249.

Fakan, S. 1978. High resolution autoradiography studies on chromatin functions. *In* The Cell Nucleus. Vol. 5. H. Busch, editor. Academic Press, Inc., New York. 3–53.

Fakan, S., and R. Hancock. 1974. Localization of newly-synthesized DNA in a mammalian cell as visualized by high resolution autoradiography. *Exp. Cell Res.* 83:95–102.

Fey, E. G., G. Krochmalnic, and S. Penman. 1986. The non-chromatin substructures of the nucleus: The ribonucleoprotein (RNP)-containing and RNP-depleted matrices analyzed by sequential fractionation and resinless section electron microscopy. *J. Cell Biol.* 102:1654–1665.

Foster, K. A., and J. M. Collins. 1985. The interrelations between DNA synthesis rates and DNA polymerases bound to the nuclear matrix in synchronized HeLa cells. *J. Biol. Chem.* 260:4229–4235.

Gautschi, J. R., Burkhalter, M., and P. Rheinhard. 1977. Semi-conservative DNA replication in vitro. II. Replicative intermediates of mouse P-815 cells. *Biochim. Biophys. Acta.* 474:512–523.

Gilbert, D. M. 1986. Temporal order of replication of Xenopus laevis 5S ribosomal RNA genes in somatic cells. *Proc. Natl. Acad. Sci. USA.* 83:2924–2928.

Goldman, M. A., G. P. Holmquist, M. C. Gray, L. Caston, and A. Nag. 1984. Replication timing of genes and middle repetitive sequences. *Science (Wash. DC).* 224:686–692.

Gratzner, H. G. 1982. Monoclonal antibody to 5-bromo and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science (Wash. DC).* 218:474–475.

Hand, R. 1975. Regulation of DNA replication on subchromosomal units of mammalian cells. *J. Cell Biol.* 64:89–97.

Hand, R. 1978. Eucaryotic DNA: organization of the genome for replication. *Cell.* 15:317–325.

Hand, R., and J. R. Gautschi. 1979. Replication of mammalian DNA in vitro. Evidence for initiation from fiber autoradiography. *J. Cell Biol.* 82:485–493.

Hand, R., and I. Tamm. 1974. Initiation of DNA replication in mammalian cells and its inhibition by reovirus infection. *J. Mol. Biol.* 82:175–183.

Hay, E. D., and J. P. Revel. 1966. The fine structure of the DNP component of the nucleus. An electron microscopic study utilizing autoradiography to localize DNA synthesis. *J. Cell Biol.* 16:29–51.

Holmquist, G., M. Gray, T. Porter, and J. Jordan. 1982. Characterization of Giemsa dark and light band DNA. *Cell.* 31:121–129.

Hori, T.-A., and K. G. Lark. 1974. Autoradiographic studies of the replication of satellite DNA in the kangaroo rat. Autoradiographs of satellite DNA. *J. Mol. Biol.* 88:221–232.

Jackson, D. A., and P. R. Cook. 1986*a*. Replication occurs at a nucleoskeleton. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1403–1410.

Jackson, D. A., and P. R. Cook. 1986*b*. A cell cycle-dependent DNA polymerase activity that replicates intact DNA in chromatin. *J. Mol. Biol.* 192:65–67.

Jalouzot, R., B. Toublan, M. L. Wilhelm, and F. X. Wilhelm. 1985. Replication timing of the H4 histone genes in Physarum polycephalum. *Proc. Natl. Acad. Sci. USA.* 82:6475–6479.

Jones, C., and R. T. Su. 1982. DNA polymerase alpha from the nuclear matrix of cells infected with simian virus 40. *Nucleic Acids Res.* 10:5517–5532.

Kondra, P. M., and M. Ray. 1978. Analysis of DNA replication patterns of human fibroblast chromosomes. The replicon map. *Hum. Genet.* 43:139–149.

Kornberg, A. 1988. DNA replication. *J. Biol. Chem.* 263:1–4.

Langer, P. R., A. A. Waldrop, and D. C. Ward. 1981. *Proc. Natl. Acad. Sci. USA.* 78:6633–6637.

Latt, S. A. 1975. Fluorescence analysis of late DNA replication in human metaphase chromosomes. *Somatic Cell Genet.* 1:293–321.

Lau, Y.-F., and F. E. Arrighi. 1981. Studies of mammalian chromosome replication. II. Evidence for the existence of define chromosome replicating units. *Chromosoma.* 83:721–741.

Madsen, P., and J. E. Celis. 1985. S-phase patterns of cyclin (PCNA) antigen staining resemble topographical patterns of DNA synthesis. A role for cyclin in DNA replication? *FEBS (Fed. Eur. Biochem. Soc.). Lett.* 193:5–11.

Marcus, M., A. Fainsod, and G. Diamond. 1985. The genetic analysis of mammalian cell cycle mutants. *Annu. Rev. Genet.* 19:389–421.

McCready, S. J., J. Godwin, D. W. Mason, I. A. Brazell, and P. R. Cook. 1980. DNA is replicated at the nuclear cage. *J. Cell Sci.* 46:365–386.

Meer, B., H. Hameister, and M. Cerrillo. 1981. Early and late replication patterns of increased resolution in human lymphocyte chromosomes. *Chromosoma.* 82:315–319.

Mikhailov, V. S., and R. Tsanev. 1983. Intranuclear localization of DNA polymerase alpha and beta in regenerating rat liver. *Int. J. Biochem.* 15:855–859.

Miller, M. R., C. Seighman, and R. G. Ulrich. 1985. Inhibition of DNA replication and DNA polymerase alpha activity by monoclonal anti-(DNA polymerase alpha) immunoglobulin G and F(ab) fragments. *Biochemistry.* 24: 7440–7445.

Milner, G. R. 1969. Nuclear morphology and the ultrastructural localization of deoxyribonucleic acid synthesis during interphase. *J. Cell Sci.* 4:569–582.

Mullinger, A. M., and R. T. Johnson. 1983. Units of chromosome replication and packing. *J. Cell Sci.* 64:179–193.

Nakamura, H., T. Morita, S. Masaki, and S. Yoshida. 1984. Intracellular localization and metabolism of DNA polymerase alpha in human cells visualized with monoclonal antibody. *Exp. Cell Res.* 151:123–133.

Nakamura, H., T. Morita, and C. Sato. 1986. Structural organizations of replicon domains during DNA synthetic phase in the mammalian nucleus. *Exp. Cell Res.* 165:291–297.

Nishizawa, M., K. Tanabe, and T. Takahashi. 1984. DNA polymerases and DNA topoisomerases solubilize from nuclear matrices of regenerating rat liver. 124:917–924.

Olins, D. E., and A. L. Olins. 1987. In vitro DNA synthesis in the macronuclear replication band of Euptotes eurystomus. *J. Cell Biol.* 104:1125–1132.

Painter, R. B. 1978. Inhibition of DNA replicon initiation by 4-nitroquinoline 1-oxide, adriamycin and ethyleneimine. *Cancer Res.* 38:4445–4449.

Painter, R. B., and B. R. Young. 1976. Formation of nascent DNA molecules during inhibition of replicon initiation in mammalian cells. *Biochim. Biophys. Acta.* 418:146–153.

Pardoll, D. M., Vogelstein, B., and D. S. Coffey. 1980. A fixed site of DNA replication in eucaryotic cells. *Cell.* 19:527–536.

Pardue, M. L., and J. G. Gall. 1970. Chromosomal localization of mouse satellite DNA. *Science (Wash. DC).* 168:1356–1358.

Pierron, G., Durica, D. S., and H. W. Sauer. 1984. Invariant temporal order of replication of the four actin gene loci during the naturally synchronous mitotic cycles of Physarum polycephalum. *Proc. Natl. Acad. Sci. USA.* 81:6393–6397.

Prelich, G., C.-K. Tan, M. Kostura, M. B. Mathews, A. G. So, K. M. Downey, and B. Stillman. 1987. Functional identity of proliferating cell nuclear antigen and a DNA polymerase–auxiliary protein. *Nature (Lond.).* 326:517–520.

Rheinhard, P., M. Burkhalter, and J. Gautschi. 1977. Semi-conservative DNA replication in vitro. I. Properties of two systems derived from mouse P-815 cells by permeabilization or lysis with Brij-58. *Biochim. Biophys. Acta.* 378:333–343.

Schempp, W., and W. Vogel. 1978. Decrease of DNA synthesis in amniotic fluid cells during the middle part of S-phase revealed by differential chromosome staining after incorporation of BUdR. *Chromosome.* 67:193–199.

Smith, H. C., and R. Berezney. 1980. DNA polymerase alpha is tightly bound to the nuclear matrix of actively replicating liver. *Biochem. Biophys. Res. Commun.* 97:1541–1547.

Smith, H. C., and R. Berezney. 1982. Nuclear matrix-bound deoxyribonucleic acid synthesis. A in vitro system. *Biochemistry.* 21:6751–6761.

Smith,. H. C., E. Puvion, L. A. Buchholtz, and R. Berezney. 1984. Spatial

distribution of DNA loop attachment and replicational sites in the nuclear matrix. *J. Cell Biol.* 99:1794–1802.

Staufenbiel, M., and W. Deppert. 1984. Preparation of nuclear matrices from cultured cells: Subfractionation of nuclei *in situ*. *J. Cell Biol.* 98:1886–1894.

Stubblefield, E. 1975. Analysis of the replication pattern of Chinese hamster chromosomes using 5-bromodeoxyuridine suppression of 33258 Hoechst fluorescence. *Chromosome*. 53:209–221.

Tubo, R. A., and R. Berezney. 1987*a*. Pre-replicative association of multiple replicative enzyme activities with the nuclear matrix during rat liver regeneration. *J. Biol. Chem.* 262:1148–1154.

Tubo, R. A., and R. Berezney. 1987*b*. Nuclear matrix-bound DNA primase. Elucidation of an RNA priming system in nuclear matrix isolated from regenerating rat liver. *J. Biol. Chem.* 262:6639–6642.

Tubo, R.A., and R. Berezney. 1987*c*. Identification of 100 and 150S DNA polymerase alpha-primase megacomplexes solubilized from the nuclear matrix of regenerating rat liver. *J. Biol. Chem.* 262:5857–5865.

Tubo, R. A., H. C. Smith, and R. Berezney. 1985. The nuclear matrix continues DNA synthesis at in vivo replicational forks. *Biochim. Biophys. Acta.* 825:326–334.

Valenzuela, M. S., G. C. Mueller, and S. Dasgupta. 1983. Nuclear matrix DNA complex resulting from EcoR1 digestion of HeLa nucleoids is enriched for DNA replicating forks. *Nucleic Acids Res.* 11:2155–2164.

van der Velden, H. M. W., M. Poot, and F. Wanka. 1984*a*. In vitro DNA replication in association with the nuclear matrix of permeable mammalian cells. *Biochim. Biophys. Acta.* 782:429–436.

van der Velden, H. M. W., G. van Willigen, R. H. W. Wetzels, and F. Wanka. 1984*b*. Attachment of origins of replication to the nuclear matrix and the chromosome scaffold. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 171:13–16.

van der Velden, H. M. W., and F. Wanka. 1987. The nuclear matrix. Its role in the spatial organization and replication of eucaryotic DNA. *Mol. Biol. Rep.* 12:69–79.

Vogelstein, D., D. M. Pardoll, and D. S. Coffey. 1980. Supercoiled loops and eucaryotic DNA replication. *Cell.* 22:79–85.

Wood, S. H., and J. M. Collins. 1986. Preferential binding of DNA primase to the nuclear matrix in HeLa cells. *J. Biol. Chem.* 261:7119–7122.

Yamamoto, S., T. Takahashi, and A. Matsukage. 1984. Tight association of DNA polymerase alpha with granular structures in the nuclear matrix of chick embryo cell: immunocytochemical detection with monoclonal antibody against DNA polymerase alpha. *Cell Struct. Funct.* 9:83–90.