

Dynamic Properties of Nuclear Lamins: Lamin B Is Associated with Sites of DNA Replication

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Abstract. The nuclear lamins form a fibrous structure, the nuclear lamina, at the periphery of the nucleus. Recent results suggest that lamins are also present as foci or spots in the nucleoplasm at various times during interphase of the cell cycle (Goldman, A. E., R. D. Moir, M. Montag-Lowy, M. Stewart, and R. D. Goldman. 1992. *J. Cell Biol.* 104:725-732; Bridger, J. M., I. R. Kill, M. O'Farrell, and C. J. Hutchison. 1993. *J. Cell Sci.* 104:297-306). In this report we demonstrate that during mid-late S-phase, nuclear foci detected with lamin B antibodies are coinci-

dent with sites of DNA replication as detected by the colocalization of sites of incorporation of bromodeoxyuridine (BrDU) or proliferating cell nuclear antigen (PCNA). The relationship between lamin B and BrDU is not maintained in the following G1 stage of the cell cycle. Furthermore, the nuclear staining patterns seen with antibodies directed against lamins A and C in mid-late S-phase do not coalign with the lamin B/BrDU-containing structures. These results imply that there is a role for lamin B in the organization of replicating chromatin during S phase.

THE nuclear lamina is a thin fibrous structure that underlies the inner nuclear membrane (Gerace and Burke, 1988; Nigg, 1992; Moir and Goldman, 1993). The proteins of the lamina, the nuclear lamins, comprise the type V intermediate filament (IF)¹ proteins. The lamins share the common structural features of cytoskeletal IF proteins, including a highly conserved central alpha helical rod domain and two nonalpha-helical flanking domains (Steinert and Parry, 1985; Stewart, 1993). There may be up to five different nuclear lamin proteins in the lamina polymer, depending on cell type (Lehner et al., 1986; Kauffmann, 1989). These different lamins are classified into two groups: the A type which are found only in differentiated cells and the B type which are found in both undifferentiated and differentiated cell types. In mammalian cells there are two major A-type lamins (lamins A and C) produced by alternate splicing of the same gene. These lamins have identical sequences, except for a 90-amino acid extension at the COOH terminus of lamin A. The B type lamins are termed B1 and B2 (Nigg, 1992). The nature of the molecular interactions between the different lamins and the structure of the assembled polymer that results have not been clearly established (Stewart, 1993). In rare cases, such as in the nucleus of the oocyte of *Xenopus laevis*, the lamina appears as a dense fibrous network occasionally having a lattice-like appear-

ance (Aebi et al., 1986). In other cell types the lamina network does not form regular arrays, but may be discontinuous and as such may cover at most half of the inner nuclear membrane (Paddy et al., 1990). However, this may not be true of all cell types (Belmont et al., 1993).

The lamina is generally thought to serve a structural role in maintaining and modulating nuclear shape and volume (Gerace and Burke, 1988). It also appears to interact with other nuclear structures and consequently may play an important role in various nuclear functions. For example, the lamina interacts with and may be involved in the organization of chromatin (Nigg, 1992). In support of this, three dimensional reconstructions of nuclei show that a fraction of chromatin appears to be in close proximity to the lamina during interphase (Paddy et al., 1991) and the thickness of the lamina is correlated with the proximity of chromatin (Belmont et al., 1993). Furthermore, the binding of lamins to decondensing chromosomes after mitosis may be an initial, critical step in nuclear reassembly in both mammalian and *Drosophila* cell-free systems (Burke and Gerace, 1986; Glass and Gerace, 1990; Ulitzur et al., 1992; Foisner and Gerace, 1993), and it has been shown that lamin A binds to chromatin in vitro (Yuan et al., 1991). Recently, lamin B1 has been shown to bind nuclear-matrix associated DNA sequences (MARS) in vitro, suggesting a role for this lamin in chromatin organization (Luderus et al., 1992). In addition, an intact lamina is required for DNA replication in nuclei formed from extracts of *Xenopus laevis* oocytes. If the *Xenopus* oocyte nuclear lamin protein is removed from the extracts, nuclei still form but are unable to replicate DNA (Newport et al., 1990; Meier et al., 1991; but see Lourim and Krohne, 1993).

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1. *Abbreviations used in this paper:* BrDU, bromodeoxyuridine; CaaX, COOH-terminal recognition sequence; IF, intermediate filament; MARS, nuclear-matrix associated DNA sequences; PCNA, proliferating cell nuclear antigen.

The lamins undergo several posttranslational modifications that affect the dynamics of lamina assembly (Nigg, 1992). Lamins A and B contain the consensus COOH-terminal recognition sequence (CaaX) for isoprenylation and undergo the same sequence of modifications that other CaaX-containing proteins such as Ras undergo (Nigg, 1992). The three amino acids at the COOH-terminal end are removed and the cysteine residue becomes farnesylated and O-methylated (Nigg, 1992). These modifications appear to be required for proper targeting of the lamins to the nuclear envelope. Transfection with cDNA or microinjection of mRNA in which the cysteine is mutated results in the abnormal formation of nuclear deposits of the expressed protein (Holtz et al., 1989; Krohne et al., 1989; Kitten and Nigg, 1991). The treatment of cells with the isoprenylation inhibitor lovastatin also leads to an accumulation of nuclear lamin A in the nucleoplasm (Lutz et al., 1992). In the case of lamin A, the modified cysteine as well as an additional 14 COOH-terminal residues are removed, apparently after the protein becomes integrated into the lamina (Gerace et al., 1984; Weber et al., 1989).

The phosphorylation state of the nuclear lamins influences their assembly properties. For example, the depolymerization of the lamina at mitosis is mediated by p34^{cdc2} phosphorylation (Nigg, 1992). The lamins are phosphorylated primarily at two sites flanking the ends of the central rod domain and cells transfected with cDNAs mutated at these sites cannot disassemble their nuclei in mitosis (Heald and McKeon, 1990). Furthermore, phosphorylation of lamin B by p34^{cdc2} prevents its self-association *in vitro* (Peter et al., 1991). There is also evidence for other mitotic lamin kinases which have yet to be identified (Luscher et al., 1991). Other well-characterized kinases such as protein kinase C and protein kinase A have been shown to influence lamin assembly during interphase in different ways (Fields et al., 1987; Lamb et al., 1991; Lourim and Lim, 1992; Hennekes et al., 1993).

To study the properties of nuclear lamins during interphase, we have developed an *in vivo* assay involving the microinjection of bacterially expressed human lamin A (Moir et al., 1991) into mouse 3T3 cells. In this fashion, we have been able to determine directly the pathway of incorporation of lamin A into the endogenous polymerized network comprising the lamina (Goldman et al., 1992). The injected protein is rapidly transported into the nucleus where it accumulates in nuclear foci before becoming incorporated into the lamina at the periphery of the nucleus. Similar foci have also been described in uninjected 3T3 cells and in human epidermal fibroblasts at different stages of the cell cycle by indirect immunofluorescence using lamin antibodies (Goldman et al., 1992; Bridger et al., 1993). These foci did not appear to be the result of invaginations of the nuclear envelope as shown by staining with a membrane dye (Bridger et al., 1993). However, a recent report argued that such apparent foci were invaginations, based on confocal microscopy showing colocalization of nuclear pore and lamin staining (Belmont et al., 1993).

Other nuclear constituents frequently appear as nuclear foci by indirect immunofluorescence. For example, foci are seen with antibodies directed against components of the kinetochore (Ouspenski et al., 1992) and snRNP particles (Fu and Maniatis, 1990). Similar foci containing DNA replication complexes can be detected using antibodies against

proliferating cell nuclear antigen (PCNA; Kill et al., 1991), DNA methyltransferase (Leonhardt et al., 1992), and bromodeoxyuridine (BrDU) (Kill et al., 1991; O'Keefe et al., 1992). The presence of these different foci suggest that nuclear components and processes are segregated into highly organized structural domains, although the relationships amongst these different domains is not clear.

Based on these considerations, it is important to establish whether there are relationships between nuclear lamin foci (Goldman et al., 1992; Bridger et al., 1993; Belmont et al., 1993; Mancini et al., 1994) and those containing other nuclear components. In this study, we demonstrate that there is a relationship between lamin B foci, as detected by lamin B specific antibodies, and sites of DNA replication as detected either by BrDU incorporation (O'Keefe et al., 1992) or antibodies directed against PCNA (Kill et al., 1991). The coincidence between lamin B and sites of DNA replication is most obvious in mid to late S phase and does not appear to be maintained in G1. Lamins A/C do not colocalize with BrDU and PCNA.

Materials and Methods

Cell Culture

Subconfluent cultures of mouse 3T3 (Todaro and Green, 1963) and BHK-21 cells (Rosevear et al., 1990) were grown on 22-mm square #1 coverslips in DMEM (GIBCO BRL, Gaithersburg, MD), containing 10% bovine calf serum (Hyclone Laboratories, Logan, UT), 50 U/ml penicillin and 50 mg/ml streptomycin (GIBCO BRL). PtK2 cells (Miller et al., 1991) were grown in MEM (GIBCO BRL) with the same supplements. Coverslip cultures were maintained in 35-mm dishes at 37°C in a humidified CO₂ incubator.

For studies of the organization of the nuclear lamins in the G1 phase of the cell cycle, mitotic 3T3 cells were obtained in the absence of drugs by the mechanical shake-off method described by Rosevear et al. (1990). Approximately 4×10^5 cells were obtained from one 150-cm² culture flask, of which greater than 90% were mitotic as shown by staining of fixed cells with DAPI (2 ng/ml; Rosevear et al., 1990) to reveal condensed chromosomes (data not shown). The cells were sedimented by centrifugation (1,000 g, 1 min at 4°C) and washed with ice cold medium. Approximately 4×10^4 cells were placed on coverslips. After 3 h, the coverslip cultures were fixed to obtain cells enriched in G-1 for use in immunofluorescence as described below.

For the determination of BrDU incorporation patterns, cells were also synchronized in S phase using methods described elsewhere (O'Keefe et al., 1992). Briefly, cells were plated at low density in serum-free medium for 72–96 h at which time all cells were in G-0 as indicated by the absence of mitotic figures. The growth medium was then replaced with medium containing 10% FCS and the cultures were incubated for 4 h at 37°C which allowed the cells to enter G1. Hydroxyurea was subsequently added to a final concentration of 1.5 mM for 14–16 h in order to block cells at the G1/S boundary. These cells did not incorporate BrDU (see below) as shown by immunofluorescence (data not shown). The cells were then released from this block by washing with and incubation in normal growth medium. Subsequently cells were fixed at 1-h time intervals to obtain cells in various stages of S phase.

Visualization of sites of DNA synthesis was accomplished by the (Sigma Chem. Co., St. Louis, MO) labeling of synchronous and asynchronous cell cultures with 10 μ M BrDU for 30 min at 37°C (O'Keefe et al., 1992).

Antibodies

A rabbit polyclonal antibody (Moir, R. D., and M. Stewart, unpublished; a gift of Dr. Murray Stewart, Medical Research Council, Cambridge, England) directed against human lamin B1 was used in the majority of our experiments. The lamin B1 protein used as antigen was produced by expression of lamin B1 cDNA (Pollard et al., 1991) in *E. coli* using the pET system (Moir, R. D., H. M. Kent, and M. Stewart, unpublished observations).

The specificity of the anti-human lamin B was determined by Western blotting of whole 3T3 cell protein as well as enriched nuclear fractions. These latter fractions were prepared using the method of Dagenais et al. (1984). The initial crude nuclear fraction was purified by centrifugation at 124,000 *g* for 30 min at 4°C through a 2.3-M sucrose cushion (Newport and Spann, 1987). The resulting nuclear pellets were boiled in sample buffer in preparation for SDS-PAGE using the method of Laemmli (1970). After electrotransfer of the nuclear proteins, the nitrocellulose filter was incubated for 1 h in PBS containing 5% dry milk as a blocking agent (Dessev et al., 1989). The primary rabbit antiserum was then added at a 1:1,000 dilution in PBS with 1% dry milk for 2 h at room temperature. Subsequently the filter was washed with PBS containing 0.1% Tween 20 (Sigma Chem. Co.) several times over a 30-min period. The filter was incubated in goat anti-rabbit IgG conjugated with horseradish peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) at 1:400 dilution in PBS with 1% dry milk for 1 h at room temperature. The filter was washed again as described above and developed with the chloronaphthol reaction (Dessev et al., 1989). We also prepared a second rabbit antibody using bacterially expressed human lamin A as an antigen (Moir et al., 1991). The specificity of this antibody is also shown in Fig. 1. We have used an antibody to B type lamins produced in guinea pigs (kindly provided by Dr. L. Gerace, Scripps Research Institute, San Diego, CA.; see Ottaviano and Gerace, 1985).

Other antibodies used in these studies include the SP Crest human auto-immune serum recognizing a component of kinetochores (a gift of Dr. Bill Brinkley, Baylor School of Medicine, Houston, TX; Ouspenski et al., 1992). A human auto-immune serum directed against the nuclear pore antigen gp210 (a gift of H. Worman, Mount Sinai Hospital, New York; Nickowitz and Worman, 1993). This antiserum does not stain mitochondria. Mouse monoclonal antibodies directed against BrDU and PCNA were obtained commercially (Boehringer Mannheim Biochemicals, Indianapolis, IN), and were used as recommended by the manufacturer. Secondary antibodies used for immunofluorescence were rhodamine conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Kirkegaard and Perry Labs, Inc.), as well as rhodamine conjugated donkey anti-mouse IgG and fluorescein conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Labs., West Grove, PA).

Fixation and Antibody Staining Protocols

Several fixation protocols were used in these studies and each gave very similar results. Cells were fixed in 1% or 4% formaldehyde (Electron Microscopy Grade, Sigma Chem. Co.) in PBS for 5 or 10 min, respectively, at room temperature. This was followed by permeabilization in two changes of 0.1% NP-40 in PBS, each for 3 min at room temperature (Lutz et al., 1992) or with a single treatment of 0.1% Triton-X-100 for 10 min at room temperature (Leonhardt et al., 1992).

After formaldehyde fixation of cells treated with BrDU, cellular DNA was denatured according to the manufacturer's instructions for the use of anti-BrDU (Boehringer Mannheim Biochemicals). Briefly, the fixed cells were treated with 2N HCl from 15–60 min at room temperature, and then neutralized with 0.1 M sodium borate, pH 8.5, for 10 min at room temperature. Regardless of the time in HCl, the same immunofluorescence patterns were seen with anti-lamin antibodies for each of the formaldehyde procedures used. When the PCNA antibody was used, cells were fixed in methanol for 10 min at –20°C and subsequently placed in PBS without air drying as required for preservation of this antigen (Kill et al., 1991).

Primary antibodies for single and double immunofluorescence were used at dilutions of 1:20 to 1:100 in PBS depending on the antibody (for details see Yang et al., 1985). Coverslips were incubated in the primary antibody solutions for 30 min at 37°C, and then washed with three 2-min changes of PBS containing 0.1% Tween 20 at room temperature. Secondary antibodies were used at a 1:20 dilution in PBS. Incubations and washings were carried out as described above. The coverslips were mounted in gelvatol with DAPCO (Sigma Chem. Co.) for microscopic observations (Yang et al., 1985).

Light Microscopic Procedures

Cells were studied either by conventional epifluorescence or confocal microscopy. Conventional epifluorescence was carried out on a Zeiss Axio-phot epifluorescence optical system equipped with a 100 Watt High Pressure Mercury Lamp, narrow band filters for observing fluorescein and rhodamine, and 63× (1.4 NA) and 100× (1.3 NA) oil immersion objectives. Photomicrographs were recorded on 35-mm Plus-X film (Eastman Kodak

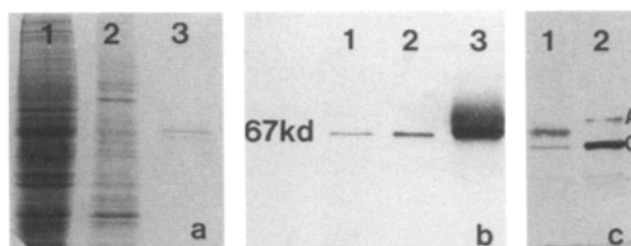


Figure 1. (a) SDS-gel stained with Coomassie blue. (Lane 1) Total cell protein prepared from an extract of 3T3 cells. (lane 2) Enriched nuclear fraction from 3T3 cells. (lane 3) Purified human lamin B made by expression in *E. coli*. (b) Corresponding Western blot of the gel in a using a rabbit polyclonal anti-human lamin B. The antibody reacts with a single major band of the same molecular weight as the expressed lamin B in each of the lanes. Lamins A and C show no crossreaction with this antibody (data not shown). (c) Western blot using a polyclonal antibody directed against bacterially expressed human lamins A/C. (lane 1) Enriched nuclear fraction obtained from 3T3 cells; (lane 2) Bands corresponding to bacterially expressed lamins A and C are indicated. The bacterially expressed lamin A is the unprocessed form of lamin A and migrates slightly slower than the processed lamin A found in nuclei (Weber et al., 1990). In addition to the two bands corresponding to lamins A and C, this antibody detects a third weakly staining band at 45 kD which represents a proteolytic cleavage product of lamins A/C that has been documented by Collard et al. (1992).

Co., Rochester, NY). Cells were also studied with a Zeiss Laser Scan confocal optical system equipped with a 488 λ argon laser, a 543 λ helium-neon laser and 63× (1.4 NA) and 100× (1.3 NA) oil immersion objectives. Digital brightness and contrast functions were used for image enhancement and before recording, confocal images were subjected to line averaging of 8 or 16 times to reduce the effects of random noise. Images were stored on optical discs and micrographs were made either from 35-mm Plus-X photos taken on a Matrix Multicolor High Resolution Monitor (Matrix Instruments, Inc., Orangeberg, NY) or a Sony UP-8000 Video Printer.

Results

Some Interphase Cells Exhibit Lamin B Nuclear Foci Which Correspond to Sites of DNA Synthesis

Most of our immunofluorescence studies were carried out with a rabbit polyclonal antibody directed against bacterially expressed human lamin B (Pollard et al., 1991). The specificity of this antibody was determined by immunoblotting of whole 3T3 cell protein, as well as enriched nuclear fractions (Fig. 1). In both the 3T3 cell fractions the lamin B antibody reacts with a single band which coelectrophoreses with the bacterially expressed lamin B.

Several recent morphological studies have demonstrated that nuclear lamins are present in both nuclear foci, as well as in the nuclear lamina at specific stages of the cell cycle using nuclear lamin antibodies (Goldman et al., 1992; Bridger et al., 1993). Confocal microscopic images of three different 3T3 cells showing nuclear foci following staining with the lamin B antibody are seen in (Fig. 2). A series of different focal planes within the same nucleus emphasizes that several of the foci are not continuous throughout (Fig. 2, a–c). The possibility that these foci might represent invaginations of the nuclear surface was examined by double indirect immunofluorescence with antibodies to lamin B and

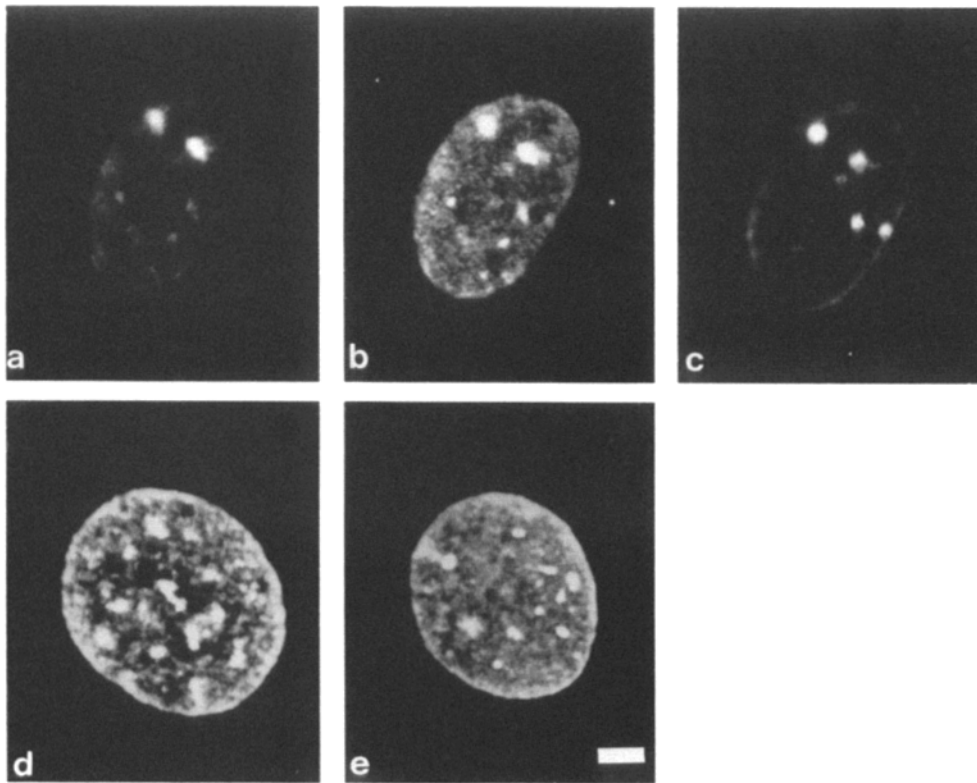


Figure 2. Confocal microscope images of 3T3 cell nuclei stained with the lamin B antibody characterized in Fig. 1. Cells were fixed using the 4% formaldehyde procedure and processed for immunofluorescence (Materials and Methods). (a-c). Confocal sections taken 1 μm apart of the same nucleus. A rim at the nuclear periphery and nuclear foci are evident within the nucleus. (d and e). Confocal sections of the nuclear regions through two different cells. The sections were taken through the mid-region of the nucleus. Nuclear foci are present in addition to the peripheral staining. Bar, 10 μm .

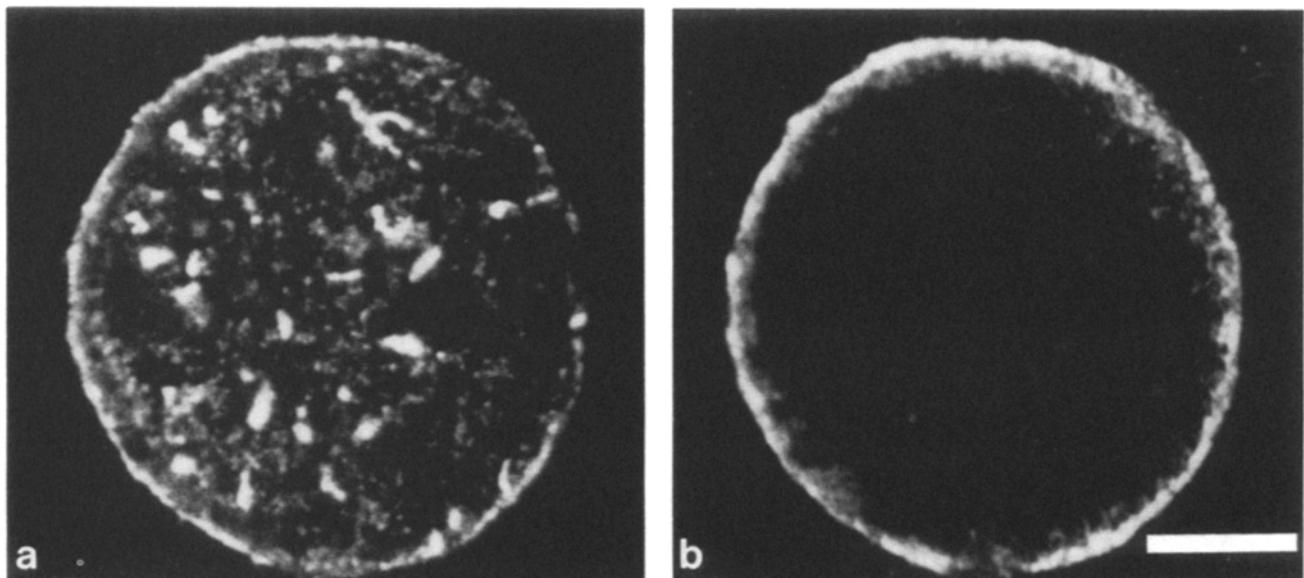
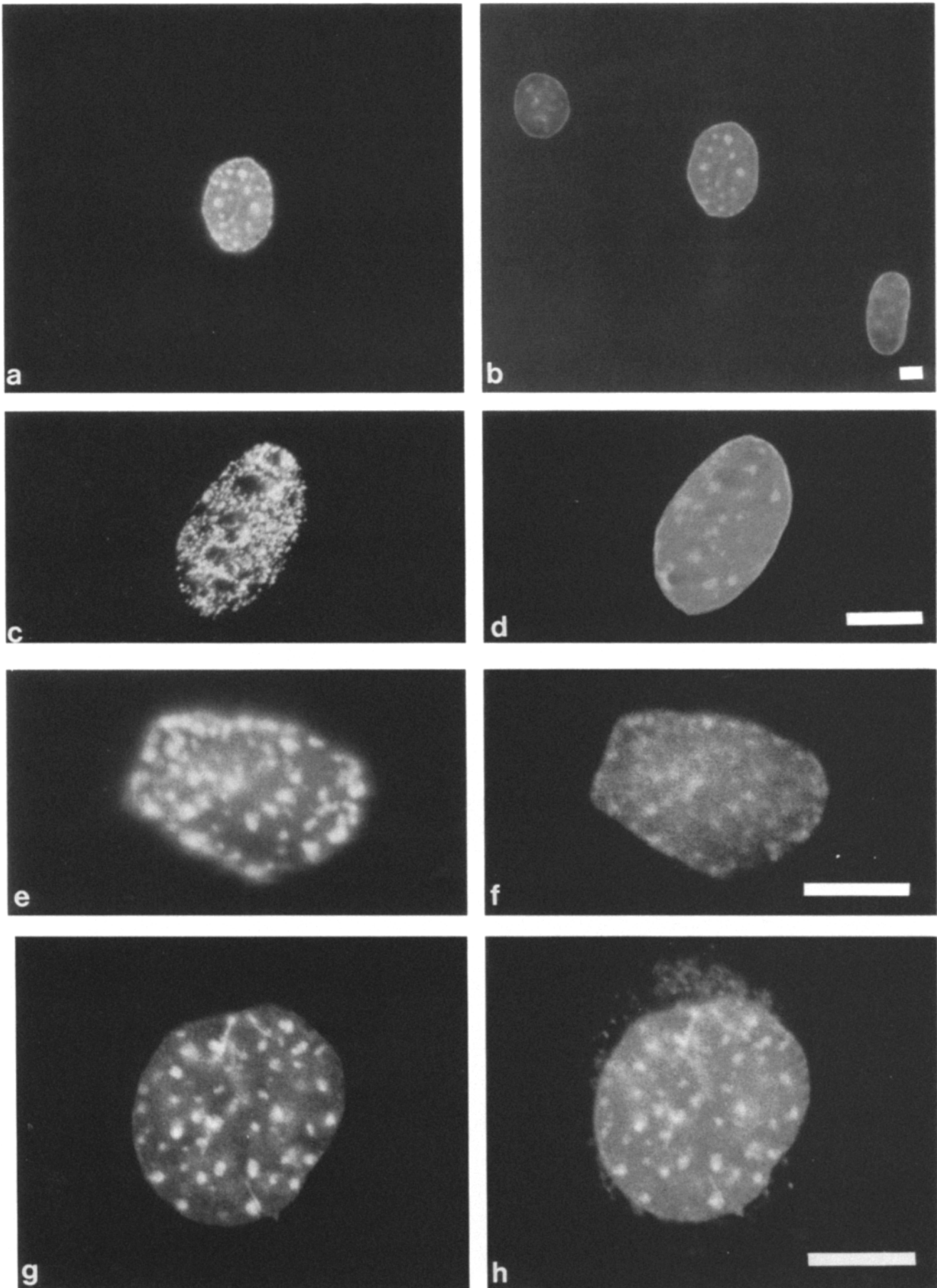


Figure 3. Confocal microscope images of a 3T3 cell fixed with methanol and prepared for double label immunofluorescence with (a) anti-lamin B and (b) the human autoantibody directed against the nuclear pore component, gp210 (Materials and Methods). Most of the foci seen with the nuclear lamin antibody are not detected with the nuclear pore antibody. Bar, 10 μm .

Figure 4. (a and b) 3T3 cells exposed to BrDU, and subsequently fixed by the 1% formaldehyde procedure and prepared for double immunofluorescence with anti-BrDU (a) and anti-lamin B (b) (see Materials and Methods). The cell in the center of the field has incorporated BrDU indicating that it is in S-phase of the cell cycle. Large nuclear foci are obvious. In the same field, the nuclei of three cells are visible with anti-lamin B (b). All three cells have lamin foci and rims. Note that the nuclear foci in the BrDU positive cell coalign with the lamin B foci. (c and d). The nucleus of a 3T3 cell exposed to BrDU and subsequently fixed with the 1% formaldehyde procedure, followed by double immunofluorescence with anti-BrDU and anti-lamin B. This nucleus displays numerous small foci containing BrDU (c) and larger foci containing lamin B (d). The two sets of foci show no obvious coalignment. (e and f). PtK2 cell in S phase exposed to BrDU and



subsequently fixed by the 4% formaldehyde procedure, followed by double indirect immunofluorescence with anti-BrDU (*e*) and anti-lamin B1 (*f*). Note the coalignment of the foci. (*g* and *h*) 3T3 cells exposed to BrDU, and subsequently fixed by the 4% formaldehyde procedure and prepared for double immunofluorescence with anti-BrDU (*g*) and a different anti-lamin B provided by Dr. Larry Gerace (*h*) (see Materials and Methods). Bars, 10 μ m.

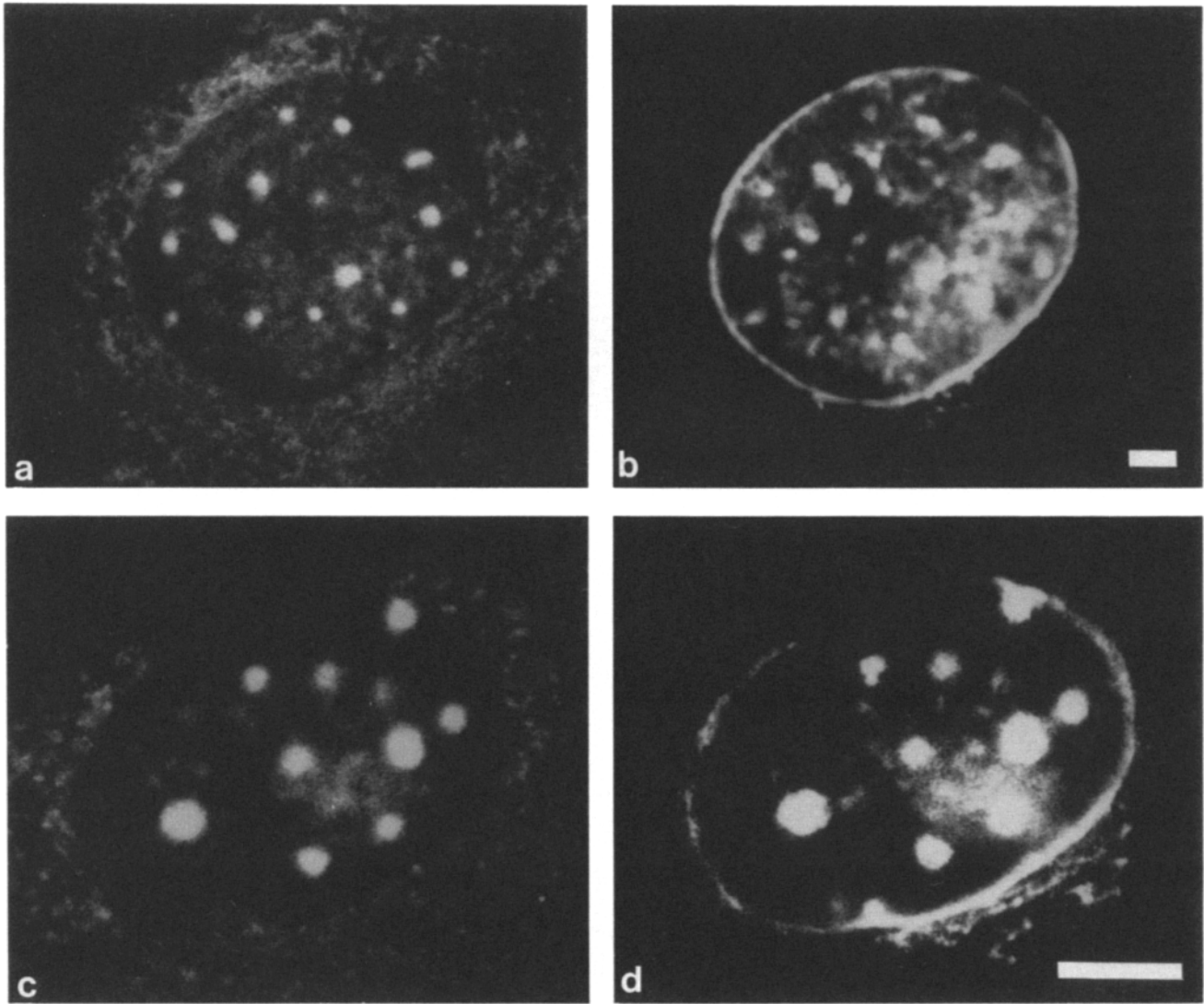


Figure 5. 3T3 cells fixed with methanol and stained for double indirect immunofluorescence with anti-PCNA (*a* and *c*) and anti-lamin B (*b* and *d*) (Materials and Methods). These are confocal microscope images. In addition to a distinctive lamin rim, there are numerous foci that align with the PCNA staining. Bars, 1 μm (*a* and *b*) and 10 μm (*c* and *d*).

the nuclear pore complex protein gp210. As shown in Fig. 3, there is no obvious correlation between the two staining patterns.

The nuclear focal patterns seen with anti-lamin B are reminiscent of the nuclear staining patterns seen with a variety of other antibodies directed against other nuclear components. One such component is that associated with DNA replication centers as revealed either by determining the sites of BrDU incorporation (Fox et al., 1991; O'Keefe et al., 1992) or by staining with an antibody directed against PCNA (Kill et al., 1991). Therefore we examined the possibility that there might be a relationship between lamin B foci and those revealed by anti-BrDU and/or anti-PCNA.

We carried out double label indirect immunofluorescence, following the incorporation of BrDU into replicating DNA. Fig. 4, *a* and *b* are taken from a microscope field containing three 3T3 cells from an asynchronous culture treated with BrDU for 30 min, and then fixed and processed for double

indirect immunofluorescence with anti-BrDU (Fig. 4 *a*) and anti-lamin B (Fig. 4 *b*). The cell in the center of the field is stained with both antibodies revealing that it is in the S phase of the cell cycle. The lamin B pattern shows a characteristic nuclear lamin rim at the periphery, and prominent foci. These foci show almost perfect coalignment with the nuclear foci revealed with the BrDU antibody. The two other cells in the field of view exhibit nuclear lamin staining with rims and foci, but show no staining with anti-BrDU. Some nuclei contain lamin B and BrDU foci which do not exhibit obvious coalignment as shown in Fig. 4, *c* and *d*. Although 3T3 cells are employed throughout the remainder of this study, we wish to point out that we have also observed the lamin B/BrDU coalignment in several other cell types, including BHK-21 and PtK2 (Fig. 4, *e* and *f*). Results similar to those seen in Fig. 4, *a* and *b* and *e* and *f* are also obtained with the lamin B antibody provided by Dr. Larry Gerace (Ottaviano and Gerace, 1985; Fig. 4, *g* and *h*).

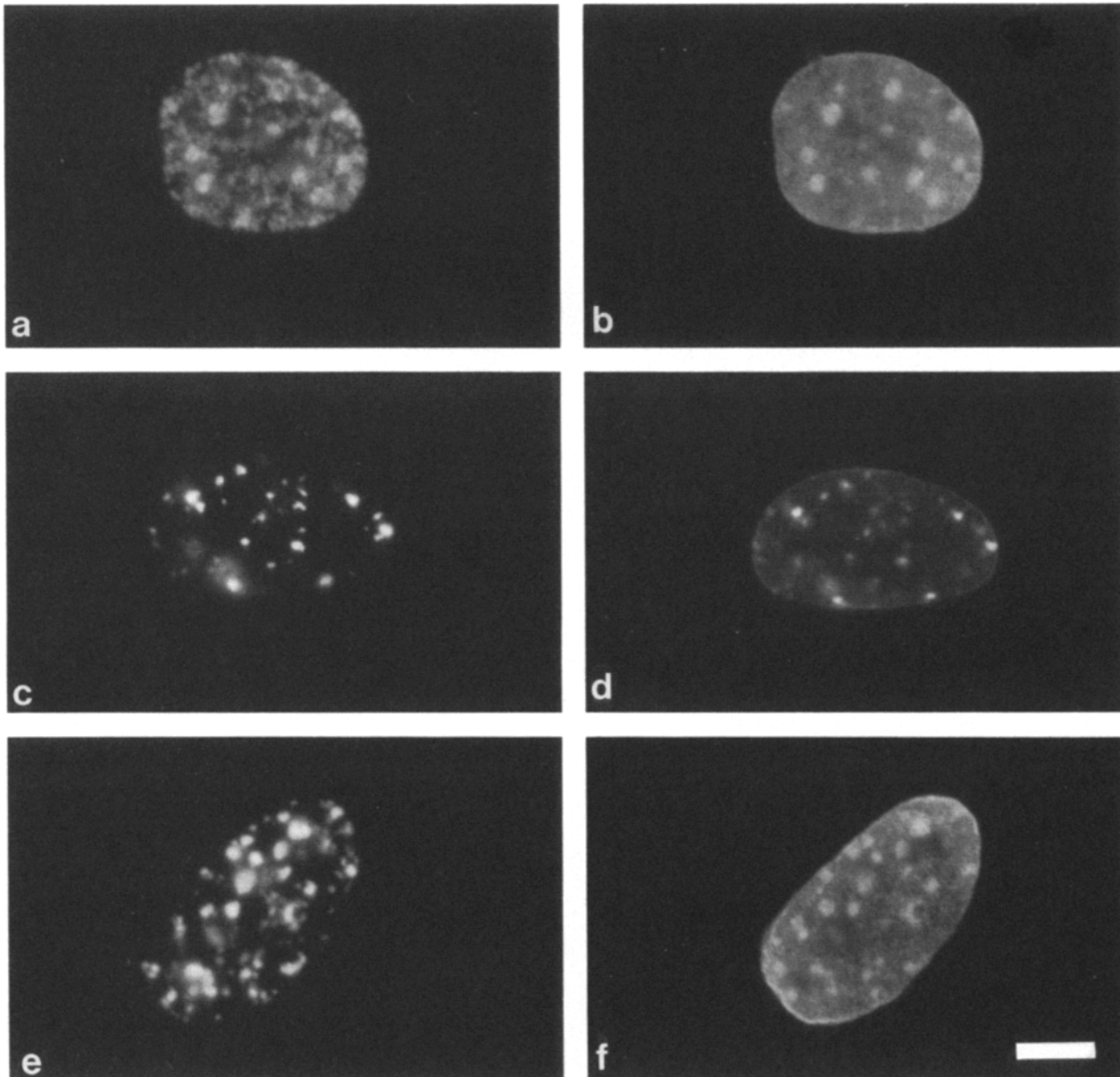


Figure 6. Examples of nuclei in three 3T3 cells fixed with the 1% formaldehyde procedure and subsequently stained with anti-BrDU (*a*, *c*, and *e*) and anti-lamin B (*b*, *d*, and *f*) and examined by conventional immunofluorescence. These nuclei are in mid to late S phase as indicated by their BrDU patterns of large foci (see O'Keefe et al., 1992). These large foci show extensive coalignment with the lamin B foci. Bar, 10 μ m.

The Coalignment of Lamin B Foci Also Occurs with PCNA But Not Other Nuclear Antigens

In the processing of cells exposed to BrDU, 2N HCl is used to denature genomic DNA in order to detect nuclear incorporation sites. It is possible that despite initial fixation in relatively high concentrations of formaldehyde, lamin B becomes solubilized and redistributed within the nucleus by the acid treatment. Therefore as an independent confirmation of the association of lamin B with replication foci, we used PCNA antibody (proliferating cell nuclear antigen) which is also found in replication foci during S phase (Kill et al., 1991). The detection of PCNA is carried out after fixation in methanol for 10 min at -20°C (Kill et al., 1991). As shown in Fig. 5, we observed cells with virtually identical distributions of lamin B and PCNA nuclear foci. These are

morphologically indistinguishable from the lamin B/BrDU double-labeled preparations. Therefore these observations demonstrate that there are no obvious alterations in lamin B distribution due to the acid treatment required for the visualization of sites of BrDU incorporation.

We also stained cells with an autoimmune serum directed against kinetochores, which also yields a pattern of nuclear foci (Ouspenski et al., 1992). However, we could not detect obvious coalignments of the lamin B and kinetochore staining patterns (data not shown).

The Most Obvious Coalignment of Nuclear Lamin B and BrDU Foci Occurs during Mid-Late S Phase

The detection of sites of DNA synthesis with BrDU immunofluorescence reveals several different staining patterns

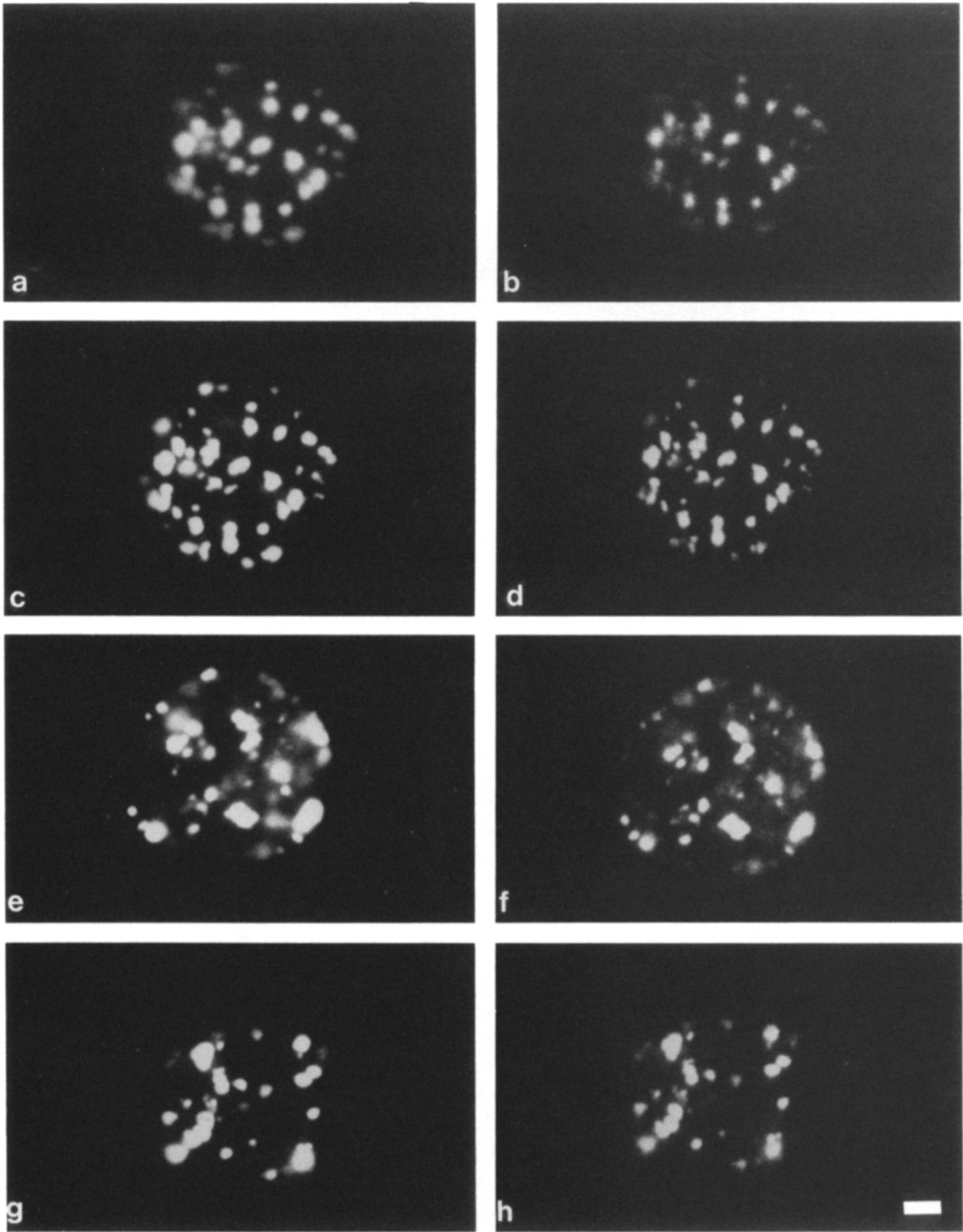


Figure 7. Confocal sections of a single nucleus fixed by the 1% formaldehyde procedure and subsequently processed for double-label immunofluorescence with anti-BrDU (*a*, *c*, *e*, and *g*) and anti-lamin B (*b*, *d*, *f*, and *h*). Confocal microscope sections were taken $0.5\ \mu\text{m}$ apart. Both antibodies detect coaligned nuclear foci. There is not a prominent lamin B staining rim at the nuclear periphery in this nucleus. Bar, $1\ \mu\text{m}$.

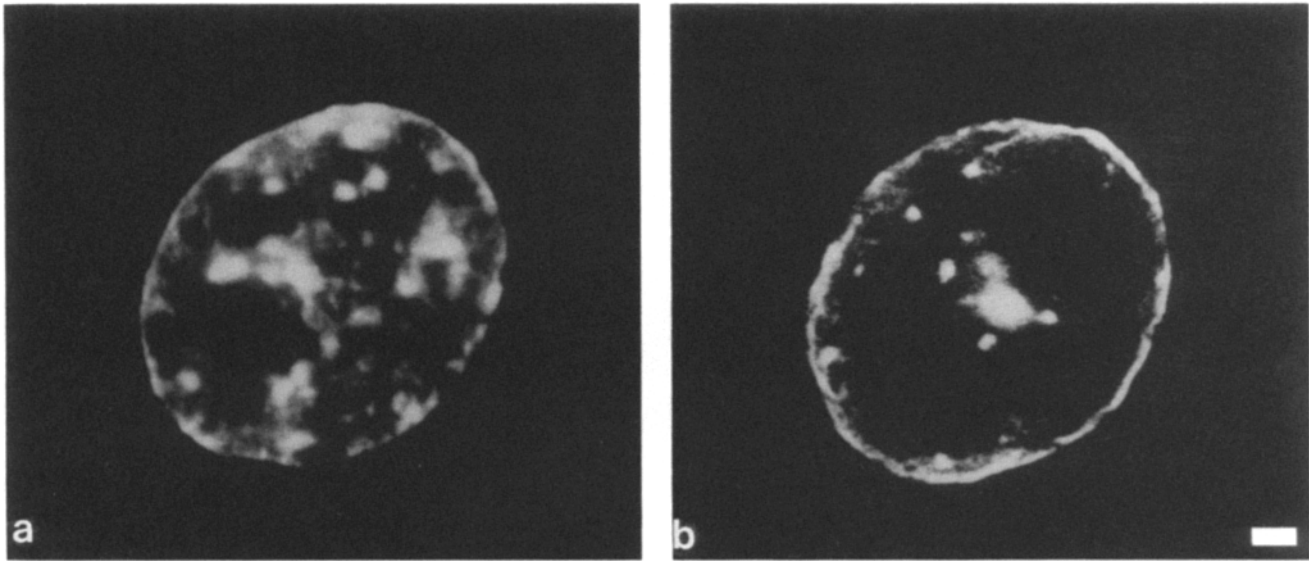


Figure 8. Confocal microscope image of a 3T3 nucleus stained with anti-BrDU (*a*) and anti-lamin A/C (*b*) (see Materials and Methods). This nucleus has large BrDU foci indicative of a mid to late S phase cell. The lamin staining has a distinctive lamin rim and also lamin foci. However, the lamin and BrDU foci are not coincident. Bar, 1 μ m.

(O'Keefe et al., 1992). These patterns reflect the replication of different DNA populations at different stages of the S phase of the cell cycle (Fox et al., 1991; O'Keefe et al., 1992). In mouse 3T3 cells we were able to identify the five different patterns of BrDU staining which have been reported for other cell types and confirm the order in which they appear during S phase using cell synchronization techniques (data not shown; O'Keefe et al., 1992).

In this fashion, we have determined that the coalignment between the lamin B and BrDU patterns occurs most frequently when large nuclear foci are present. In the case of BrDU such foci typify mid to late S phase cells (O'Keefe et al., 1992). In asynchronous cultures, these types of BrDU patterns are seen in $\sim 20\%$ of S phase cells and we have observed that $\sim 60\%$ of the cells possessing these patterns show an obvious coalignment. At the present time, however, we cannot exclude the possibility that there is also a relationship between DNA replication foci and lamin B at other stages of S phase. Fig. 6 contains several examples of cells with nuclei exhibiting the coincident staining patterns of BrDU and lamin B in mid to late S phase. A confocal series of lamin B and BrDU staining taken through a mid to late S phase nucleus (Fig. 7) further emphasizes the close relationship of the two staining patterns and demonstrates that the foci are not continuous throughout the nucleus.

No Obvious Relationship Is Seen between Lamin A/C and BrDU

We also used an antibody specific for lamins A and C (see Fig. 1 *c*) for double immunofluorescence with the BrDU method. We found no obvious coalignment between the staining patterns in mid-late S phase 3T3 cells (Fig. 8, *a-b*). Similar results were obtained in cells double labeled with lamin A/C and PCNA antibodies (data not shown).

The Lamin B/BrDU Relationship Is Not Displayed in G1

Previous results have suggested that lamin foci are present in G1 in 3T3 cells (Goldman et al., 1992) and in human epidermal fibroblasts (Bridger et al., 1993). Therefore, we determined whether or not the close association between lamin B and BrDU-labeled foci seen in mid-late S phase could also be detected in the G1 stage of the cell cycle. We incubated an asynchronously growing culture in BrDU-containing medium for 30 min. Subsequently, the cells were rinsed several times with and incubated in normal culture medium. After 16 h, we obtained mitotic cells by mechanical shakeoff as previously described (Goldman et al., 1992; Rosevear et al., 1990). We replated these cells, and after 2–3 h, they were fixed and processed for double indirect immunofluorescence with lamin B and BrDU antibodies. We photographed pairs of recently divided daughter cells connected with a distinct midbody (as seen by phase contrast) to ensure that they were in the early G1 phase of the cell cycle. In some cells, the BrDU patterns consist of large foci similar to those seen in S phase, as well as some filamentous structures (Fig. 9 *a*). One hundred pairs of G1 cells were examined in this fashion and in no instance were we able to find an obvious relationship between the lamin B and BrDU patterns (for an example see Fig. 9).

Discussion

In this paper, we describe results showing that there is a relationship between nuclear foci containing lamin B and sites of DNA replication, as detected by two independent markers, BrDU and PCNA. The coincidence of lamin B with BrDU staining occurs most obviously in the case of DNA replication patterns typifying mid to late S phase cells. We

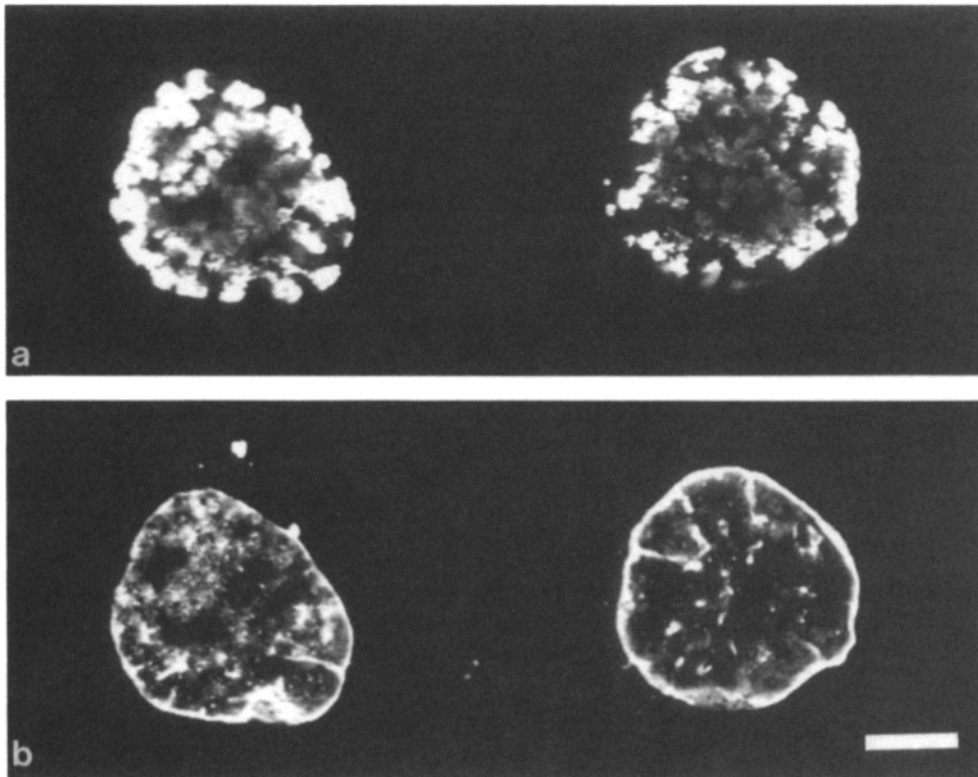


Figure 9. An example of cells in the G1 stage of the cell cycle stained with both (a) anti-BrDU and (b) anti-lamin B. An asynchronous culture of cells was incubated with BrDU for 30 min (see Materials and Methods), and subsequently the BrDU was removed by washing with fresh medium. Cells in the G1 stage of the cell cycle were obtained by replating mitotic cells obtained by mechanical shakeoff. Cells containing an obvious midbody as seen with phase contrast were examined. Nuclear foci are revealed with both antibodies, but there is not an obvious relationship between them. Bar, 10 μ m.

were unable to find a similar association when cells were labeled in S phase with BrDU, and then fixed and stained in the subsequent G1 phase of the cell cycle.

There does not appear to be a similar colocalization between lamins A/C and BrDU. Furthermore lamin B, but not A/C, aligns with replication foci seen with anti-PCNA in cells fixed with methanol, rather than the formaldehyde-acid treatment required for the BrDU method. In a previous report on nuclear lamin/BrDU staining, a coalignment of nuclear lamin and BrDU staining patterns was not reported, although some associations were seen at the nuclear periphery (Fox et al., 1991). This discrepancy may be related to the fact that in our study, the most striking examples of coalignment occur in a relatively small fraction of asynchronous cells stained with lamin B specific antibody.

Several recent reports have described the presence of lamins within the nucleus and not exclusively at the nuclear periphery (Goldman et al., 1992; Bridger et al., 1993; Mancini et al., 1994; Belmont et al., 1993). In one case, these lamin accumulations did not colocalize with a dye which stains nuclear membranes, suggesting that they were not the result of nuclear membrane invaginations (Bridger et al., 1993). However, a recent report, using nuclear pore and lamin B antibodies, argued that internal lamin foci were the consequence of membrane invaginations (Belmont et al., 1993). In this study, we have been unable to find convincing double staining of lamin B foci and anti-gp210, a human nuclear pore complex autoantibody. Therefore based on our data, we believe that the lamin B foci do not represent invaginations of the nuclear envelope. However, we cannot exclude the possibility that such invaginations may be below the limit of light microscopic resolution. For this reason, it will be

necessary to carry out detailed ultrastructural immunolocalization analyses of the relationship between the replication foci and the nuclear lamins.

Despite these concerns regarding the precise nature of the lamin rich nuclear foci, we have been able to clearly observe an association between lamin B and chromatin during mid to late S phase, when heterochromatin is being duplicated. Other morphological evidence supports the view that the peripheral nuclear lamina is also associated with heterochromatin (Paddy et al., 1991). Furthermore, heterochromatin undergoes reorganization during interphase which appears to involve translocations over considerable intranuclear distances (Vour'ch et al., 1992). It is conceivable that the nuclear lamins may play a role in this reorganization, as suggested by the observations of associations between lamins A/C and heterochromatin in early G-1 phase cells by Bridger et al. (1993). In further support of this possibility, the observations reported in this study suggest that nuclear lamin B may function in the reorganization of chromatin during DNA synthesis.

Results from in vitro nuclear reassembly experiments using extracts of *Xenopus* oocytes have shown that an assembled nuclear lamina and nuclear envelope are required for DNA replication. If the majority of the nuclear lamin of this system is depleted from the extracts by immunoprecipitation, nuclei form but are unable to replicate their DNA (Newport et al., 1990; Meier et al., 1991). Based on these observations, in addition to our own, it seems likely that the requirement for the presence of a lamina is a structural one. In this regard, the lamina may mediate interactions between the nucleoskeleton/nuclear matrix, chromatin, and the replication machinery. It is also interesting to note that lamin B

has recently been shown to bind MARS (Luderus et al., 1992). Therefore, it is conceivable that lamin B may be involved in regulating the organization of chromatin during S phase through its interaction with MARS.

Our results indicate that nuclear lamins are reorganized during various interphase events such as DNA synthesis. Thus it appears that the nuclear lamina is a dynamic karyoskeletal network which is restructured and reorganized at different stages of the cell cycle. This is quite different from the conventional view of the nuclear lamina. This view suggests that it is a relatively static protein polymer system localized at the nuclear periphery throughout interphase which only alters its state of assembly when it is hyperphosphorylated and disassembled in mitosis (Nigg, 1992; Moir and Goldman, 1993). Changes in the supramolecular organization of individual lamins have also been proposed in other experimental systems. For example, lamin A has been shown to undergo rapid alterations in its synthesis and organization during differentiation in HL60 cells. Initially, lamin A is present in a small nuclear "cap", but becomes more evenly distributed throughout the periphery of the nucleus over a period of several hours after induction of the differentiation process (Collard et al., 1992). It is possible that the nuclear lamin B foci reported in this study may also represent sites of assembly of newly synthesized protein. In support of this, there is some evidence for the specific synthesis of lamin B during S phase (Foisy and Bibor-Hardy, 1988; but see Gerace et al., 1984).

It should be possible to determine more specifically the functional significance of the dynamic properties of the nuclear lamins during the cell cycle through attempts to perturb lamin-chromatin interactions. For example, transfection of lamin B mutant cDNA might disrupt chromatin organization and perhaps even DNA replication during mid-late S phase. If true, this result would establish a functional role for the lamins in the regulation and in the organization of chromatin.

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