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Internal lamin structures within G_1 nuclei of human dermal fibroblasts

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SUMMARY

The nuclear lamina is a mesh-like network of fibres subjacent to the inner nuclear membrane that is believed to be involved in the specific spatial reorganisation of chromatin after mitosis. To determine how the lamina might be involved in chromatin reorganisation, we have performed indirect immunofluorescence studies on quiescent and proliferating human dermal fibroblasts (HDF). Two monoclonal antibodies recognising human lamins A and C and three different fixation methods were employed. In indirect immunofluorescence studies, cultures of quiescent cells displayed a uniform perinuclear distribution of the antibodies. In proliferating cultures two distinct populations of cells were observed: one population displayed a typical perinuclear antibody distribution, while the second population displayed an unusual pattern consisting of a series of spots and fibres within the nucleus. By inducing cell-cycle synchrony in cultures we were able to determine that the unusual internal distribution of the lamin antibodies was restricted to cells in G_1. Optical sectioning and 3-D reconstruction of the lamina structures in G_1 nuclei was performed with a confocal laser scanning microscope (CLSM). This revealed that the internal lamin structures consisted of small foci and fibres proliferating throughout the nucleus. These structures were shown to be closely associated with areas of condensed chromatin but not nuclear membrane. As cells progress towards S phase the internal lamin foci disappear.

Key words: nuclear lamina, G_1 phase, confocal microscopy, lamin/chromatin association, monoclonal antibodies

INTRODUCTION

In eukaryotic cells the nuclear envelope comprises three structural components: the nuclear membrane, the nuclear pores and the nuclear lamina (reviewed by Newport and Forbes, 1987). The nuclear lamina is a mesh-like network of fibres (Gerace, 1986), subjacent to the inner nuclear membrane (Franke et al., 1981; Georgatos and Blobel, 1987; Aebi et al., 1986), consisting of lamins (Aaronson and Blobel, 1975; Dwyer and Blobel, 1976) which are members of the intermediate filament family of proteins (Franke, 1987; Aebi et al., 1986; Fisher et al., 1986; Lehner et al., 1986; McKeon et al., 1986). The lamina of most mammalian cells is composed of three polypeptides of M_r 70, 65 and 60×10^3, which have been termed, respectively, lamins A, B and C. Sequence analysis of cDNA clones encoding human, avian and amphibian lamins has revealed that whereas lamins A and C are highly related, being derived by alternative splicing of the same primary transcript (Fisher et al., 1986), lamin B is distinct. Thus lamins have been classified into two groups termed A-type and B-type (Hoger et al., 1988; Krohne et al., 1987; Lehner et al., 1986; Peter et al., 1989; Vorburger et al., 1989). Of these, one or two B-type lamins appear to be expressed constitutively in most somatic cells (Lehner et al., 1986; Vorburger et al., 1989; Wolin et al., 1987) and embryos (Lehner et al., 1986; Rober et al., 1989; Stewart and Burke, 1987; Wolin et al., 1987), while expression of A-type lamins is highly regulated during development, implying a role in cell differentiation (Benavente et al., 1985; Stick and Hausen, 1985; Lehner et al., 1986; Rober et al., 1989).

Lamins have been implicated in a number of nuclear functions including nuclear envelope assembly (Burke and Gerace, 1986; Burke, 1990; Dabauvalle et al., 1990; Glass and Gerace, 1990), DNA replication (Meier et al., 1991) and higher order chromatin organisation (Franke, 1974; Franke et al., 1981; Hochstrasser et al., 1986; Lebkowski and Laemmli, 1982), but none of these putative functions have been fully characterised. In cell-free extracts of CHO cells, A-type lamins have been shown to associate with metaphase and telophase chromosomes in the absence of nuclear membranes (Burke and Gerace, 1986; Burke, 1990; Glass and Gerace, 1990). This association is believed to promote chromosome decondensation (Benavente and Krohne, 1986) and the reassembly of the nuclear envelope (Burke and Gerace, 1986). B-type lamins appear to have two functions in nuclear reassembly. Since they are associated with membrane vesicles during mitosis (Gerace and Blobel, 1980; Stick et al., 1988), they may target these vesicles to lamin A/C-coated chromosomes during nuclear envelope reassembly (Burke and Gerace, 1986; Lebel and Raymond, 1984) and anchor the lamina to the nuclear membrane once it is formed (Gerace et al., 1984; Gerace and Blobel, 1980; Stick et al., 1988). In cell-free extracts of *Xenopus* eggs, assembly of the single lamin species, Liii, into a lamina is not required for either chromatin decon-

densation or nuclear membrane assembly (Meier et al., 1991; Newport et al., 1990), but may be required for chromatin organisation, as DNA binding proteins display unusual distributions in lamin depleted nuclei (Meier et al., 1991). Recent studies on *Drosophila*, human CNS cells and plant cells indicate that chromosomes occupy discrete domains within interphase nuclei (Heslop-Harrison and Bennett, 1990; Hochstrasser and Sedat, 1987; Hochstrasser et al., 1986; Manuelidis and Borden, 1988; Mathog et al., 1984). It has been suggested that the three dimensional organisation and segregation of chromosomal domains may be maintained or altered by the nuclear pore complexes, the lamina or the nuclear matrix (Blobel, 1985). Indeed, in a large number of cell types, heterochromatic regions are directly associated with the lamina (Lebkowski and Laemmli, 1982; Franke et al., 1981; Franke, 1974; Hochstrasser et al., 1986) and it has been proposed that the expression of A-type lamins during cell differentiation increases the capacity of the lamina to bind heterochromatin (Nigg, 1989), which is reflected in the increased temporal control of replication zones in somatic nuclei when compared to embryonic nuclei (Kill et al., 1991).

Despite this evidence, it is not yet clear how the lamins are involved in the spatial organisation of chromatin. During interphase, lamins continue to enter the nucleus via the nuclear pores (Loewinger and McKeon, 1988). Indeed, prior to S phase the number of nuclear pores in the nuclear envelope doubles (Maul et al., 1972), which presumably results in significant reorganisation of both the lamina and chromatin. It is not clear whether lamins entering the nucleus during the G_1-phase of the cell-cycle play a role in this reorganisation or if it involves the A/C type lamins that may associate with telophase chromosomes prior to nuclear envelope reformation. A systematic study of lamin/chromatin associations during different phases of the cell-cycle has not been performed; however, the availability of confocal microscopy permits such analyses in lightly fixed samples. Indeed, deconvoluted images of *Drosophila* nuclei have already been used to determine whether chromatin domains are directly associated with the lamina (Paddy et al., 1990). In this study we have used monoclonal antibody reagents which react with A-type lamins, and confocal laser scanning microscopy (CLSM), to investigate lamin/DNA associations during the progress of the cell-cycle. Our results indicate that during the G_1-phase of the cell-cycle, A-type lamins form discontinous filaments which possibly associate with partially decondensed chromatin deep within the nucleus. By S phase these structures have disappeared and lamins are restricted to the perinuclear region. It is possible that these structures represent associations with areas of heterochromatin and that as the nucleus expands, the lamins relocate to the nuclear periphery, thus drawing heterochromatin to the nuclear envelope.

MATERIALS AND METHODS

Cell culture and synchronisation

Human dermal fibroblasts (HDF) were isolated from neonate and juvenile foreskins and termed 3DD and 2DD respectively. Strain 1BR HDF was also used (Arlett et al., 1975). For nuclear isolation and immunoblotting, cells were seeded on 90 mm tissue culture dishes at a density of 3×10^3 cells/cm^2, while for indirect immunofluorescence, cells were seeded at the same density on 13 mm glass coverslips. The cells were grown in Dulbecco's modified Eagles medium (DMEM, Gibco) with 10% (v/v) newborn calf serum (NCS) and antibiotics (10 units/ml penicillin, 50 μg/ml streptomycin). The cultures were allowed to proliferate for 48 h prior to use. Cells were made quiescent by washing twice with serum free DMEM and then refeeding with DMEM containing 0.5% (v/v) NCS for 7 days. Synchronisation of cells plated onto 13 mm glass coverslips was achieved by two methods: firstly cells were released from quiescence by refeeding with DMEM containing 10% (v/v) NCS; secondly cells were released from quiescence as above then, after 8 h, 1 mM hydroxyurea (HU) was added to the culture medium for 10 h to accumulate the cells at the G_1/S boundary. The cells arrested at the G_1/S boundary were then allowed to progress through the cell cycle by extensive washing to remove the HU. For the 36 h synchronisation experiments, cells on coverslips were taken every two hours, washed with phosphate buffered saline (PBS) and prepared for indirect immunofluorescence.

Indirect immunofluorescence

After fixation (see below) cells were prepared for indirect immunofluorescence. Cells in S phase were detected with a human autoimmune serum to the proliferating cell nuclear antigen, as previously described (Kill et al., 1991). To observe the distribution of the lamins A and C, two monoclonal antibodies (mAb) NZ1.D6 and L6.8A7 were employed. HDF were covered with 10 μl of either NZ1.D6 (final dilution 1:10 in PBS/1% (v/v) NCS) or L6.8A7 (final dilution 1:1000 in PBS/1% (v/v) NCS) and incubated overnight at 4°C or for one hour at RT. The cells were washed in PBS and covered with 10 μl of the secondary antibody (TRITC-rabbit anti-mouse Ig or FITC-rabbit anti-mouse Ig (Dakopatts), both at a final concentration of 1:30 (v/v) in PBS/1% (v/v) NCS), and incubated for 1 h at RT. The distribution of nuclear membranes was detected with either one of two fluorescent lipophillic dyes, 3,3′-dihexylocarbocyanine (DHCC), which fluorescences in the fluorescein channel, or nile red which fluoresces in the rhodamine channel. DHCC was applied at 2 μg/ml in PBS for 10 min at RT or nile red was applied at 1 μg/ml in PBS for 30 min, following the secondary antibody incubation. Propidium iodide (PI) (which fluoresces in the rhodamine channel) was used to detect the distribution of DNA. Cells were treated with 2.5 mg/ml RNase A during the primary antibody incubation. Propidium iodide was added with the secondary antibody at a concentration of 1 μg/ml. Following extensive washing, coverslips were mounted onto microscope slides in either 50% (v/v) glycerol containing 1 μg/ml DAPI and 2.5% (w/v) isopropylgallate or 30% (v/v) glycerol containing 12% (w/v) Mowiol (Hoechst), 1 μg/ml DAPI and 2.5% (w/v) 1,4-diazobicyclo-[2.2.2]-octane (DABCO, Sigma). Slides were viewed with either a Zeiss Axioskop microscope or with a Biorad MRC 600 confocal laser scanning microscope (CLSM).

Fixation procedures

Cells on coverslips were prepared for indirect immunofluoresence using three different fixation procedures. (1) Cells were washed three times with PBS and fixed with methanol:acetone (1:1, v/v) for 4 min at 4°C and then washed with PBS. (2) Cells were washed with PBS and placed in ethanol:acetic acid (19:1, v/v) for 5 min at −20°C and then rewashed with PBS. (3) Cells were washed, incubated in Tris-buffered saline (TBS) containing 0.5% (v/v) Triton X-100 (Sigma), 5 units units/ml RNase inhibitor (Sigma) and 50 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) for 10

min at 4°C, washed with PBS and placed in 3.7% formaldehyde diluted 1:10 in PBS (v/v) for 30 min at 4°C and finally washed with PBS.

Nuclear isolation

To isolate nuclei, cell cultures were washed once with PBS, twice with nuclear isolation buffer (NIB, 10 mM Tris/HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% (v/v) Triton X-100) at 4°C and removed from the dish by scraping with a rubber policeman. Nuclei were released from the cells by homogenisation, using a tight fitting pestle in a Dounce homogeniser, and collected by centrifugation at 1700 revs per min for 10 min through a 30% (w/v in NIB) sucrose cushion. The isolated nuclei were examined by phase contrast microscopy before being resuspended in double strength SDS-sample buffer (Laemmli, 1970).

Immunoblotting

Isolated nuclei in double strength sample buffer were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 8% polyacrylamide gels using a BioRad minigel apparatus. The gels were equilibrated in blot transfer buffer (BTB; 25 mM Tris, 192 mM glycine, 20% (v/v) methanol for 15 min) and the proteins transferred to a nitrocellulose membrane using a Biorad Mini-Trans Blot apparatus for 1 h in BTB. The membranes were washed in blot rinse buffer (BRB; 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% (v/v) Tween 20) and blocked by incubation in 4% (w/v) dried milk in BRB for 8 h at 4°C. After a second wash the membranes were incubated in primary antibody overnight at 4°C. NZ1.D6 was used at a dilution of 1:10 in PBS/1% (v/v) NCS, L6.8A7 and LN43.2 were both used at a dilution of 1:1000 in PBS/1% (v/v) NCS and IFA was used undiluted. The membranes were washed again with BRB and incubated with a peroxidase-conjugated, rabbit anti-mouse secondary antibody Ig diluted 1:400 (Dakopatts) in PBS/1% (v/v) NCS for 4 h at 4°C. Visualisation of the protein bands recognised by the antibodies was with 1 mg/ml 3,3′-diaminobenzidine and hydrogen peroxide (0.3% (v/v)).

RESULTS

Monoclonal antibodies to human lamins A and C reveal unusual internal lamin structures within the nuclei of human dermal fibroblasts

In this study two monoclonal antibodies which recognise human lamins A and C have been employed, NZ1.D6 and L6.8A7. NZ1.D6 is a hybridoma derived from a fusion between splenocytes from an NZB × W hybrid autoimmune mouse and murine myeloma cells. L6.8A7 was originally isolated as a monoclonal antibody that reacted with *Xeno - pus laevis* lamin Li, Lii and Liii (Stick and Hausen, 1985); however, it also cross-reacts with human lamins A and C (Fig. 1c).

In order to confirm the specificity of the two mAbs to human lamins A/C, western blotting was performed (Fig. 1). Nuclei were isolated from proliferating and quiescent HDFs, subjected to polyacrylamide gel electrophoresis (PAGE) on 8% gels, transferred to nitrocellulose membranes and probed with the relevent antibodies. NZ1.D6 reacted with both full-length human lamins A and C expressed in *Escherichia coli* from a pET-1 vector (Fig. 1a, tracks 4 and 3 respectively) (Moir et al., 1991). It also reacted with two bands only, of M_r 71.5 and 61×10^3, in nuclei isolated from quiescent (track 1) and proliferating (track 2) HDF. The M_r of these bands closely correspond to the predicted M_r of human lamins A and C respectively. A multi-channel blotting apparatus allowed comparison between the bands recognised by NZ1.D6, bands recognised by IFA, a monoclonal antibody that reacts with intermediate filament proteins, and LN43.2, a monoclonal antibody to human lamin B (Fig. 1b). The IFA antibody recognised three bands (track 3). The M_r of the upper two bands were identical to those of the two bands recognised by NZ1.D6 (track 1). In contrast, LN43.2 recognised a single band of M_r 67×10^3 (lamin

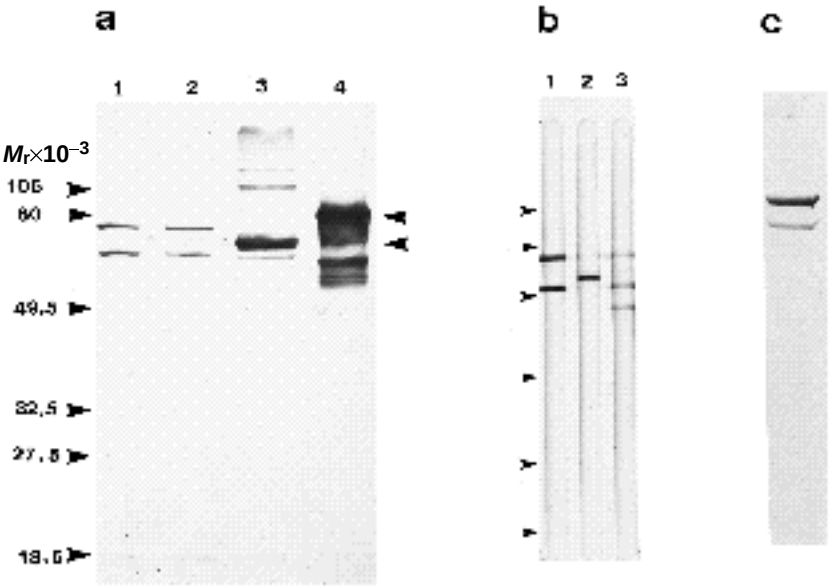


Fig. 1. Western blot analysis to confirm the specificity of the two monoclonal antibodies NZ1.D6 and L6.8A7 to human lamins A and C. (a) illustrates a western blot probed with NZ1.D6. Tracks 1 and 2 are whole nuclei isolated from quiescent (1) and proliferating (2) human dermal fibroblasts (HDF), strain 2DD. The M_r of the two bands recognised by NZ1.D6 were calculated as 71.5 and 61×10^3. Tracks 3 and 4 contain human lamins A (4) and C (3) expressed in *Escherichia coli* from a pET-1 vector, of M_r 82 and 68×10^3, respectively. The lower bands in track 4 are products of proteolysis. (b) shows a western blot analysis of whole nuclei isolated from quiescent cells which have been resolved in one large well and probed with three different antibodies using a multi-channel blotting apparatus. Two bands were detected by NZ1.D6 (1), one band was detected by a monoclonal antibody to human lamin B LN43.2 (2), and three bands were detected by a monoclonal antibody to intermediate filaments IFA (3). The two bands detected by NZ1.D6 were identical in M_r to the upper bands detected by IFA but non-identical to the band detected by LN43.2. The lower band detected by IFA in track 3 is vimentin (data not shown). (c) is a western blot analysis of whole isolated nuclei from quiescent cells, strain 3DD probed with L6.8A7. Two bands were detected, of M_r equivalent to those predicted for lamins A and C.

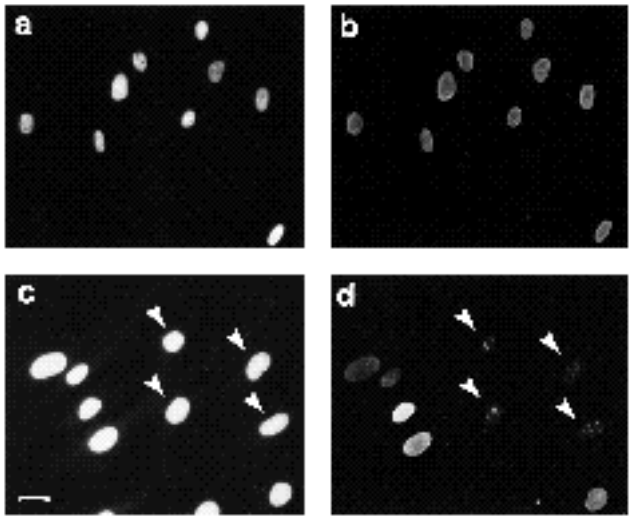


Fig. 2. Micrographs of quiescent (a,b) and proliferating (c,d) HDF, strain 1BR stained with 4,6-diamino-2-phenylindole (DAPI) (a,c) and NZ1.D6 (b,d) to display the anti-lamin distribution. In the quiescent cells the anti-lamin fluorescence gives a typical perinuclear distribution (b), whereas in the proliferating culture NZ1.D6 recognises two populations of cells with respect to anti-lamin fluorescence, the first with the perinuclear distribution, the second having structures within the nucleus (arrowed, c and d). Bar, 20 μm.

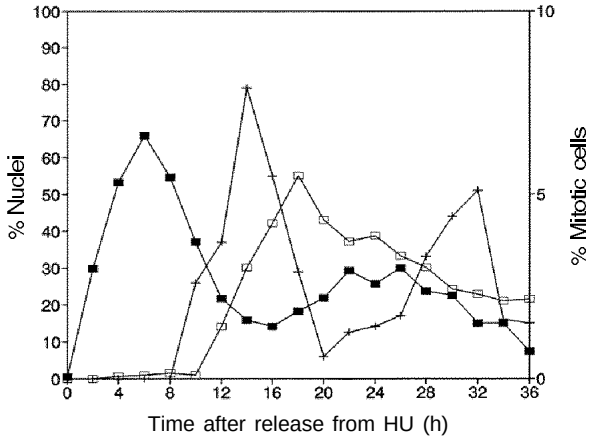


Fig. 3. Graph showing results from a 36 h experiment on synchronously proliferating HDF. HDF were synchronised by release from quiescence then reversibly blocked at the G_1/S boundary using hydroxyurea (HU). Following the release from the HU-induced block, cells were analysed by indirect immunofluorescence. The distribution of a human antibody to proliferating cell nuclear antigen (PCNA) (closed boxes) was used to detect cells in S phase, while the estimation of the fraction of cells in mitosis was achieved by observing typical mitotic figures in DAPI-stained cells (crosses). Cells were co-stained with NZ1.D6 to determine the timing within the cell cycle of the unusual anti-lamin distribution (open boxes). Each point represents percentages from scores of at least 1000 cells.

B) (track 2). L6.8A7 also reacted with expressed lamins (not shown) as well as with two bands in nuclei isolated from quiescent HDF. The M_r of both bands were eqivalent to those of human lamins A and C (Fig. 1c). These results confirmed that both antibodies, NZ1.D6 and L6.8A7, are specific for human lamins A and C in human dermal fibroblasts and do not react with human lamin B.

The distribution of the A/C lamins in both quiescent and proliferating HDF cultures was analysed by indirect immunofluorescence. Cells in G_0 displayed a typical uniform distribution of antibody at the nuclear periphery (Fig. 2b). In contrast, cultures of proliferating cells displayed two distinct patterns of anti-lamin staining. In 70% of the cells the usual uniform peripheral distribution was observed, but in 30% of the cells an unusual pattern of anti-lamin distribution was detected (Fig. 2d), in which there was little staining at the nuclear periphery but instead a series of spots and fibres within the nucleus.

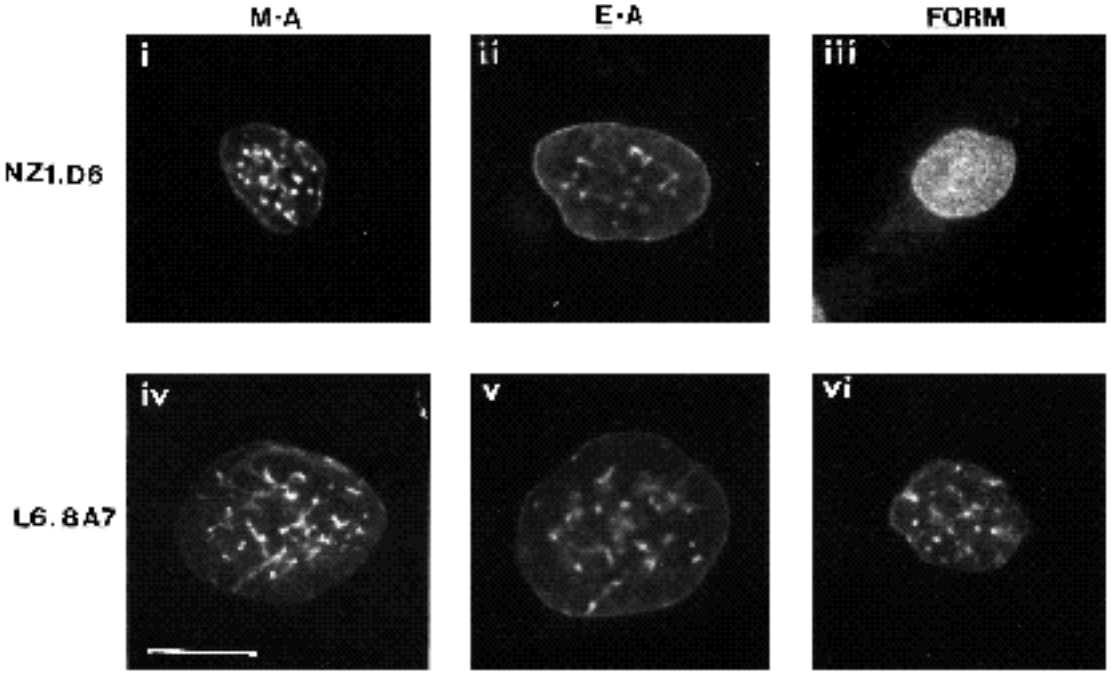
The unusual anti-lamin distribution is restricted to cells in the G_1 phase of the cell cycle

Cells that displayed the unusual lamin pattern were usually smaller than cells with the typical uniform distribution of antibody, and appeared in pairs, sometimes with a bridge of cytoplasm between the two (not shown). The pairs of cells appeared to be daughter cells and the nuclei with the internal lamin structures were therefore thought to be in G_1 phase of the cell cycle.

To determine if the unusual anti-lamin pattern was cell cycle-related, it was necessary to synchronise division in cultures of HDF. Synchronously proliferating cultures were assayed over a 36 h period which allowed the cells to complete 1.5 cell cycles. Two methods of synchrony were used. Initially, cells were made quiescent for 7 days by serum starvation and then restimulated by addition of complete medium. Alternatively, synchrony was achieved by first releasing quiescent cells from serum starvation but then blocking them at the G_1/S boundary with HU. The cells were subsequently released from this block by addition of fresh medium with no HU. Indirect immunofluorescence was performed at 2 h intervals on cells which had been prepared by fixation with methanol:acetone for 4 min at 4°C. Cells in S phase were detected with human autoimmune serum to proliferating cell nuclear antigen (PCNA) (Kill et al., 1991). DAPI staining of chromatin was used to identify cells in mitosis by observing typical mitotic figures. Cells were co-stained with NZ1.D6 to determine the timing of the unusual anti-lamin distribution in the cell cycle.

When cell synchrony was induced by restimulation from quiescence, a peak of 60% of cells in first S phase was observed. Under these conditions the unusual lamin pattern was detected at 24 h, just after the first mitosis (data not shown). 70% of cells released from the HU G_1/S boundary block entered first S phase synchronously; however, during the second cell cycle the fraction of cells entering S phase synchronously had fallen to 30%. Given this limitation on synchrony a distinct peak of the unusual lamin staining was observed at 18 h, just after first mitosis and prior to second S phase (Fig. 3). This result was confirmed in several cell synchrony experiments. Hence, both methods indicate that the unusual lamin pattern is restricted to cells progressing from mitosis to S phase via G_1. Furthermore, internal lamin foci were not detected in S phase cells, as determined by PCNA co-staining (data not shown).

a



bi

OVERNIGHT 4°C

	METH:ACETONE 4°C 5 MINS	ETHAN:ACETIC -20°C 5 MINS	TRITON EXT. FORMALDEHYDE
NZ1.D6	+	+	-
L6.8A7	+	+	+

ii

ONE HOUR, ROOM TEMP.

	METH:ACETONE 4°C 5 MINS	ETHAN:ACETIC -20°C 5 MINS	TRITON EXT. FORMALDEHYDE
NZ1.D6	/	-	-
L6.8A7	-	+	-

Fig. 4. (a) illustrates micrographs of cells displaying the G_1 distribution of the two monoclonal anti-lamin antibodies NZ1.D6 and L6.8A7 incubated overnight at 4°C. Indirect immunofluorescence was performed with the two mAbs in conjunction with three different fixation procedures. a(i) and (iv) illustrate the unusual G_1 distribution of the anti-lamin A/C antibodies observed when cells were fixed with methanol:acetone (1:1 (v/v)) for 5 min at 4°C. a(ii) and (v) illustrate the similar G_1 distribution of the anti-lamin A/C antibodies when cells were fixed with ethanol:acetic acid (19:1 (v/v)) at −20°C for 5 min. a (iii) and (vi) illustrates cells treated with 0.5% Triton X-100 and then fixed with 3.7% formaldehyde. (b) illustrates the differences in the anti-lamin antibody distibution when the overnight incubations of primary antibody at 4°C (bi) were compared with incubations at room temperature for one hour (bii). Internal lamin structures revealed by indirect immunofluorescence are denoted by +, perinuclear anti-lamin distribution is denoted by −, and absence of staining is denoted by /.

The internal lamin structures are detected with three different fixation procedures

The unusual distribution of anti-lamin antibody seen at G_1 was initially identified following fixation with methanol:acetone (1:1 (v/v)) for 4 min at 4°C. To exclude the possibility that the G_1 anti-lamin pattern was the product of a fixation artifact, cells were fixed using two other fixation procedures: ethanol:acetic acid (19:1 (v/v)) for 5 min at −20°C, and Triton extraction followed by 3.7% formaldehyde for 30 min at 4°C. Fig. 4 illustrates typical distributions of both mAbs in G_1 nuclei fixed using the three methods. L6.8A7 detected internal lamin foci with all three fixation procedures, whereas NZ1.D6 only detected these structures following fixation with organic solvents and not with formaldehyde. Since the G_1 internal lamin structures were identified with two different antibodies and three different fixation methods, we concluded that these structures were not artifactual. Since two different antibodies recognised the structures it also seemed unlikely that they represented a unique epitope which may be present in only a sub-set of lamins. Since the internal lamins were first observed using prolonged primary antibody incubations (NZ1.D6 overnight at 4°C), shorter incubations were also tried. No staining was detected when the primary antibody incubations with NZ1.D6 were performed using methanol:acetone-fixed cells at room temperature (RT) for

one hour (see Fig. 4bii). When the other two fixation methods were employed in conjunction with NZ1.D6 for one hour at room temperature, a peripheral distibution of the lamin antibody was observed in cells synchronised for G_1 by restimulation from quiescence. Similarly, the distribution of L6.8A7 was also perinuclear in G_1 cells which had been fixed with methanol:acetone or formaldehyde. However, cells fixed with ethanol:acetic acid did display internal lamin structures when stained with L6 8A7 for 1 h at RT (see Fig. 4bii). Thus it appeared that internal lamins were preferentially revealed following prolonged antibody incubations at 4ºC or by fixation with ethanol:acetic acid at −20°C. NZ1.D6 secretes IgM whereas L6 8A7 secretes IgG. As IgM is very large it may not penetrate the nucleoplasm during short incubation periods. Similarly, ethanol:acetic acid may not dehydrate cells to the same extent as methanol:acetone and might therefore allow L6 8A7 to penetrate the nucleoplasm during short incubation periods. Whatever the explanation, the observation that one fixation method generates two distinct patterns implies that the internal lamin foci are revealed rather than created by specific fixation procedures.

The internal lamin structures are found deep within the nucleus

Using conventional fluorescence light microscopy it is not possible to observe the exact position or precise detail of the internal lamin structures. G_1 nuclei were therefore analysed by CLSM, which allows optical sectioning without physical disruption of material. A series of images is produced which allows observation of detail throughout a three dimensional object. Optical sectioning of G_1 nuclei was performed at 0.5 μm intervals (Fig. 5). This revealed that the lamin structures were distributed throughout the nucleus in all planes of sectioning. Many of the structures were observed in the mid-section indicating their presence in the centre of the nucleus. Some lamin foci were observed in only a single optical plane; however, others were seen in several consecutive planes of sectioning. This implied that while some of the internal lamin structures were small discrete spots, other structures seen through several focal planes, were asymmetric in shape and continuous over relatively long distances, giving the impression of fibrillar structures. To confirm this, three dimensional optical reconstructions of anti-lamin stained G_1 nuclei were made. Z-series were collected and the individual images of this series were recombined with a 2 pixel shift to give a three dimensional view of the distribution of the lamin structures within the nucleus (Fig. 6). When these three dimensional images are observed with red-green stereo glasses, lamin fibrils can be seen proliferating throughout the nucleus.

Internal lamin structures appear to be associated with regions of condensed chromatin and not nuclear membrane

Double indirect immunofluorescence and CLSM allows putative associations between two fluorescently labelled components of a particular structure to be analysed. Two structures within the same organelle are labelled with different fluorochromes, one fluorescing in the fluorescein channel, the other in the rhodamine channel. Both structures are then scanned in a single optical plane and the images saved. Optical reconstruction is then performed by superimposing one image over the other. Where the two structures co-localise the image appears yellow (Kill et al., 1991).

It was necessary to ascertain if the internal lamin structures were simply invaginations of the nuclear envelope due to fixation. We assumed that if this were so, then nuclear membrane would be associated with the internal lamin foci. Two fluorescent lipophyllic dyes were used to visualise the nuclear membrane: 3,3′-dihexylacarbocyanine (DHCC) which fluoresces in the fluorescein channel, and nile red which fluoresces in the rhodamine channel. For these analy-

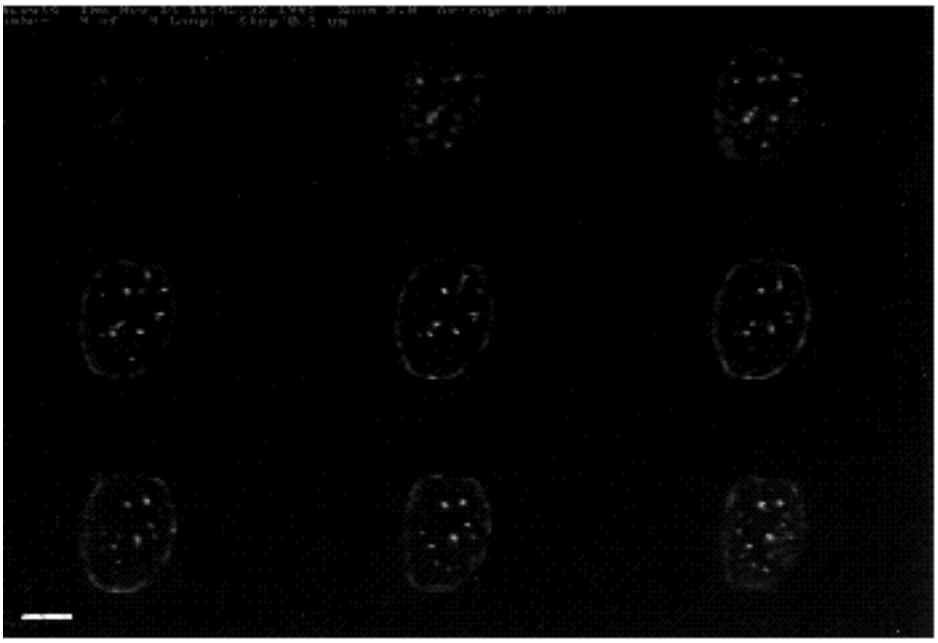


Fig. 5. G_1 HDF nucleus stained with NZ1.D6 and analysed by CLSM. Nine consecutive optical sections were obtained at 0.5 μm intervals, the image at the top left hand corner being the top of the nucleus. To collect these images the pinhole aperture was adjusted to its minimum diameter. Optical reconstruction was performed on images obtained from averaged values of 30 scans using a Kalman programme. Bar, 5 μm.

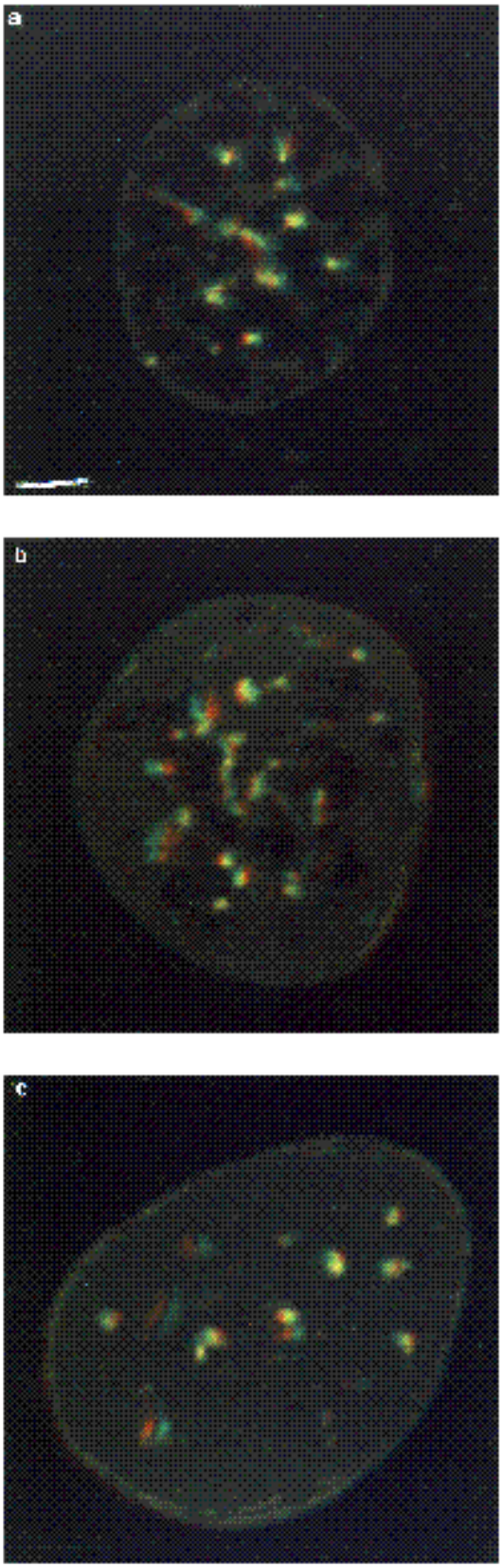


Fig. 6. Three dimensional reconstructions of optically sectioned G_1 nuclei on the CLSM stained with NZ1.D6. These images need red-green stereo glasses to observe the 3-D effect on the internal lamin structures.

ses G_1 nuclei were co-stained with NZ1.D6. Fig. 7 illustrates optically reconstructed images of mid-sections through two such G_1 nuclei. Panels a and c display membranous material stained by DHCC and nile red respectively. Panels b and d display the corresponding sections in which images of membrane fluorescence have been merged with images of anti-lamin fluorescence. The anti-lamin distribution is displayed in red in Fig. 7b and green in Fig. 7d. At the nuclear periphery, the merged images of both nuclei display a yellow coloration indicating that the lamina and nuclear membrane co-localise. However, the internal lamin foci in both nuclei display no co-localisation with membrane. This suggests that the internal lamin structures were not invaginations of the nuclear envelope. It should be noted that the G_1 nucleus in Fig. 7b has a continous peripheral lamina with fewer internal foci, whereas the nucleus in Fig. 7d has a discontinuous peripheral lamina but extensive internal anti-lamin foci. These relative distributions were typical of the nuclei which we have observed.

If the internal lamin structures play a role in chromatin reorganisation after mitosis an association with condensed chromatin would be expected. Propidium iodide (PI), which fluoresces in the rhodamine channel, was used to stain the DNA (Fig. 8a,e). Confocal images of PI staining illustrate typical distributions of chromatin in early G_1 nuclei. To over-emphasise areas of condensed chromatin, the PI fluorescence due to decondensed chromatin was removed using an image enhancement facility termed basing. The weakest 17% of fluorescence was removed by this method to provide an image in which condensed DNA could be seen throughout the nucleus as fibres which surrounded the nucleoli. Fig. 8d-f illustrates the distribution anti-lamin fluorescence in the same mid-sections, superimposed over the PI confocal image. A number of such nuclei were analysed and without exception the anti-lamin foci abutted fibres of partially decondensed chromatin and the material surrounding the nucleoli. Anti-lamin fluorescence was never observed in areas from which the PI fluorescence, representing fully decondensed chromatin, had been removed by using an image enhancement facility termed basing. Hence, we would like to suggest that the internal lamin structures were associated with the partially condensed chromatin.

DISCUSSION

The nuclear lamina has been studied extensively by indirect immunofluorescence and by confocal microscopy. Without exception the distribution of the lamins has been described as perinuclear (Gerace et al., 1978, 1984; Benavente and Krohne, 1986; Paddy et al., 1990; Hutchison et al., 1988; Fox et al., 1991). Here we report results using two different monoclonal antibodies and three fixation techniques which reveal lamin structures in the interior of G_1 nuclei. It is difficult to resolve the discrepancy between our results and previous studies. However, in some studies using confocal microscopy, embryonic nuclei which have rapid cell-cycles and no G_1 period (Hutchison et al., 1988; Paddy et al., 1990) and S phase nuclei (Fox et al., 1991) were used. In both cases, based on our results, inter-

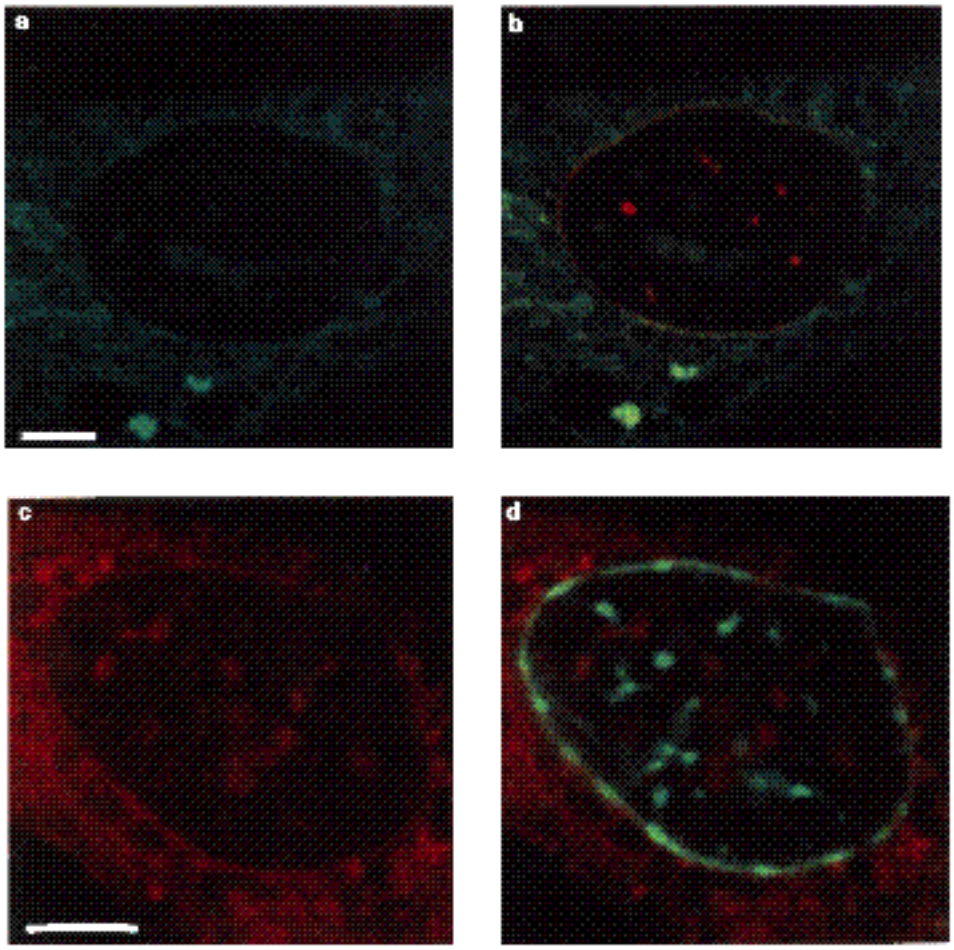


Fig. 7. Optical reconstructions from CLSM mid-sections of G_1 nuclei which were co-stained with NZ1.D6 and a lipophillic dye. (a) displays a nucleus stained with DHCC. In merged images (b) where anti-lamin fluorescence is superimposed over DHCC fluorescence, the lamin fluorescence appears red. (c) displays a nucleus stained with nile red. In merged images (d) anti-lamin fluorescence appears green. In both merged images (b and d), coincident fluorescence appears yellow. Bar, 5 μm.

nal lamin structures would not be expected. Other studies involved rat liver nuclei, the majority of which would be derived from G_0 cells and again would not be expected to display internal lamin foci (Gerace et al., 1978, 1984). In addition to our observation that internal lamins are restricted to the G_1 phase of the cell-cycle, we also report that they are preferentially revealed by fixation with ethanol:acetic acid at −20°C or following primary antibody incubations at 4°C. Investigations using other fixation methods, or more importantly short incubation times with primary antibodies, would again fail to reveal these structures (Gerace et al., 1978, 1984; Paddy et al., 1990; Benavente and Krohne, 1986; Fox et al., 1991).

Three dimensional reconstructions from z-series clearly show that bright foci of anti-lamin fluorescence are detected in the middle of G_1 nuclei. In merged images of nuclei co-stained with anti-lamin antibodies and lipophilic dyes, the internal lamin foci are not associated with membrane structures. This implies that the structures are not simply points of invagination of the nuclear envelope. If the internal lamin foci are not fixation artefacts how do they arise, what is their fate and, more importantly, what is their function?

Internal lamin foci are observed in the nuclei of cells progressing from mitosis and through G_1 and predominate in cells that are in early G_1. Until recently the predominant view was that nuclear lamina assembly preceded and was required for nuclear membrane assembly (Burke and Gerace, 1986; Benavente and Krohne, 1986). Furthermore, experiments in which A-type lamins were observed to polymerize around telophase and metaphase chromosomes in the absence of nuclear membranes apparently confirmed the hypothesis that lamina assembly is essentially completed at telophase (Glass and Gerace, 1990; Burke, 1990). However, more recent studies have shown that while lamins may be required for nuclear membrane assembly (Dabauvalle et al., 1990) lamin polymerisation is not (Meier et al., 1991; Newport et al., 1990) and that extensive lamina assembly requires nuclear transport (Dabauvalle et al., 1990; Newport et al., 1990). It is well established that A-type lamins have nuclear localisation sequences which, when mutated, cause them to assemble as cytoplasmic filaments (Loewinger and McKeon, 1988). It has therefore been assumed that possession of a nuclear localisation sequence, in conjunction with isoprenylation or methylation of a C-terminal motif CaaX, directs lamins to the nuclear envelope where they are inserted into the lamina (Kitten and Nigg, 1991; Krohne et al., 1989). However, a detailed analysis of the fate of lamins as they are transported from the cytoplasm to the nucleus has not been reported. Recent studies in which biotinylated, bacterially expressed human lamin A were microinjected into the cytoplasm of 3T3 cells showed that these lamins are transported into the nucleus at a faster rate than they are assembled into a lamina. As a consequence labelled lamins accumulate as large foci in

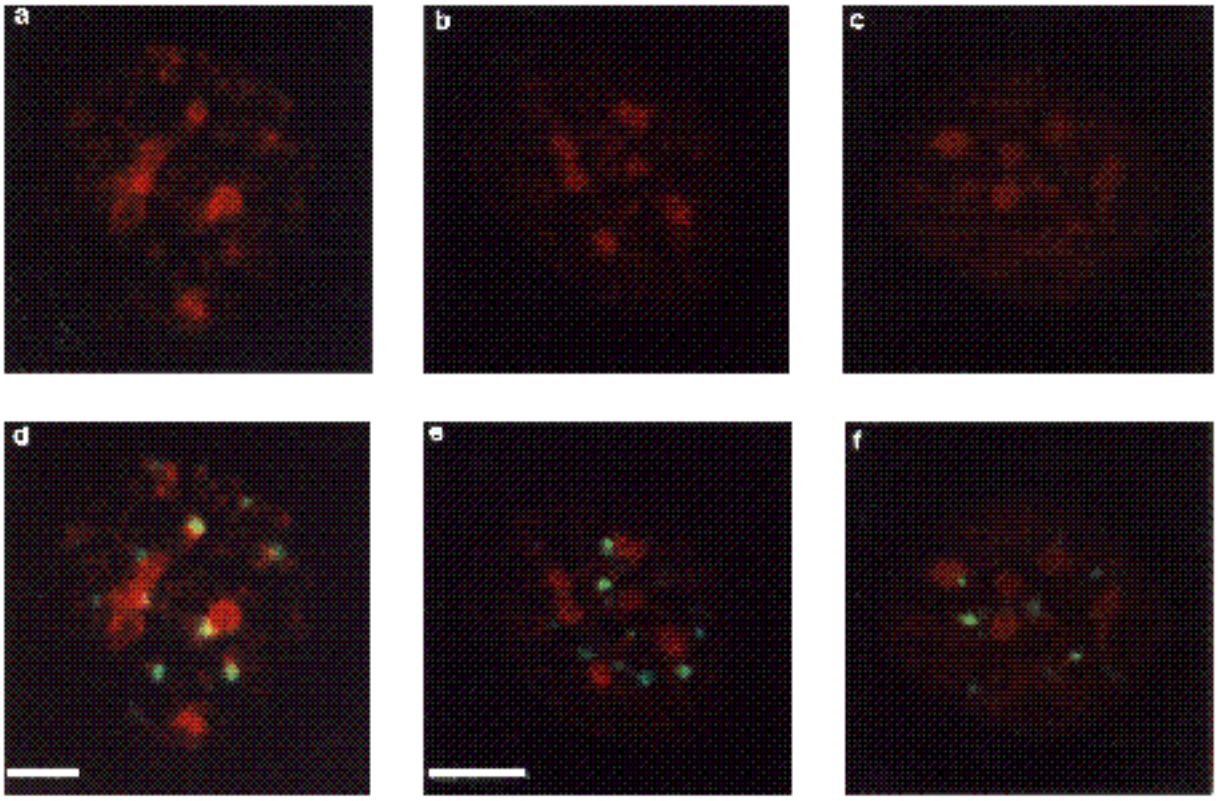


Fig. 8. Optical reconstructions from CLSM mid-sections of G_1 nuclei co-stained with NZ1.D6 and propidium iodide. (a-c) display the distribution of propidium iodide. In each image background fluorescence has been removed to over-emphasise the distribution of condensed chromatin. This was achieved by employing an image enhancement facility on the CLSM termed base. The image was based by a value of 40, which removed the weakest 17% of flourescence. When NZ1.D6 immunofluorescence is superimposed over PI fluorescence (d-f), lamin foci which lie directly above areas of condensed chromatin appear yellow. Bar, 5 μm.

the nucleoplasm before slowly relocating to the nuclear envelope (Goldman et al., 1992). As stated above, when we observe extensive internal lamin foci in a nucleus, the peripheral lamina is usually discontinuous, the corollary of this being that when the peripheral lamina is continuous few or no internal foci are seen. This would be expected if the majority of A-type lamins inherited by a daughter cell accumulate rapidly in the nucleoplasm following nuclear membrane assembly but then relocate more slowly to the nuclear envelope. The lamin foci which we observed were large and resistant to extraction with Triton X-100, suggesting that they were not a soluble pool but rather structural elements. However, the lamin foci could represent a storage pool of insoluble lamin protein which had aggregated and polymerized in the nucleoplasm. A-type lamins polymerize efficiently in the presence of chromosomes at concentrations which are 8-fold lower than are required for self-assembly (Glass and Gerace, 1990). As lamins are transported efficiently from the cytoplasm to the nucleus (Krohne et al., 1989) nucleoplasmic lamins may be expected to be present at local concentrations exceeding that which is required for polymerisation. Hence lamins which are translocated from the cytoplasm to the nucleus may well polymerize into small filaments before they appear at the nuclear envelope. Optical sectioning of dual labelled images has revealed that lamin foci always appear over or adjacent to regions of condensed chromatin. This would be predicted from previous studies which suggest a high affinity between A-type lamins and chromatin (Glass and Gerace, 1990).

The internal lamin structures appeared to associate with dense areas of chromatin, in CLSM sections (Fig. 8). While this localisation might be expected for storage particles, an alternative explanation for the exsistence of internal lamin foci is also suggested. Previous studies have implied that heterochromatin is anchored to the nuclear periphery via the lamina (Nigg, 1989). Perhaps lamins which migrate into the early nucleus preferentially polymerize on heterochromatic regions of the genome. Since these internal lamins relocate to the nuclear periphery, the heterochromatin may be relocated with them. To examine this hypothesis, the mechamism by which internal lamin structures relocate to the nuclear periphery must first be elucidated.

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