

Localisation of the Ki-67 antigen within the nucleolus

Evidence for a fibrillar-deficient region of the dense fibrillar component

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SUMMARY

The Ki-67 antigen is detected in proliferating cells in all phases of the cell division cycle. Throughout most of interphase, the Ki-67 antigen is localised within the nucleolus. To learn more about the relationship between the Ki-67 antigen and the nucleolus, we have compared the distribution of Ki-67 antibodies with that of a panel of antibodies reacting with nucleolar components by confocal laser scanning microscopy of normal human dermal fibroblasts in interphase stained in a double indirect immunofluorescence assay. During early G₁, the Ki-67 antigen is detected at a large number of discrete foci throughout the nucleoplasm, extending to the nuclear envelope. During S-phase and G₂, the antigen is located in the nucleolus. Double indirect immunofluorescence studies have revealed that during early to mid G₁ the Ki-67 antigen is associated with reforming nucleoli within discrete domains which are distinct from domains containing two of the major nucleolar antigens fibrillar and RNA polymerase I.

Within mature nucleoli the Ki-67 antigen is absent from regions containing RNA polymerase I and displays only partial co-localisation within domains containing either fibrillar or B23/nucleophosmin. Following disruption of nucleolar structure, induced by treatment of cells with the drug 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole or with actinomycin D, the Ki-67 antigen translocates to nucleoplasmic foci which are associated with neither fibrillar nor RNA polymerase I. However, in treated cells the Ki-67 Ag remains associated with, but not co-localised to, regions containing B23/nucleophosmin. Our observations suggest that the Ki-67 antigen associates with a fibrillar-deficient region of the dense fibrillar component of the nucleolus. Integrity of this region is lost following either nucleolar dispersal or nucleolar segregation.

Key words: Ki-67, Nucleolus, Anti-nuclear antibody, Confocal microscopy, Fibrillar

INTRODUCTION

The Ki-67 antigen (pKi-67) was first identified by virtue of its reactivity with Ki-67 antibodies (Gerdes et al., 1983). pKi-67 is detected in the nucleus of proliferating cells in all active phases of the cell division cycle, but is absent in non-proliferating cells (Gerdes et al., 1984; Braun et al., 1988; Kill et al., 1994). During interphase, pKi-67 is localised mainly in the nucleolus (Verheijen et al., 1989a). During mitosis pKi-67 is present on all chromosomes (Gerdes et al., 1983, 1984), forming a reticulate structure surrounding metaphase chromosomes (Verheijen et al., 1989b).

Ki-67 antibodies have been used widely for the estimation of the growth fraction of clinical samples of human neoplasms (Brown and Gatter, 1990; Gerdes, 1990; Hall and Levison, 1990; Hall and Woods, 1990) and of normal cells in culture (Kill et al., 1994). Thus, Ki-67 antibodies can be valuable as a prognostic indicator. Molecular and functional characterisation of pKi-67 is necessary for a greater understanding of the role of the antigen in the cell cycle of normal and of neoplastic cells.

Western blotting has revealed that Ki-67 reacts with two polypeptides of 345 and 395 kDa (Gerdes et al., 1991). A full-length cDNA coding for pKi-67 has been cloned by immunoscreening a λgt11 library of human IM-9 cells (Schlüter et al.,

1993). Analysis using the cDNA has identified two differentially spliced mRNAs encoding polypeptides of predicted molecular masses of 359 kDa and 320 kDa. Both sequences contains 16 'Ki-67 repeat' sequences, each of which includes a conserved 66 bp 'Ki-67 motif'. The deduced amino acid sequence of the cDNA has revealed two potential nuclear localisation signals, a number of potential PEST sequences, implicated in proteolysis of the antigen (Rogers et al., 1986) and numerous potential sites for phosphorylation, amidation, N-myristoylation and ATP/GTP binding. Despite recent advances in the molecular characterisation of pKi-67, a detailed analysis of the function of the antigen is yet to be determined. Significantly, incubation of IM-9 cells with synthetic antisense deoxyoligonucleotides complementary to the deduced translation start site of pKi-67 prevents incorporation of [³H]thymidine, suggesting that pKi-67 is necessary for cell cycle progression (Schlüter et al., 1993). Most recently, a cDNA clone likely to represent the murine homologue of pKi-67 has been described (Starborg et al., 1996).

Indirect immunofluorescence has revealed that a large number of antigens are located exclusively in the nucleolus of interphase cells (Kistler et al., 1984; Tan, 1989; Hernandez-Verdun, 1991). A number of antigens which are located in the nucleolus during interphase associate with chromosomes

during mitosis (Gautier et al., 1992a,b). Furthermore, a small number of antigens are found in the nucleolus of proliferating cells only (Freeman et al., 1988 and references therein; Waseem and Lane, 1990). However, pKi-67 appears unique in that it associates with nucleoli of proliferating cells only and with chromosomes during mitosis.

The nucleolus contains rRNA gene repeats and is the site of ribosome synthesis. Nucleoli display a unique higher-order structure (reviewed by Scheer and Benavente, 1990). During the cell cycle, nucleoli undergo characteristic reorganisation (Ochs et al., 1985a; Jimenez-Garcia et al., 1989). During nucleologenesis, prenucleolar bodies form around nucleolar organising regions (NORs) which subsequently fuse to form the interphase nucleolus (Jimenez-Garcia et al., 1994). Early in mitosis, nucleoli disperse, and nucleolar antigens become redistributed (Sommerville, 1986). Some nucleolar antigens remain associated with nucleolar domains (for example NORs), whilst others are dispersed throughout the mitotic cytoplasm or distributed around chromosomes (Gautier et al., 1992a). Ultrastructural analysis of mature nucleoli has defined three distinct nucleolar sub-structures, each associated with distinct nucleolar functions (for a review see Hernandez-Verdun, 1991).

The fibrillar centres (FC) are surrounded by the dense fibrillar components (DFC). The fibrillar components of the nucleolus are embedded within the granular components (GC). Many nucleolar proteins are compartmentalised within nucleolar domains. For instance, fibrillarin is localised within the DFC (Ochs et al., 1985b), nucleophosmin/B23 is localised within the GC (Spector et al., 1984; Schmidt-Zachmann et al., 1987) whilst DNA topoisomerase I (Guldner et al., 1986) and RNA polymerase I (Reimer et al., 1986) are localised within the FC. Compartmentalisation of proteins within the nucleolus reflects compartmentalisation of specific nucleolar functions. Since pKi-67 is located within nucleoli of proliferating cells only, it has been suggested that the antigen may regulate nucleolar metabolism by, for example, increasing rates of ribosomal synthesis required by rapidly dividing cells (Chatterjee et al., 1987). However, it is not entirely clear with which nucleolar compartment pKi-67 is associated. Immunoelectron microscopy has revealed that Ki-67 antibodies stain regions surrounding the FC, probably localised within the DFC (Verheijen et al., 1989a). Ki-67 staining is reported to be absent from the FC, GC and nucleolar interstices.

To learn more about the relationship between pKi-67 and the nucleolus, we have compared the distribution of Ki-67 antibodies with that of a panel of antibodies reacting with nucleolar components by confocal laser scanning microscopy (CLSM) of normal human dermal fibroblasts in interphase stained in a double indirect immunofluorescence assay. Our results suggest that pKi-67 behaves largely independently of the other nucleolar antigens which we have tested, perhaps reflecting a unique function of the antigen in nuclear metabolism.

MATERIALS AND METHODS

Antibodies

All antibodies were diluted in phosphate buffered saline containing 1% (v/v) newborn calf serum (PBS/NCS). Ki-67 (anti-proliferating cells; Dako Ltd, UK) is a rabbit polyclonal antibody raised against a

synthetic polypeptide of pKi-67, and used at 1:150. Anti-PCNA (human polyclonal; Alpha labs, UK) was used at 1:10. AAC 15 (anti-centromere) and AAC 22 (anti-ribonuclear protein) were obtained from the Protein Reference Unit, Royal Hallamshire Hospital, Sheffield, and used at 1:200. L6 8A7 (a kind gift from Dr Reimer Stick, University of Goettingen, Germany) a mouse monoclonal antibody which reacts with A-type lamins in human cells (Bridger et al., 1993) was used at 1:500. S4 (anti-fibrillarin) and S18 (anti-RNA polymerase I), kind gifts from Prof. Dr Ulrich Scheer, University of Wurzburg, Germany, were used at 1:100. NPM (a kind gift from Dr Pui Chan, Baylor College of Medicine, Texas, USA) a mouse monoclonal antibody which reacts with B23/nucleophosmin was used at 1:30. mAb 72B9 (a kind gift from Prof. Eng Tan, Scripps Clinic, California, USA) reacts with fibrillarin and was used at 1:30 for co-staining with S18.

Cells and indirect immunofluorescence

Normal human dermal fibroblasts (strain 2DD) were grown in Dulbecco's modification of Eagles medium supplemented with 10% (v/v) NCS and antibiotics as described previously (Bridger et al., 1993). For indirect immunofluorescence, cells on coverslips were washed three times with PBS then fixed by treatment with methanol/acetone (1:1, v/v) at 4°C for 4 minutes (for anti-PCNA staining) or by treatment with 3.7% formaldehyde in PBS for 7 minutes at room temperature then treated with methanol/acetone as described above. Cells were incubated with 10 µl of primary antibody solutions for 1 hour at room temperature or overnight at 4°C in an humidified chamber. After washing three times in PBS, cells were incubated with secondary antibodies for 1 hour at room temperature or overnight at 4°C in an humidified chamber. Cells were then washed three times with PBS and once with water then mounted onto glass slides in 30% (v/v) glycerol containing 12% (w/v) Mowiol (Aldrich, UK), 1 µg ml⁻¹ DAPI and 2.5% (w/v) 1,4-diazobicyclo-[2.2.2]-octane (DABCO; Sigma, UK). Slides were viewed using a Bio-Rad MRC 600 confocal laser scanning microscope (CLSM), using COMOS software. Images were collected using a 63× oil-immersion lens (NA 1.25). The pinhole aperture for each fluorescence channel was adjusted manually to achieve optimal confocality. The gain controls were then adjusted to achieve a similar range of output signal intensities for each channel. Individual images were normalised by using the command 'Contrast Stretch'. Confocal images and composite confocal and phase contrast images were assembled using Adobe Photoshop 3.0. Triple stained cells were viewed using a Zeiss Axioskop fitted with a 100× oil immersion lens. Images were photographed using Tmax 400 film rated at 6400 ASA.

Synchronisation of HDF

Cultures of HDF were arrested in metaphase using a triple block procedure. Firstly, cultures of HDF were made quiescent by transferring to DMEM containing 0.5% NCS for 4 days. Cultures were then restimulated by transferring to DMEM containing 10% NCS. After 10 hours, 1 µM hydroxyurea (HU) was added to the medium for 14 hours to arrest cells in S-phase. Cells were released from the S-phase block by extensive washing with DMEM containing 10% NCS and allowed to proceed through the cell cycle. After 10 hours, cultures were treated with 10 µM nocadazole to accumulate cells in metaphase. After 10 hours, cultures were washed extensively and allowed to proceed through the cell cycle. Cells on coverslips were removed from dishes and prepared for indirect immunofluorescence at the times indicated for up to 12 hours following release from metaphase arrest.

5,6-Dichloro-β-D-ribofuranosylbenzimidazole treatment of HDF

5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; Sigma, UK) was added to the culture medium of HDF at a concentration of 50 µg ml⁻¹. After incubation for 6 hours, treated cells were prepared for indirect immunofluorescence as described above.

Actinomycin D treatment of HDF

Actinomycin D (AMD; Sigma, UK) was added to the culture medium of HDF at a concentration of $2 \mu\text{g ml}^{-1}$. After incubation for 4 hours cells were prepared for indirect immunofluorescence.

RESULTS

Subcellular localisation of Ki-67 in normal HDF during interphase

Initially, we determined the distribution of pKi-67 within interphase cells by indirect immunofluorescence of normal HDF stained with Ki-67 antibodies, followed by confocal laser scanning microscopy (CLSM). Throughout interphase, Ki-67 staining is restricted to the nucleus (Fig. 1). At passage 4, 56.1% of cells display Ki-67 staining. However, we noted that the staining pattern in individual interphasic nuclei conforms to one of two distinct patterns of distribution. Firstly, a small percentage of nuclei (8.3%, or 15.0% of all Ki-67-positive cells) display numerous small, discrete foci of staining (Fig. 1A) herein referred to as pattern type I. Often these nuclei are found in pairs in close proximity with each other, suggesting that these nuclei are in early G₁. Examination of these nuclei co-stained with L6 8A7 antibodies, which react with A-type lamins in human cells, reveals a discontinuous perinuclear lamin stain with a number of discrete internal foci of lamin staining (Fig. 1B, arrows). This pattern of lamin

staining is restricted to early G₁ cells (Bridger et al., 1993). The merged image reveals that all regions of Ki-67 staining are contained within the boundary of the perinuclear lamin stain. However, some regions of Ki-67 staining are found in close association with the lamina (Fig. 1C, arrows). Comparison of the distribution of Ki-67 staining with the phase contrast image (Fig. 1D-F) reveals a good correlation between regions of Ki-67 staining and phase-dense regions of the nucleus (Fig. 1F). A different pattern of Ki-67 staining is present in a larger fraction of nuclei (47.0%, or 84.0% of all Ki-67-positive cells) in which Ki-67 staining is restricted to nucleoli (Fig. 1G), herein referred to as pattern type II. Furthermore, Ki-67 staining of nucleolar regions exhibits variation in the intensity of the stain.

To determine the pattern of Ki-67 distribution in cells which have progressed beyond G₁, cells were co-stained with anti-PCNA antibodies (Fig. 1G-I). Indirect immunofluorescence of cells fixed with methanol/acetone followed by staining with anti-PCNA antibodies reveals S-phase cells only (Bravo and Macdonald-Bravo, 1987; Kill et al., 1994). At passage 4, 12.2% of cells display a positive stain with anti-PCNA antibodies (Fig. 1H). All PCNA-positive cells also display a positive stain with Ki-67. The pattern of anti-PCNA staining shown in Fig. 1H is typical of cells in early S-phase (Kill et al., 1991). In cells displaying positive reactions with both Ki-67 and anti-PCNA antibodies, the distribution of Ki-67 antibodies is exclusively nucleolar (Fig. 1I). Examination of the

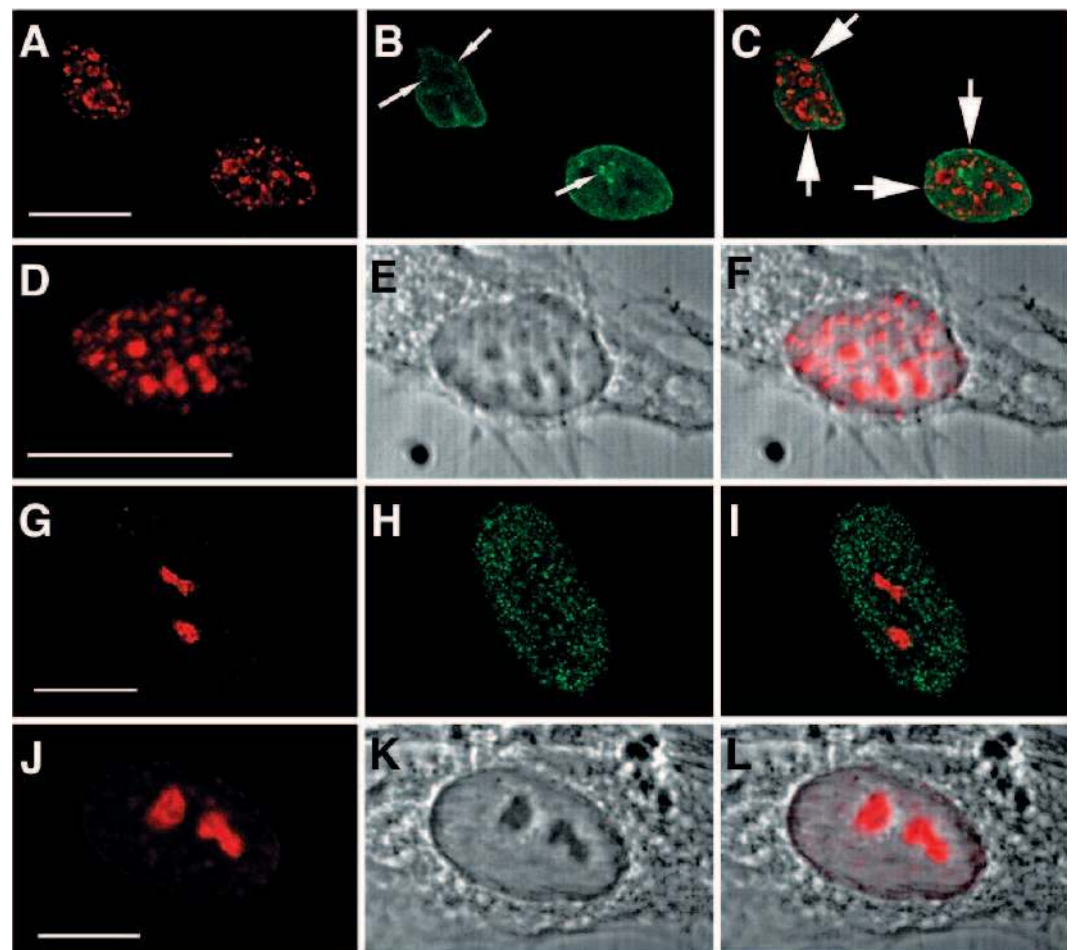


Fig. 1. Distribution of the Ki-67 antigen in G₁ and S-phase HDF. Confocal mid-section of HDF in G₁ (A-F) and S-phase (G-L) stained with Ki-67 (A,D,G,J) and either L6 8A7 (anti-lamin A, arrows in B) or anti-PCNA antibodies (H). (C and I) Corresponding merged images. Some regions of Ki-67 staining are found in close association with the lamina (arrows in C). Phase contrast images of HDF in G₁ (E) and HDF displaying mature nucleoli (K). (F and L) Superimposition of corresponding Ki-67 immunofluorescence with phase contrast. Bars, 10 μm .

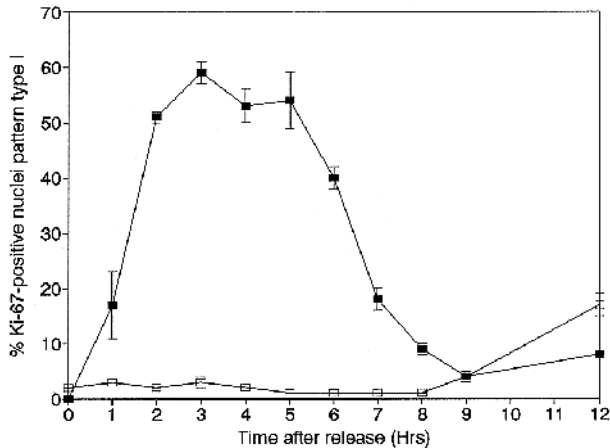


Fig. 2. Timing of the transition from Ki-67 staining pattern type I to type II. Graph showing the percentage of Ki-67-positive cells displaying staining pattern type I (■) and the percentages of cells displaying S-phase PCNA (□) in HDF at times following release from metaphase block. Bars indicate s.e.m ($n=3$).

phase contrast image with the distribution of Ki-67 staining confirms the localisation of pKi-67 within nucleoli (Fig. 1J-L).

Timing of the transition from Ki-67 staining pattern type I to type II

To estimate the extent of G_1 during which pKi-67 pattern type I is present, cultures of HDF were arrested in metaphase using a triple block procedure then released from arrest and prepared for indirect immunofluorescence at the times indicated in Fig. 2. Synchronised cells were stained using Ki-67 and anti-PCNA antibodies. Following release from metaphase arrest, we observe a rapid increase in the percentage of Ki-67 positive cells displaying pattern type I staining. By three hours after release, the percentage of Ki-67 positive cells (type I) reaches a maximum value of about 60% which persists for a further 3 hours, then levels decline to about 5% by 9 hours following release. At 12 hours following release, we observe a 60-fold increase (0.3% to 17%) in the fraction of cells displaying PCNA staining, indicating that cells are entering S-phase by this time.

Association of pKi-67 with other nuclear antigens

Since Ki-67 staining reveals distinct patterns of immunofluorescence during cell cycle progression, we wished to determine whether other nucleolar antigens display similar patterns of distribution, and to determine the relationship between pKi-67 and other nuclear antigens.

Description of the antibodies used for co-staining

To compare the distribution of pKi-67 with the distribution of other antigens within characteristic nucleolar domains, we obtained the following antibodies: S4 is a human autoimmune serum which contains antibodies reacting with fibrillar and stains the DFC (Ochs et al., 1985b). S18 is a human autoimmune serum which contains antibodies reacting with RNA polymerase I (pol I) and stains FCs predominantly (Reimer et al., 1986). NPM is a mouse monoclonal antibody reacting with B23/nucleophosmin and stains GCs (Chan et al., 1987;

Schmidt-Zachmann et al., 1987). Two further human autoimmune sera were obtained from the Protein Reference Unit, Sheffield Hospital, and had been characterised previously as anti-centromere (AAC15) and anti-ribonuclear protein (anti-RNP, AAC22). Both of these sera were screened using HDF and indirect immunofluorescence microscopy at 1:200 dilution. The patterns of staining are shown in Fig. 3.

Distribution of pKi-67 located in nucleoli compared with the localisation of other nuclear antigens

HDF were co-stained with Ki-67 antibodies and one of each antibody described above (Fig. 3). Stained cells were analyzed by CLSM. Each panel shows a confocal mid-section through a nucleus displaying representative patterns of staining. Ki-67 staining is shown in red (Fig. 3A,D,G,J,M) and co-staining antibodies shown in green (Fig. 3B,E,H,K,N). CLSM allows the precise imposition of one image over another. In this case, regions of co-localisation of antibodies are revealed in yellow, with areas of unique staining shown in red or green, respectively (Fig. 3C,F,I,L,O).

S4 (fibrillar) is detected in the nucleolus only, as a number of large 'clumpy' foci with areas of weaker fluorescence between these areas (Fig. 3B). The merged image reveals that the clumpy regions of S4 staining often complement regions deficient in Ki-67 staining (Fig. 3C). Ki-67 staining is most prominent within regions flanking S4-stained domains, although some regions of S4 staining extend beyond the Ki-67-stained boundary (arrows). S18 (RNA pol I) displays fine aggregated staining of the nucleolar interior (Fig. 3E). The merged image reveals that S18 staining is restricted to the nucleolar interior in the absence of Ki-67 staining, whilst Ki-67 alone stains regions surrounding the nucleolar interior (Fig. 3F). In contrast to S4 staining, S18 does not complement Ki-67 staining, revealed by the presence of unstained regions within the interior bounded by Ki-67 staining. Typically, NPM (B23/nucleophosmin) displays an intense stain of the nucleolar periphery (Fig. 3H), although some cells display weaker staining. The merged image reveals only limited co-localisation of NPM with Ki-67 at the nucleolar periphery (Fig. 3I). However, Ki-67 staining is also prominent within the nucleolar cortex in the absence of NPM staining.

AAC15 displays punctate staining typical of anti-centromeric (kintochore) antigens (Fig. 3K). The merged image reveals that in a small number of cases AAC15 co-localises with regions of Ki-67 staining in the nucleolus (Fig. 3L, arrows). However, most of the AAC15 stained foci present in the nucleus are not associated with any Ki-67 staining. AAC22 displays bright staining of a number of clumped regions with weak staining within the surrounding nucleoplasm (Fig. 3N). AAC22 antibodies are excluded from a small number of large regions within nuclei. The merged image reveals that Ki-67 staining is present within the regions excluding AAC22 antibodies, and exhibits no co-localisation at other sites (Fig. 3O).

Association of pKi-67 with other nuclear antigens during G_1

During most of interphase, Ki-67 is present within nucleoli. In these cells there are only a limited number of cases in which the other nuclear antigens which we have tested, that are present in the nucleolus, co-localise to sites that are stained with Ki-67. However, during G_1 pKi-67 is distributed at a

number of foci throughout the nucleus (Fig. 4A,D,G,J,M). To determine whether or not the associations between pKi-67 and other nucleolar antigens observed in cells exhibiting mature nucleoli is present during nucleologenesis we identified G₁ cells stained with both Ki-67 antibodies and other anti-nuclear antibodies and analyzed them using CLSM. Each panel shows a single nucleus of a G₁ pair.

During G₁, S4 antibodies are present at a small number of sites of variable size throughout the nucleoplasm (Fig. 4B). The merged image reveals an association of Ki-67 and S4 antibodies at a number of sites, mostly within larger clumps of Ki-67 staining (Fig. 4C). However, both Ki-67 and S4 antibodies are present at unique sites displaying no co-localisation. S18 staining is restricted to a small number of bright clumps (Fig. 4E). Ki-67 staining in this image is revealed as a large number of clumps and spots. However, the merged image reveals that most S18-stained clumps are associated with Ki-67 staining, but are not co-localised. In these regions Ki-67 staining appears to surround regions of S18 staining. Other Ki-67-stained regions show no such association with S18 staining and thus represent

sites which are unique to pKi-67 (Fig. 4D). NPM staining is localised to a number of large structures and smaller foci within the nucleoplasm (Fig. 4H). The merged image reveals extensive association of Ki-67 within and/or bridging the larger NPM-stained structures. Furthermore, there is a similar distribution of both NPM and Ki-67-stained small foci but a number of these sites display Ki-67 staining alone (Fig. 4I).

During G₁, AAC15 antibodies are present at a number of focal sites throughout the nucleoplasm (Fig. 4K). The merged image reveals a complex association between Ki-67 and AAC15 antibodies (Fig. 4L). Both Ki-67 and AAC15 antibodies are present at a small number of unique sites. At other sites, AAC15 antibodies are located within large Ki-67-stained clumps. In some cases, AAC15 antibodies are adjacent to and overlap regions of Ki-67 staining (arrows). AAC22 staining is present at a small number of clumped sites surrounded by weaker nucleoplasmic staining, with a number of regions exhibiting no stain (Fig. 4N). The merged image reveals that Ki-67 staining is restricted to regions excluding AAC22 staining (Fig. 4O).

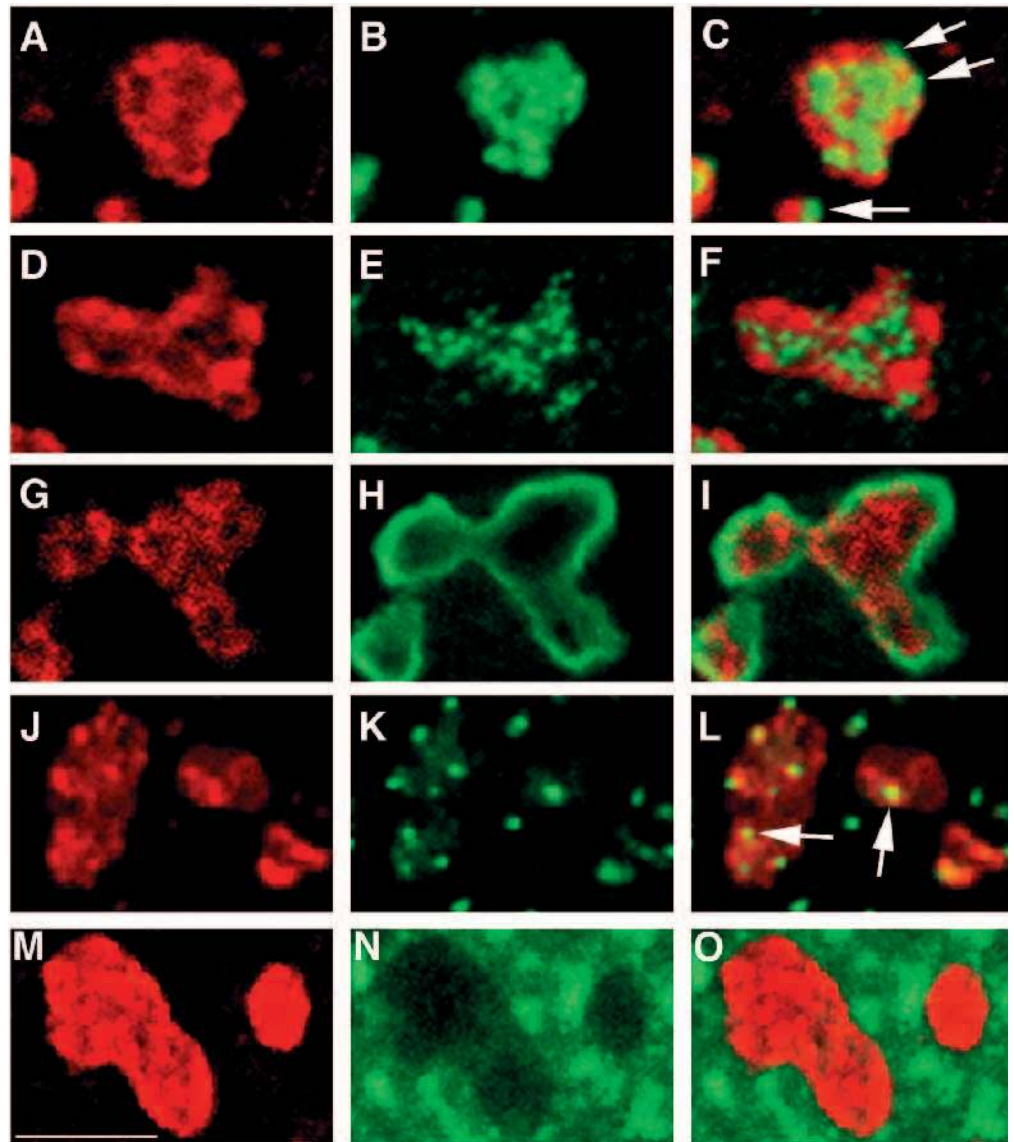


Fig. 3. CLSM analysis of HDF possessing mature nucleoli co-stained with Ki-67 and other anti-nuclear antigen antibodies. Confocal mid-sections of HDF co-stained with Ki-67 (A,D,G,J,M) and anti-fibrillarin (B); anti-RNA pol I (E); anti-B23/nucleophosmin (H); anti-centromere (K) or anti-RNP (N). (C,F,I,L,O) Corresponding merged images. Some regions of S4 staining extend beyond the Ki-67-stained boundary (arrows in C). In a small number of cases AAC15 co-localises with regions of Ki-67 staining in the nucleolus (arrows in L). Bar, 10 μ m.

Distribution of pKi-67 and other nuclear antigens following nucleolar disruption induced by treatment of cells with the drug DRB

Indirect immunofluorescence has revealed that Ki-67 antibodies are located within mature nucleoli. Co-staining experiments have revealed that during this time, other nucleolar antigens are associated with Ki-67 staining but reveal only limited co-localisation. To determine whether associations between pKi-67 and other antigens is maintained following disruption of nucleolar structures, we treated cells in culture with the drug DRB, then performed double indirect immunofluorescence.

The adenosine analogue DRB inhibits both RNA polymerase II-mediated transcription and casein kinase 2 activity (Meggio et al., 1990). Treatment of cells with DRB results in disruption to nucleolar integrity (Granik, 1975; Scheer et al., 1984). In DRB-treated cells, pKi-67 is present throughout

nuclei at a large number of sites of variable size and intensity of fluorescence (Fig. 5A,D,G,J and M). This pattern of Ki-67 staining is somewhat reminiscent of the pattern of staining observed in G₁ cells.

S4 antibodies are distributed at discrete punctate sites within nuclei displaying varied intensities (Fig. 5B). The merged image reveals that sites stained with S4 antibodies exclude Ki-67 staining (Fig. 5C). Similarly, S18 staining within DRB-treated cells is restricted to focal points with weaker staining between them (Fig. 5E). S18 stained regions excludes Ki-67 staining (Fig. 5F). NPM antibodies are distributed throughout the nucleoplasm with stronger staining at a small number of circular structures (Fig. 5H, arrows). Since NPM staining is present throughout the nucleoplasm, the merged image can provide little information regarding specific co-localisation of Ki-67 stained structures and NPM-stained regions (Fig. 5I). However, it is clear that Ki-67 staining is excluded from

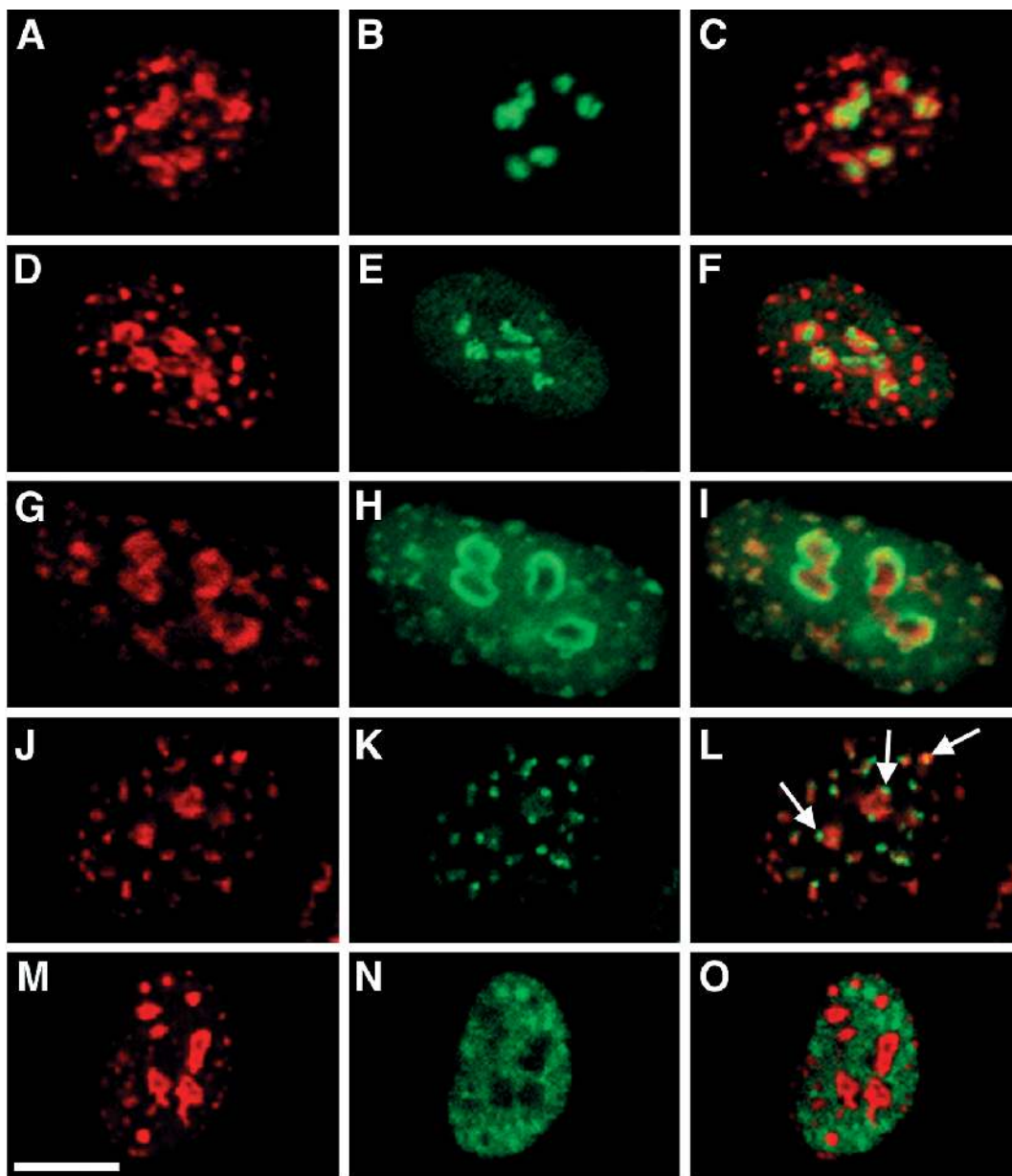
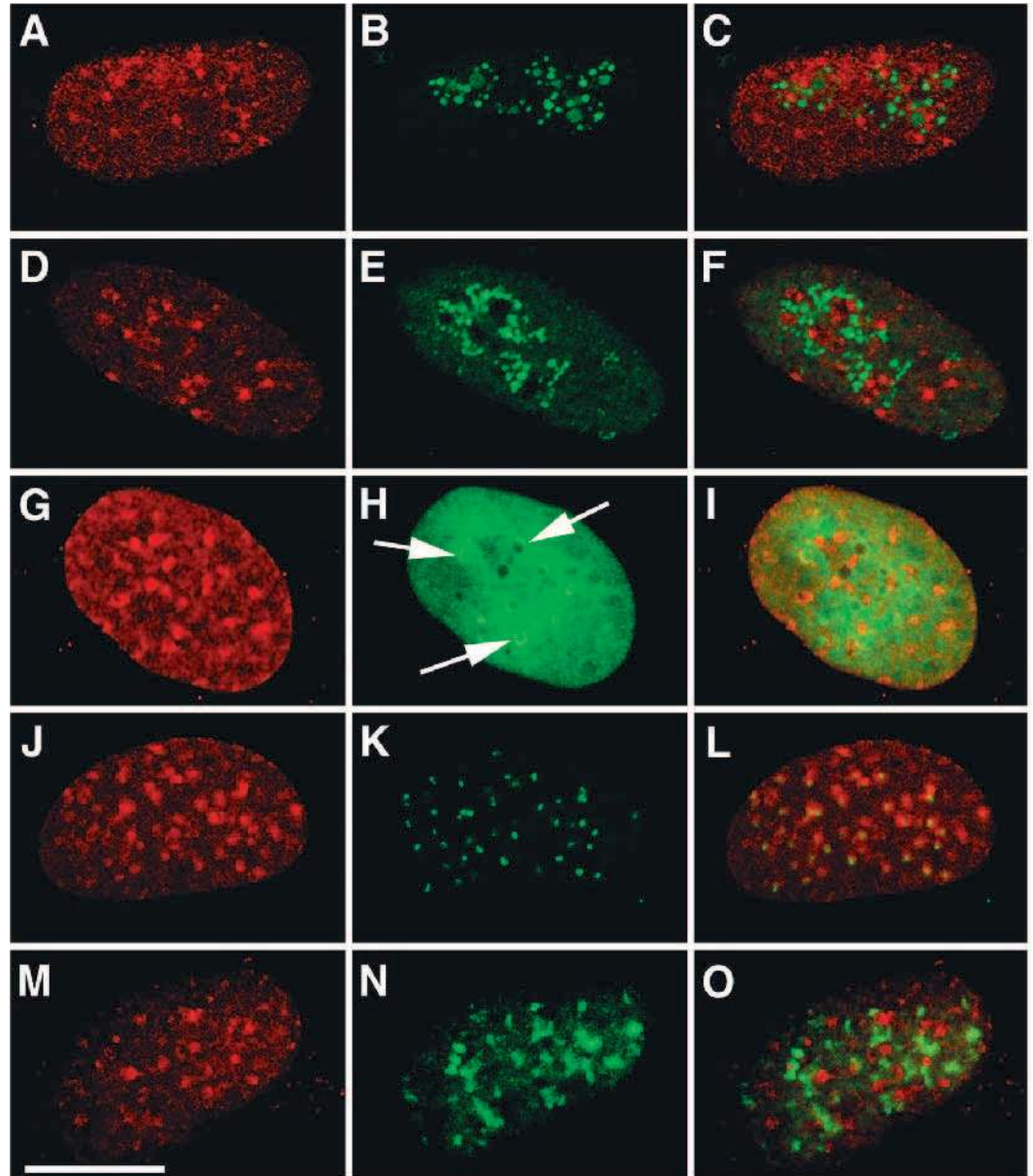


Fig. 4. CLSM analysis of HDF in G₁ co-stained with Ki-67 and other anti-nuclear antigen antibodies. Confocal mid-sections through HDF co-stained with Ki-67 (A,D,G,J,M) and anti-fibrillarin (B); anti-RNA pol I (E); anti-B23/nucleophosmin (H); anti-centromere (K) or anti-RNP (N). (C,F,I,L,O) Corresponding merged images. In some cases AAC15 antibodies are adjacent to and overlap regions of Ki-67 staining (arrows in L). Bar, 10 μ m.

Fig. 5. CLSM analysis of HDF treated with DRB co-stained with Ki-67 and other anti-nuclear antigen antibodies. Confocal mid-sections of HDF treated with DRB for 6 hours then co-stained with Ki-67 (A,D,G,J,M) and anti-fibrillarin (B); anti-RNA pol I (E); anti-B23/nucleophosmin (H); anti-centromere (K) or anti-RNP (N). Arrows in H indicate stronger staining of NPM antibodies at a small number of circular structures. (C,F,I,L,O) Corresponding merged images. Bar, 10 μ m.



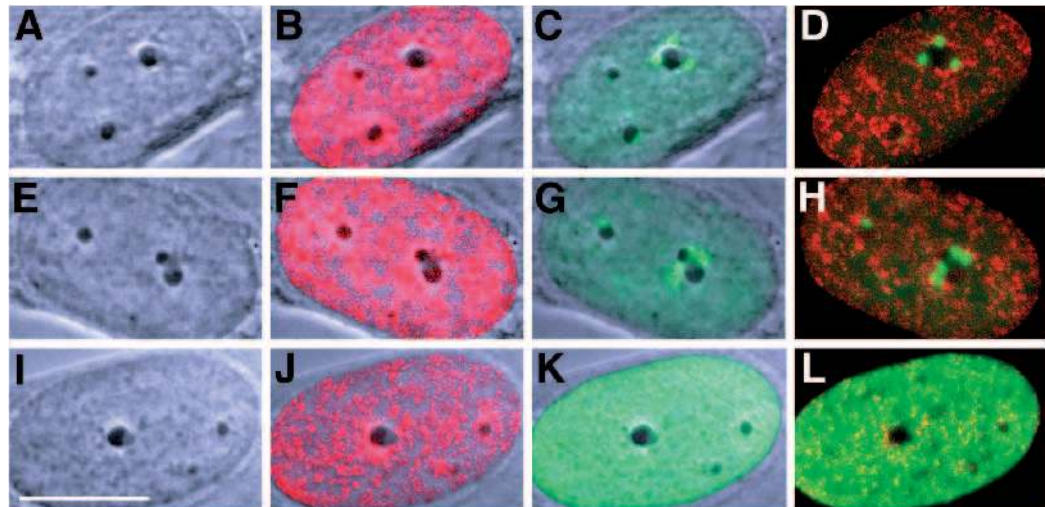
regions within the circular structures stained with NPM antibodies, although in some cases Ki-67 staining is associated with and bridges these structures.

In DRB-treated cells, AAC15 antibodies are distributed at a large number of focal sites, reminiscent of the staining pattern observed in cells in all phases of interphase (Fig. 5K). However, the merged image reveals that in every case, foci of AAC15 antibody staining are associated with, and frequently co-localise to, clumps of Ki-67 antibody staining (Fig. 5L). Z-series optical sectioning through these nuclei reveals that the association between pKi-67 and centromeric regions is present in serial sections (data not shown). Thus the association of pKi-67 with centromeric regions is not an artifact due to the optical resolution of the confocal microscope. AAC22 staining is present within a large number of clumped regions with little or no nucleoplasmic staining (Fig. 5N). The merged image reveals that regions of AAC22 staining exclude regions of Ki-67 staining (Fig. 5O).

Distribution of pKi-67 and other nucleolar antigens following nucleolar segregation induced by treatment of cells with AMD

Treatment of cells with AMD results in segregation of the nucleolar fibrillar component from the nucleolar granular component (Reimer et al., 1986). Under these conditions, pKi-67 translocates to nucleoplasmic sites, although it is not clear whether or not pKi-67 remains associated with either developed sub-compartment (Verheijen et al., 1989a). To compare the localisation of pKi-67 with other nucleolar antigens following AMD-induced nucleolar segregation, HDF were treated with AMD for 4 hours then co-stained with Ki-67 and each of the three anti-nucleolar antigens (Fig. 6A-L). Phase contrast images reveal the distribution of the segregated nucleoli. Clearly both fibrillarin and pol I remain closely associated with segregated nucleoli (Fig. 6C,G) whilst pKi-67 is much more widely dispersed (Fig. 6B,F,J). The merged images of either Ki-67 with fibrillarin or Ki-67 with pol I reveals that

Fig. 6. Phase contrast and CLSM analysis of HDF treated with AMD co-stained with Ki-67 and other anti-nucleolar antibodies. Phase contrast images of HDF treated with AMD for 4 hours (A,E,I). Superimposition of corresponding confocal mid-sections showing Ki-67 staining (B,F,J) and anti-fibrillarin (C), anti-pol I (G) and anti-B23/nucleophosmin (K). (D,H,L) Corresponding merged images. Bar, 10 μ m.



Ki-67 is excluded from regions of the segregated nucleolus containing markers of the fibrillar components (Fig. 6D,H). The distribution of NPM within AMD-treated cells is quite diffuse although staining is excluded from phase-dense structures of segregated nucleoli (compare Fig. 6I with Fig. 6K). The merged image of Ki-67 and NPM co-staining provides little information regarding the specific co-localisation of the two antibodies (Fig. 6L), although treatment of AMD-treated cells with 0.1% (v/v) Triton X-100 for 5 minutes prior to fixation and staining reveals that nucleophosmin is largely soluble whereas pKi-67 is insoluble (data not shown).

Relative distribution of B23, fibrillarin and RNA pol I within the nucleolus

Using immuno-electron microscopy, Verheijen et al. (1989a) have localised pKi-67 within the fibrillar region, possibly the DFC. However, co-staining interphasic cells with Ki-67 and each of the three nucleolar antigens has revealed that the dis-

tribution of pKi-67 differs substantially from that of B23, fibrillarin and RNA pol I. Since, though, it is well established that fibrillarin is localised within the DFC (Ochs et al., 1985b), then it follows that pKi-67 is predominantly localised within a fibrillarin-deficient region of the DFC. Therefore, we predicted that there would be clear definition between regions stained by anti-B23 antibodies and regions stained with either anti-fibrillarin antibodies or with anti-RNA pol I antibodies. Furthermore, we predicted that there would be a close association between regions stained with anti-fibrillarin antibodies and regions stained with anti-RNA pol I antibodies. To test these predictions we co-stained cells with combinations of antibodies to reveal the relative distributions of B23/fibrillarin, B23/RNA pol I and fibrillarin/RNA pol I (Fig. 7).

The distributions of fibrillarin (Fig. 7A) and RNA pol I (Fig. 7B) are very closely aligned as revealed by the merged image (Fig. 7C). Co-staining cells with anti-B23 (Fig. 7D) and anti-RNA pol I (Fig. 7E) reveals that all of the anti-RNA pol I

Fig. 7. Relative distributions of B23, fibrillarin and RNA pol I within interphasic cells. Confocal images of HDF co-stained with: anti-RNA pol I (A) and anti-fibrillarin (B); anti-B23 (D) and anti-RNA pol I (E); anti-B23 (G) and anti-fibrillarin (H). (C,F,I) Corresponding merged images. Arrows in F indicate discrete regions in which staining with either antibody is absent or at very low levels (I) Co-stained nucleoli show regions displaying little or no staining with either antibody (small arrows). Anti-fibrillarin staining is not restricted within the nucleolar border defined by anti-B23 staining (large arrows). Bar, 5 μ m.

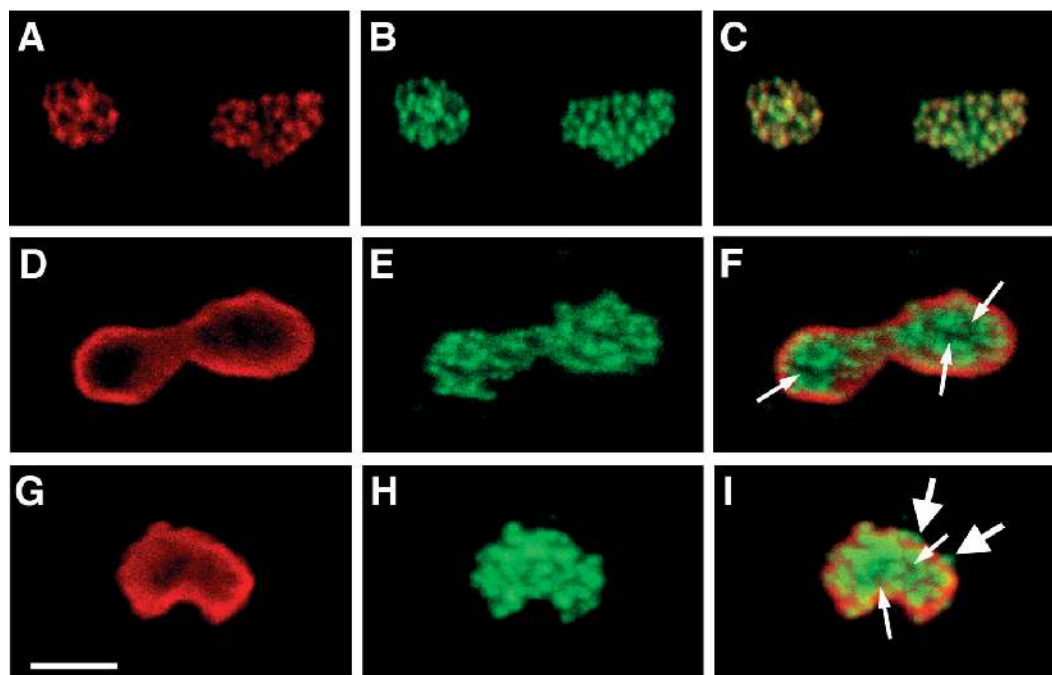
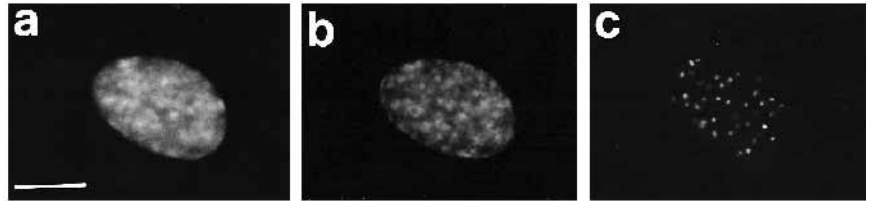


Fig. 8. Distribution of Ki-67, centromeric antigens and DNA in HDF treated with DRB. Photomicrographs of HDF stained with DAPI to reveal the distribution of DNA (A) Ki-67 (B) and AAC 15 to reveal the distribution of centromeric antigens (C). Bar, 10 μ m.



staining is contained within the nucleolar boundary marked by anti-B23 staining. Furthermore, the merged image reveals discrete regions in which staining with either antibody is absent or at very low levels (Fig. 7F, arrows). Similarly, co-staining nucleoli with anti-B23 (Fig. 7G) and anti-fibrillarin (Fig. 7H) reveals regions within the nucleolus displaying little or no staining with either antibody (Fig. 7I, small arrows). However, in contrast to anti-RNA pol I staining anti-fibrillarin staining is not restricted within the nucleolar border defined by anti-B23 staining (Fig. 7I, large arrows).

Distribution of the Ki-67 antigen compared with the distribution of DNA and centromeric antigens in cells treated with DRB

We wished to determine the relationship between the distribution of pKi-67, centromeric regions and regions of condensed DNA. HDF in culture were treated with DRB for 6 hours then prepared for immunofluorescence staining as before using Ki-67 antibodies and AAC 15 and then counterstained with DAPI to reveal DNA (Fig. 8). The distribution of Ki-67 staining closely resembles the distribution of regions of DNA densely stained with DAPI (Fig. 8B). Centromeric antigens also appear to be closely associated with regions of DNA densely stained with DAPI, presumably centromeric heterochromatin (Fig. 8C). As expected, regions stained with AAC 15 antibodies are associated with regions stained with Ki-67 antibodies.

DISCUSSION

We have examined the distribution of pKi-67 in normal HDF during interphase. We have observed two distinct patterns of Ki-67 staining within HDF. Pattern type I (speckled, nucleoplasmic) is restricted to G₁ cells, revealed by co-staining with anti-lamin A antibodies, whilst pattern type II (nucleolar) is predominant and includes all S-phase cells revealed by co-staining with anti-PCNA antibodies. Ki-67-staining of synchronised cultures demonstrates that pattern type I is predominant during early G₁ and persists for up to 6 to 7 hours following release from metaphase arrest, the time at which the maximum level of pattern type I begins to decline. Similar observations have been reported previously using image cytometry, but did not attribute specific timing to the process of pKi-67 redistribution from nucleoplasmic to nucleolar localisation (du Manoir et al., 1991). Furthermore, similar patterns of redistribution of pKi-67 have been described previously in the human breast cancer cell line MCF-7 (van Dierendonck et al., 1989). However, in these cells, the intensity of Ki-67 staining was shown to be much reduced in early S-phase cells. Clearly in normal HDF, Ki-67-staining in early S-phase cells displays no such decrease in intensity. This apparent discrepancy may reflect differences between normal and transformed

cells. However, it should be noted that van Dierendonck et al. used a mouse monoclonal Ki-67 antibody together with immunoenzymatic detection methods for their study which has been cogently criticised previously (du Manoir et al., 1991). We have used a new rabbit polyclonal anti-Ki-67 antigen antibody together with indirect immunofluorescence detection.

From early to mid-G₁, pKi-67 is detected within focal sites throughout reforming nuclei. Co-staining of these cells with anti-nucleolar antibodies reveals that many Ki-67 Ag-rich foci are associated with, but not necessarily co-localised to, sites containing nucleolar antigens. In cells co-stained with S4 and S18, many pKi-67-rich foci are segregated and distant from newly-formed regions of nucleolar reassembly. Co-staining G₁ cells with Ki-67 and NPM antibodies reveals a closer association between the two antigens. Indeed, there is a similarity between the distribution of pKi-67-rich foci and foci stained with NPM antibodies within the nucleoplasm, although NPM staining within these regions is only weak. Furthermore, pKi-67 appears to link regions stained most intensely with NPM antibodies. Clearly then, pKi-67 is localised within reforming nucleoli during G₁. This observation suggests that it is not necessary to complete nucleolar reassembly in order for pKi-67 to associate with presumptive nucleolar regions.

Compartmentalisation of proteins within discrete nucleolar domains reflects compartmentalisation of nucleolar metabolism. Although it is widely accepted that GC are the sites of maturation and storage of pre-ribosomal particles, it is still unclear where in the nucleolus transcription of ribosomal genes takes place. Nascent rRNA transcripts labelled with either ³H-uridine or Br-UTP are localised within both FC and DFC (reviewed by Hozák, 1995). Immunofluorescence data reveals

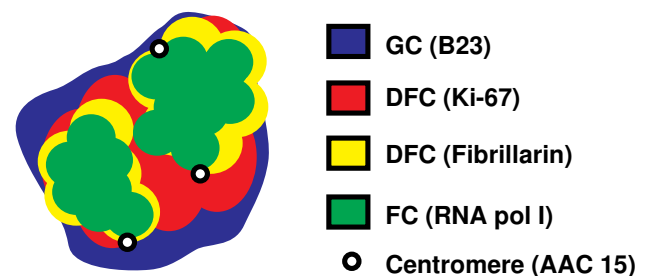


Fig. 9. Proposed scheme for nucleolar architecture. Relative localisation of nucleolar domains deduced from indirect immunofluorescence staining (not to scale). Fibrillar centres (FC), revealed by anti-pol I staining, are surrounded by the dense fibrillar components (DFC), revealed by anti-fibrillarin staining. The fibrillar components are flanked by regions of the DFC containing pKi-67 which are deficient in fibrillarin. The FC and DFC are embedded within the granular component (GC) revealed by anti-B23/nucleophosmin staining. Centromeres may be associated with nucleoli, predominantly within the DFC, revealed by AAC 15 staining.

that RNA polymerase I (pol I) is localised predominantly within the FC (Scheer and Rose, 1984), although ultrastructural studies have localised pol I also within the DFC (Raska et al., 1989). Recently it has been suggested that transcription of ribosomal genes takes place within the DFC and at the borders of the DFC and FC (Hozák, 1995). Where then, within mature nucleoli, is pKi-67 localised? Our results using double indirect immunofluorescence have revealed that within mature nucleoli, pKi-67 is localised predominantly within regions which do not contain fibrillarin, pol I or nucleophosmin/B23. We have observed that in some regions of the nucleolus, pKi-67 co-localises with nucleophosmin/B23 and fibrillarin. However, co-localisation of the respective antigens is at the borders of domains showing exclusive staining, indicating that nucleolar domains do overlap (Hozák, 1995). Previous studies using immunoelectron microscopy have localised pKi-67 within the fibrillar components of nucleoli, possibly within the DFC (Verheijen et al., 1989a). If this is the case then we conclude that pKi-67 localises to regions of the DFC which do not contain fibrillarin. Indeed, co-staining cells with anti-B23 and anti-fibrillarin antibodies reveals unstained regions between the two domains. By deduction, the unstained regions probably contain pKi-67. To our knowledge, this is the first report of fibrillarin-deficient regions of the DFC. This is shown diagrammatically in Fig. 9. We are currently seeking to confirm this observation at the ultrastructural level using dual label immunoelectron microscopy. The suggestion that the DFC may contain different functional zones has been made previously (Hozák et al., 1993, 1994), based upon ultrastructural mapping of nucleolar transcription and rDNA localization.

Even under conditions which result in disruption to nucleolar integrity, (treatment with AMD or DRB) pKi-67 localises to regions deficient in both fibrillarin and pol I. Curiously, under these conditions, pKi-67 co-localises to sites containing centromeric antigens. Association of centromeric antigens with nucleoli have been described previously although it is unclear whether or not this association reflects a role for centromeric antigens in nucleolar function (Ochs and Press, 1992). The authors suggested that centromeric antigens associate with nucleoli due to the proximity of centromeric DNA to regions of DNA which form part of nucleolar structure. We have found a reasonable correlation between the distribution of dense regions of chromatin revealed by DAPI staining and regions stained with both Ki-67 antibodies and anti-centromere antibodies. Perhaps, following release from nucleoli induced by DRB-treatment, pKi-67 associates with regions containing dense DNA, presumably heterochromatin. The association of pKi-67 with centromeric antigens can thus be explained by supposing that both antigens associate with centromeric heterochromatin. That pKi-67 has DNA-binding properties is supported by two further observations. Firstly, pKi-67 binds to chromosomes throughout mitosis (Verheijen et al., 1989b). Secondly, Ki-67 antibodies are able to bind pKi-67 in cellular extracts digested with Dnase I. However, Ki-67 antibody binding to pKi-67 is enhanced in the presence of undegraded double-stranded DNA (Lopez et al., 1994). It has been suggested that dsDNA modulates the conformation of pKi-67, which is then more reactive with Ki-67 antibodies. Recent evidence shows that the murine homologue of pKi-67

accumulates within both nucleolar and heterochromatic regions of interphase cells (Starborg et al., 1996).

Co-staining cells with Ki-67 and anti-RNP antibodies (AAC22) reveals that throughout interphase, regions staining with antibodies from each source are mutually exclusive. DRB-treatment of cells apparently causes aggregation of sites of AAC22 staining demonstrated by loss of nucleoplasmic staining. Re-organisation of RNP-containing domains in cells treated with transcriptional inhibitors (Lamond and Carmo-Fonesca, 1993) or by heat shock (Carmo-Fonesca et al., 1993) have been described previously. Even in DRB-treated cells, regions of Ki-67 and AAC22 staining are mutually exclusive demonstrating that higher-order nuclear structure is maintained in the presence of nucleolar disruption.

What then is the possible function of pKi-67? Sawhney and Hall (1992) have proposed that pKi-67 is a structural protein which maintains higher order structure of DNA during mitosis. Yasuda and Maul (1990) have proposed that certain nucleolar proteins, including pKi-67, which relocate to the mitotic chromosomal surface, the so-called perichromosomal layer, may protect the surfaces of chromosomes during mitosis. Alternatively, Chatterjee et al. (1987) have proposed that proliferation-associated nucleolar antigens may modulate nucleolar structure to favour high rates of ribosomal synthesis. pKi-67 is not required for nucleolar function per se since Ki-67 negative cells, for example senescent HDF, are viable. Furthermore, pKi-67 is not required for nucleolar assembly since serum-starved, quiescent HDF are able to support nucleolar reassembly following removal of DRB from the culture medium (I. R. Kill, unpublished data). Perhaps pKi-67 performs a number of functions within proliferating cells, maintaining nucleolar structure during interphase and chromosome structure during mitosis?

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