

Lamin proteins form an internal nucleoskeleton as well as a peripheral lamina in human cells

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SUMMARY

The nuclear lamina forms a protein mesh that underlies the nuclear membrane. In most mammalian cells it contains the intermediate filament proteins, lamins A, B and C. As their name indicates, lamins are generally thought to be confined to the nuclear periphery. We now show that they also form part of a diffuse skeleton that ramifies throughout the interior of the nucleus. Unlike their peripheral counterparts, these internal lamins are buried in dense chromatin and so are inaccessible to antibodies, but acces-

sibility can be increased by removing chromatin. Knobs and nodes on an internal skeleton can then be immunolabelled using fluorescein- or gold-conjugated anti-lamin A antibodies. These results suggest that the lamins are misnamed as they are also found internally.

Key words: cell nucleus, immunoelectron microscopy, lamina, nuclear matrix

INTRODUCTION

The nuclear lamina is a protein mesh underlying the nuclear membrane that remains associated with the residual nuclear envelope after extraction with non-ionic detergents and high concentrations of salt (Newport and Forbes, 1987; Gerace and Burke, 1988). In most mammalian cells, it is composed of the intermediate filament proteins, lamins A, B and C (Steinert and Roop, 1988). Despite its well-characterized peripheral location, lamins (and/or other intermediate filaments) have occasionally been found internally within nuclei; for example, during G₁ or S-phase, in certain pathological states, when mutated, or when overexpressed (e.g. Cardenas et al., 1990; Gill et al., 1990; Bader et al., 1991; Beven et al., 1991; Kitten and Nigg, 1991; Eckelt et al., 1992; Goldman et al., 1992; Lutz et al., 1992; Mirzayan et al., 1992; Bridger et al., 1993; Moir et al., 1994). Moreover, an intermediate-filament-like skeleton is seen in chromatin-depleted nuclei prepared using conditions close to the physiological in cells from all stages of the cycle (Jackson and Cook, 1988; Hozák et al., 1994). Lamins have also been detected within nuclear matrices (Luderus et al., 1992; Minguez and Moreno Diaz de la Espina, 1993; Mancini et al., 1994). But despite these reports, lamins, as their name suggests, are normally considered to be confined to the periphery (e.g. Stick and Hausen, 1980; Gerace et al., 1987; Gerace and Burke, 1988).

We now show that lamins also form part of a diffuse

skeleton that ramifies throughout the interior of human nuclei. Visualization of such a skeleton posed several problems. First, chromatin is so dense that it prevents access of the antibodies used for immunolabelling to any underlying skeleton; indeed, the peripheral lamin mesh is closely associated with aligned chromatin fibres (Paddy et al., 1990; Belmont et al., 1993) and internal lamins were only detected in G₁ cells after long exposures to antibodies when, presumably, they had time to penetrate into the dense chromatin (Bridger et al., 1993). Second, diffuse skeletons are visualized in the electron microscope with difficulty in the thin sections of <100 nm normally used for immunolabelling. Third, the question of whether an internal nucleoskeleton exists has a long and controversial history; skeletons seen in vitro might be artifacts generated by the unphysiological conditions used during isolation (Cook, 1988).

We minimize these problems as follows. Cells are encapsulated in agarose microbeads (diameter 25-150 µm) before cell membranes are permeabilized with Triton X-100 in a 'physiological' buffer; encapsulation protects the fragile cell contents during subsequent manipulations. Access of antibodies to an underlying skeleton is improved by removing most of the chromatin by cutting the chromatin fibre with restriction endonucleases and then removing fragments unattached to the skeleton by electrophoresis in the physiological buffer. As, under optimal conditions, such permeabilized and eluted cells synthesize RNA and DNA at in vivo rates (Jackson et al.,

1988), it seems unlikely that many nuclear components have been rearranged artifactually. We then use thick resinless sections for electron microscopy (He et al., 1990) to improve detection of diffuse skeletons.

MATERIALS AND METHODS

General procedures

Suspension cultures of HeLa cells were grown, labelled with [*methy*-³H]thymidine, encapsulated, lysed with 0.2% Triton X-100 (two 5 minute treatments) in ice-cold physiological buffer (PB), washed in PB and ~90% of the chromatin was removed by treatment with *Eco*RI + *Hae*III followed by electrophoresis as described by Hozák et al. (1993). HeLa cells in G₂ phase were also collected 20 hours after release of a nitrous oxide block used to accumulate cells in mitosis (Hozák et al., 1993), but cells were unsynchronized unless stated otherwise. Human epithelial HEp-2 cells (ATCC CCL23) were grown in MEM medium in the presence of 10% (v/v) heat-inactivated foetal bovine serum and 2 mM glutamine.

Antibodies

The monoclonal antibody 133A2 was obtained from the fusion of spleen cells from a mouse immunized with partially purified recombinant human lamin A, expressed from a pUC9 vector (McKeon et al., 1986), with mouse myeloma cells (Raymond and Gagnon, 1988). 133A2 reacted with no proteins other than the various forms of lamin A in whole-cell lysates of 10⁶ HEp-2 cells, as judged by immunoblotting (conditions described below) of two-dimensional gels (not shown).

Primary antibodies (used at 1.0 and 5-10 µg/ml for light and electron microscopy, respectively): anti-lamin A (clone 133A2, a mouse IgG3 kappa monoclonal antibody; see above); anti-lamin A/C (clone 1E4, a mouse monoclonal antibody; Loewinger and McKeon, 1988); anti-lamin A/C (clone L6.8A7; this monoclonal antibody was raised against *Xenopus* lamin III but recognizes human lamins A/C; Stick and Hausen, 1985; Bridger et al., 1993); anti-lamin B2 (clone LN43, a mouse IgG1; Bridger et al., 1993); anti-hnRNP C1/C2 monoclonal antibody (clone 4F4; Choi and Dreyfuss, 1984); anti-hnRNP A1 monoclonal antibody (clone 9H10, a gift from Dr G Dreyfuss, which is similar to clone 4B10; Pinol-Roma et al., 1988); anti-vimentin and anti-cytokeratin monoclonal antibodies (Amersham); monoclonal antibody that recognizes most intermediate filaments (TIB-131; Pruss et al., 1981); anti-DNA topoisomerase II (serum BS, a human autoantibody from Dr Earnshaw), anti-coilin (a monoclonal antibody from Dr Carmo-Fonseca), anti-Nopp 140 (Meier and Blobel, 1992).

Secondary antibodies: goat anti-mouse IgG, goat anti-rabbit or goat anti-human IgG, conjugated with FITC (Amersham; 1:400 dilution) or with 5 nm gold particles, (BioCell; human proteins absorbed 1:50 dilution).

Electrophoresis and immunoblotting

SDS-PAGE was performed as described by Laemmli (1970) or using Tricine (Sigma) as the trailing ion for low molecular mass proteins (Schägger and von Jagow, 1987). Conditions for electrophoretic transfer of proteins on to nitrocellulose sheets and immunodetection were as described (Raymond and Gagnon, 1988) except that peroxidase-conjugated anti-mouse immunoglobulins were used instead of biotin and avidin conjugates.

Preparation and purification of recombinant lamins

Full-length cDNA for human lamin A (McKeon et al., 1986), a generous gift from Dr F. McKeon (Harvard Medical School), was expressed from a pET vector (Novagen) in BL21(DE3) pLysS bacteria after IPTG induction. Truncated lamin A expression vectors

(except for NΔ463) were constructed using the polymerase chain reaction and subcloning into the TA cloning vector (Invitrogen). All constructs were verified by manual or automated (Pharmacia) DNA sequencing and inserted into the pET vector for expression. Full-length lamin A, carboxyl terminus deletion mutants and the (NΔ463) mutant were purified by preparative isoelectric focusing and continuous elution from SDS-PAGE gels (Gagnon et al., 1992). Alternatively, the second purification step was performed on regular slab gels from which lamin bands were excised and lamins recovered by electroelution. Lamin A (NΔ562) was purified by metal chelate affinity chromatography and electroelution from Tricine-SDS-PAGE gels (to be described in detail elsewhere by A.M.-J.S. and Y.R.).

Immunofluorescence

Cells were encapsulated in agarose beads, permeabilized, and then most of the chromatin was removed from half the sample by nuclease treatment followed by electroelution. Cells were then fixed (20 minutes; 4°C) in 4% paraformaldehyde in PB, washed 4× in PB, incubated (1 hour; room temperature) with primary antibody in PBS supplemented with 0.02% Tween-20 (PBT), washed 4× in PBT, incubated (1 hour at room temperature) with secondary antibody, washed 4× in PBT and beads were mounted under coverslips in Vectashield (Vector). Photographs were taken using a Zeiss Axiophot microscope. Alternatively, cells grown on coverslips were washed twice with PBS, fixed for 5 minutes in methanol followed by 10 minutes in acetone, both at -20°C. Indirect immunofluorescence and adsorption experiments using lamins in suspension were performed as described (Collard et al., 1992).

Preembedding immunolabelling and resinless sections

Encapsulated and eluted cells were lightly fixed (10 minutes; 0°C) with 0.1% glutaraldehyde or 1% paraformaldehyde in PB, washed 2× in 0.1 M Na/K phosphate Sørensen buffer (SB; pH 7.4), incubated (10 minutes) in 0.02 M glycine in SB, and rewashed in SB. After incubation (20 minutes; 20°C) with 5% normal goat serum in TBT buffer (20 mM Tris, 150 mM NaCl, 0.1% bovine serum albumin, 0.005% Tween-20, 20 mM NaN₃; pH 8.2), cells were incubated (1 hour; 20°C) with primary antibody in TBT, washed 3× in TBT and incubated (1 hour) with the gold-conjugated secondary antibody (5 nm particles; 50× dilution in TBT + 0.5% fish gelatine; BioCell). After washing 2× in TBT and once in SB, cells were fixed (20 minutes) in 2.5% glutaraldehyde in SB, washed 3× in SB, postfixed with 0.5% OsO₄ in SB, and dehydrated through an ethanol series (including 30 minute incubation in 2% uranyl acetate in 70% ethanol). Ethanol was replaced in three steps by *n*-butanol and samples were then embedded in the removable compound, diethylene glycol distearate (DGD; Poly-science) and resinless sections prepared (Fey et al., 1986; Hozák et al., 1993); beads were immersed in a DGD/*n*-butanol mixture at 60°C, impregnated with pure DGD, blocks hardened, 500 nm thick sections cut using a diamond knife and placed on Pioloform-coated grids (Agar; grids preincubated with poly-L-lysine in water), DGD removed (3× 1.5 hour incubations in *n*-butanol; 20°C), butanol replaced by acetone, and specimens critical-point dried. Sections were observed in a Jeol 100CX electron microscope (accelerating voltage 80 kV). Nuclear morphology was also observed in HeLa sections prepared without immunolabelling after Triton-permeabilization, digestion, electroelution or embedding in DGD.

Postembedding immunoelectron microscopy

Intact unencapsulated HeLa cells in G₂ phase were pelleted and fixed (20 minutes; 0°C) in 3% paraformaldehyde in PB. After washing in SB (including 10 minute incubation in 0.02 M glycine in SB), cells were dehydrated in ice-cold ethanol, embedded in LR White (polymerization 24 hours at 50°C) and ultrathin sections on gilded copper grids were immunolabelled. Unspecific binding was blocked by preincubation (30 minutes) with a 10% normal goat serum in PBS with 0.1% Tween 20 and 1% BSA (PBTB buffer; pH 7.4), sections were

incubated (40 minutes) with primary antibodies in PBTB, washed in PBT, incubated (40 minutes) with gold-conjugated secondary antibody in PBTB (pH 8.2), rewashed in PBT and contrasted with a saturated solution of uranyl acetate in water (3 minutes).

Statistical significance was assessed using Student's *t*-test. Three photographs were taken of the nuclear region and three of the cytoplasmic region of 30 different cells; the number of gold particles per μm^2 was determined and compared with controls in which the primary antibody was omitted.

Testing the stability of the nucleoskeleton

After removal of most of the chromatin from encapsulated HeLa cells by electrophoresis, beads were incubated (20 minutes; 33°C) in PB supplemented with and without DNase I (RNase-free; 6 or 70 U/ml; Boehringer) or RNase (DNase-free, 25 U/ml; Boehringer) in the presence of protease inhibitors (pepstatin 1 $\mu\text{g}/\text{ml}$; 1 mM PMSF; leupeptin 10 $\mu\text{g}/\text{ml}$).

RESULTS

Characterizing monoclonal antibody 133A2

We first characterized a monoclonal antibody (i.e. 133A2) raised against human lamin A. As 133A2 reacted against lamin A but not lamin C, we concluded that it recognized an epitope lying within the 98 amino acid C terminus that is unique to lamin A, rather than one in the N-terminal 566 amino acids common to both lamins A and C (McKeon et al., 1986; Fisher et al., 1986). Various deletion mutants of lamin A were constructed (Fig. 1A), expressed in bacteria, purified and tested by immunoblotting to see if they were recognized by 133A2. Two-thirds of the N terminus (Fig. 1B) could be deleted without affecting reactivity but analysis of C-terminal deletions showed that amino acids 598-611 were essential for reactivity (Fig. 1C).

Removing chromatin improves detection of internal lamins by immunofluorescence

Fig. 2A illustrates a photomicrograph of an encapsulated HeLa cell that has been indirectly immunolabelled using this anti-lamin A antibody. (Unsynchronized populations were used unless stated otherwise.) The peripheral lamina is labelled and there is some weak labelling within nuclei. Removing ~90% chromatin (by nucleolytic treatment and then electrophoresis of detached fragments) has little effect on the peripheral labelling but increases the 'speckly' internal labelling (Fig. 2B); removing chromatin increases accessibility.

An anti-lamin A/C antibody labels the lamina discontinuously and the interior in faint speckles (Fig. 2C); elution again enhances the internal (extra-nucleolar) signal (Fig. 2D). An anti-lamin B2 antibody predominantly labels the periphery (Fig. 2E) but elution has little effect (Fig. 2F). Elution did not increase any intranuclear signal given by antibodies directed against vimentin, DNA topoisomerase II, coilin, hnRNP proteins (i.e. C1/C2 and A1) or Nopp 140 (not shown); therefore, the elution-induced enhancement was specific to lamins A/C.

The specificity shown by monoclonal antibody 133A2 for lamin A was confirmed using the truncated lamin A proteins. The fluorescent signal was eliminated by adding proteins containing amino acids 598-611 (Fig. 3B,C) but proteins lacking this region had no effect (Fig. 3D,E). Furthermore, the

antibody reacted against no proteins other than the various forms of lamin A on immunoblots of whole cell lysates of HEP-2 cells resolved on a two-dimensional gel (see Materials and Methods).

Internal lamins visualized by immunoelectron microscopy

We next investigated the distribution of lamins by electron microscopy. Fig. 4A illustrates a micrograph of a resinless

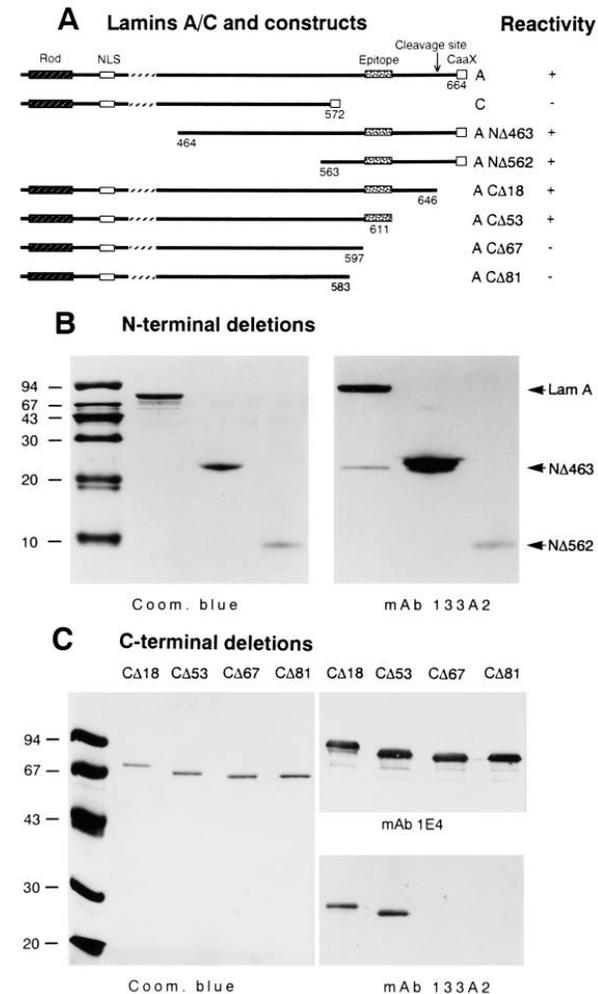


Fig. 1. Mapping the epitope recognized by monoclonal antibody 133A2. (A) Schematic diagrams of lamin A and C (which is identical to lamin A except for 6 amino acids at the C terminus) and of lamin A deletion mutants. The diagrams are not drawn to scale; central regions have been omitted (indicated by hashes) and N termini are shortened to emphasize C termini. Rod domains, nuclear localization signals (NLS), epitopes (residues 598 and 611), proteolytic cleavage sites, CaaX motifs and reactivity with 133A2 are indicated. (B) 133A2 reacts with full-length lamin A and two N-terminal deletion mutants; 2 μg protein per lane was subjected to Tricine-SDS-PAGE and either stained with Coomassie Blue (left) or blotted with 133A2 (right). (C) 133A2 reacts with two of the four C-terminal deletion mutants; 2 μg protein per lane was subjected to SDS-PAGE and either stained with Coomassie Blue (left) or blotted with 1E4 monoclonal antibody reactive against all mutants (upper right) or 133A2 (lower right). Size (kDa) markers: B and C at left.

section (500 nm thick) of a HeLa cell from which ~90% chromatin has been removed. Agarose filaments (a) surround the remnants of the cytoplasm (c). Within the nucleus, residual chromatin is strung along a diffuse nucleoskeleton (ns) that extends from the nucleolus (nu) to the lamina. This diffuse skeleton is associated with the residual ~10% chromatin, dense structures that are the sites of replication and probably sites involved in the transcription and processing of RNA (e.g. Hozák et al., 1993, 1994; Cook, 1994); therefore it is a very complex and ill-defined structure that is best described pictorially. At this stage in the analysis, we use the loose term 'diffuse nucleoskeleton' to describe all of this structure, including its 'core filaments' that we have previously shown have an axial repeat typical of intermediate filaments (Jackson and Cook, 1988; see also He et al., 1990). (In the micrographs presented below, such repeats cannot be seen as the sections have not been shadowed.)

Most intermediate filaments share a common epitope and

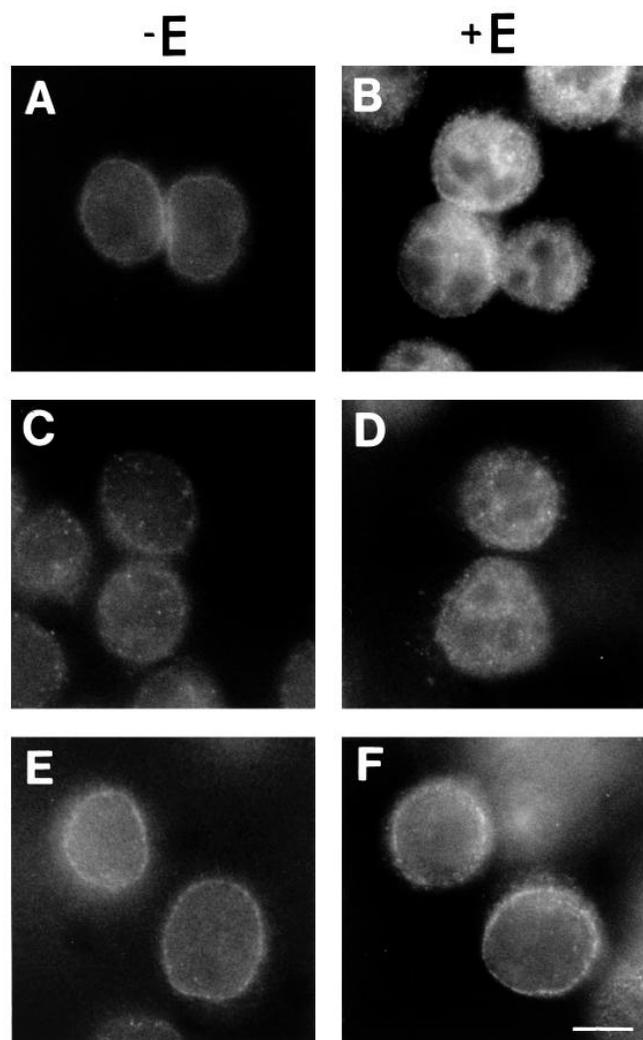


Fig. 2. Removing most of the chromatin improves detection of lamin. (A) Encapsulated HeLa cells were permeabilized and indirectly immunolabelled using antibodies against (A,B) lamin A, (C,D) lamins A/C, and (E,F) lamin B2 (using 133A2, L6.8A7 and LN43, respectively). -E (A,C,E), untreated; +E (B,D,F), treated with *EcoRI* + *HaeIII* and then subjected to electrophoresis to remove ~90% chromatin. Bar, 5 μm .

can be immunolabelled with an antibody directed against it (Pruss et al., 1981; see, however, Reimer et al., 1991), including lamins A,B and C (Lebel and Raymond, 1987); this pan-intermediate-filament antibody does not react with nuclear proteins other than lamins (Belgrader et al., 1991). Immunogold labelling (Fig. 4B,C) shows that this antibody reacts weakly with the 'core' filaments of the vimentin mesh in the cytoplasm (c), and much more strongly at nodes in this mesh (Fig. 4B). It also labels the lamina (l) and the diffuse nucleoskeleton, again weakly at the core filaments but more strongly at the nodes (Fig. 4B,C). The nucleolus (nu) in Fig. 4C is unlabelled, so labelling does not reflect unspecific labelling of high concentrations of protein or nucleic acid.

The reactivity of the pan-intermediate-filament antibody with internal material cannot result from the (accidental) misincorporation of vimentin destined for the cytoplasm into the nucleoskeleton because anti-vimentin antibodies label the cytoplasm, but not the lamina (l) or the interior of the nucleus (Fig. 4D). Although the intensity of this cytoplasmic labelling is higher than that given by the pan-intermediate-filament antibody, nodes in the cytoplasmic mesh are again more strongly labelled than the core filaments.

We next confirmed that the internal nucleoskeleton contained lamins. The anti-lamin A antibody labelled both the lamina (l) and nodes or knobs on the diffuse skeleton (Fig. 5A); neither the cytoplasm (not shown) nor nucleoli (nu) were labelled (Fig. 5A). The anti-lamin B antibody labelled the lamina (l) in all cells but generally the diffuse nucleoskeleton was unlabelled (Fig. 5B); however, a few nuclei contained labelled foci on the diffuse nucleoskeleton (Fig. 5C). These labelling patterns seen by electron microscopy are similar to those seen by light microscopy in samples from which most chromatin had been removed.

Some internal lamin A might be lost during elution or during the removal of resin from the thick sections when material unattached to the grid surface is lost. Therefore, we examined the distribution of lamin A in uneluted samples using conventional thin (LR White) sections (Fig. 5D). Gold particles are found over the lamina (l) and the nucleoplasm (n), but not the cytoplasm (c). As the diffuse nucleoskeleton is not visible in these thin sections (see Introduction), this labelling pattern is consistent with that seen in the thick sections. Statistical analysis of 30 HeLa cells in G₂ phase like those in Fig. 5D (i.e. in thin sections through the centre of nuclei) confirmed the specificity of this nuclear labelling. The densities of gold particles over the nucleoplasm (including the peripheral lamina) and the cytoplasm and over the nucleoplasm in a control sample in which the first antibody was omitted, were 10.3 ± 4.6 , 0.5 ± 0.8 , and 0.4 ± 0.7 per μm^2 , respectively (3626, 176 and 141 particles were counted over an area of $352 \mu\text{m}^2$ in each case and only nucleoplasmic labelling in the experimental sample was significant; $P < 0.001$). Nuclear labelling was split between the interior and the periphery (generously defined as a zone $0.5 \mu\text{m}$ wide), with 7.1 ± 2.9 and 23.9 ± 5.3 particles per μm^2 over the two regions, respectively. (Both values were significant ($P < 0.01$) compared to the cytoplasmic density or the control in which the first antibody was omitted.) As the internal area in a typical nuclear section is at least three times that of the lamina (defined as above), this means that there was about as much label over the interior as there was

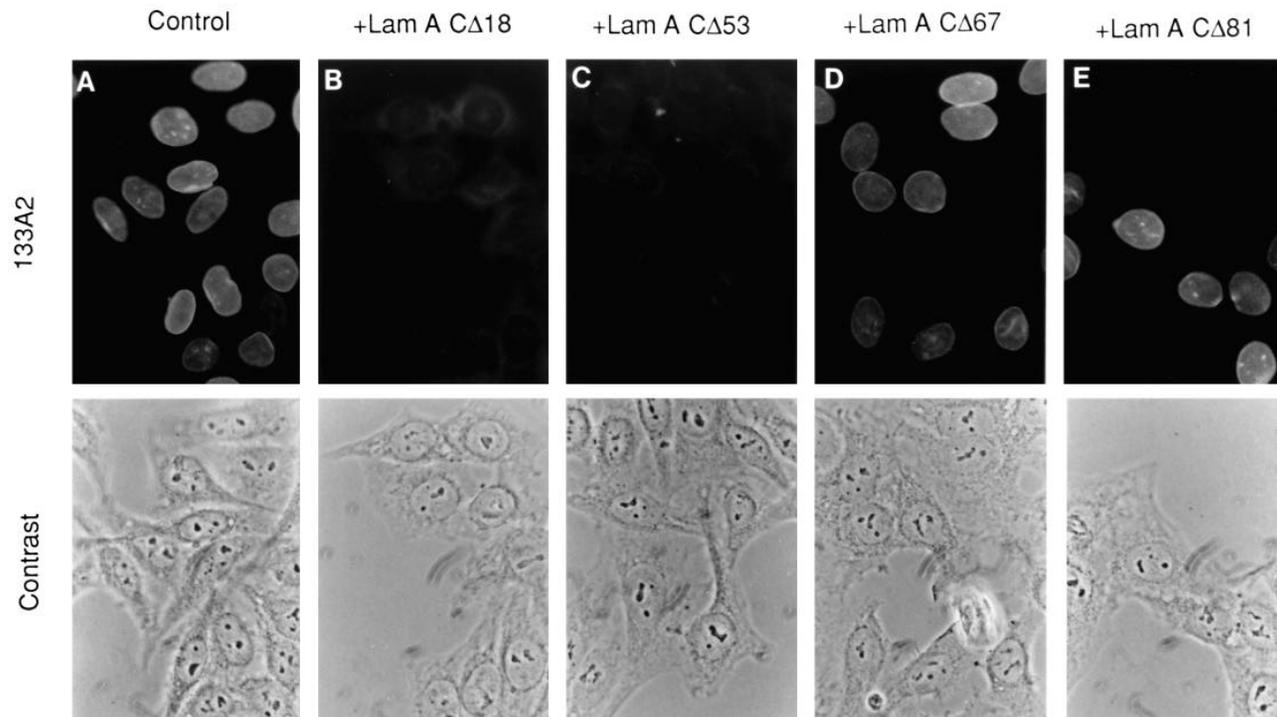


Fig. 3. Specificity of monoclonal antibody 133A2. HEp-2 cells growing on coverslips were permeabilized, indirectly immunolabelled using 133A2 and (top) fluorescence and (bottom) phase-contrast images of the same field collected. 133A2 was preincubated in the absence of lamin A (A) or in the presence of the purified deletion mutants shown (B-E). Bar, 8 μ m.

over the lamina. Note that cells synchronized in G₂ phase were used here, as: (i) it has been suggested that lamins accidentally trapped internally during nuclear re-formation might be lost by then (Bridger et al., 1993); and (ii) lamin B is found internally during S-phase at replication sites (Moir et al., 1994).

Characterization of the internal skeleton

We next investigated the nucleic acid content of the nucleoskeleton. As ~10% chromatin remains associated with the diffuse skeleton after treatment with *EcoRI* + *HaeIII* and elution, it remained formally possible that the skeleton consisted of residual chromatin fibres coated with lamins. Therefore, after removing ~90% chromatin as before, beads were treated with DNase and re-eluted to remove most of the remainder (<2% DNA remained). Despite the harsh treatment, the basic internal skeletal structure remained (Fig. 6A).

There have been many suggestions that the skeleton might be associated with RNA (e.g. Long et al., 1979; Fey et al., 1986), so we also treated chromatin-depleted beads with sufficient RNase to remove ~95% of nascent RNA (Jackson and Cook, 1988). This removed the diffuse skeleton and core filaments (Fig. 6B), consistent with RNA being part of the structure. However, nuclear morphology was so distorted that we are loath to draw any firm conclusions from such experiments; the high concentrations of charged oligonucleotides released during digestion might be expected to induce the kind of precipitation of a fragile skeleton on to the stronger lamina that is seen. Nevertheless, we pursued the idea that the diffuse skeleton might contain RNA using antibodies directed against proteins known to be associated with heterogeneous nuclear RNA (i.e. C1/C2 and A proteins; Choi and Dreyfuss, 1984;

Pinol-Roma et al., 1988). Immunogold labelling showed that C1/2 were localized on the diffuse skeleton, sometimes concentrated in foci (Fig. 6C). The distribution of A was similar, but the signal was weaker (Fig. 6D).

DISCUSSION

Lamins form part of the internal skeleton

A lamin, or lamina, is defined in the Oxford English Dictionary as 'a thin layer of bone, membrane, or other structure'. Although lamin proteins have been found internally within nuclei (see Introduction), this is generally attributed to a transient, accidental, or pathological, location of proteins destined for a peripheral lamina. Our results show that lamin A is also part of a diffuse internal skeleton that ramifies throughout the nucleus from the nucleolus to the periphery. We have previously shown that 'core filaments' in this complex structure have the axial repeat typical of intermediate filaments (Jackson and Cook, 1988). We use the loose term 'diffuse nucleoskeleton' here to include these core filaments plus all associated knobs, nodes and non-chromatin material. This skeleton is also functionally complex; it contains sites involved in transcription, RNA processing and, in S-phase cells, replication (e.g. Hozák et al., 1993, 1994; Cook, 1994).

We removed most obscuring chromatin from nuclei to leave this diffuse nucleoskeleton; then any lamins associated with it were detected using various antibodies, including one that specifically recognizes an epitope in lamin A between amino acids 598 and 611. Immunofluorescence using this specific antibody, as well as another directed against lamins A/C,

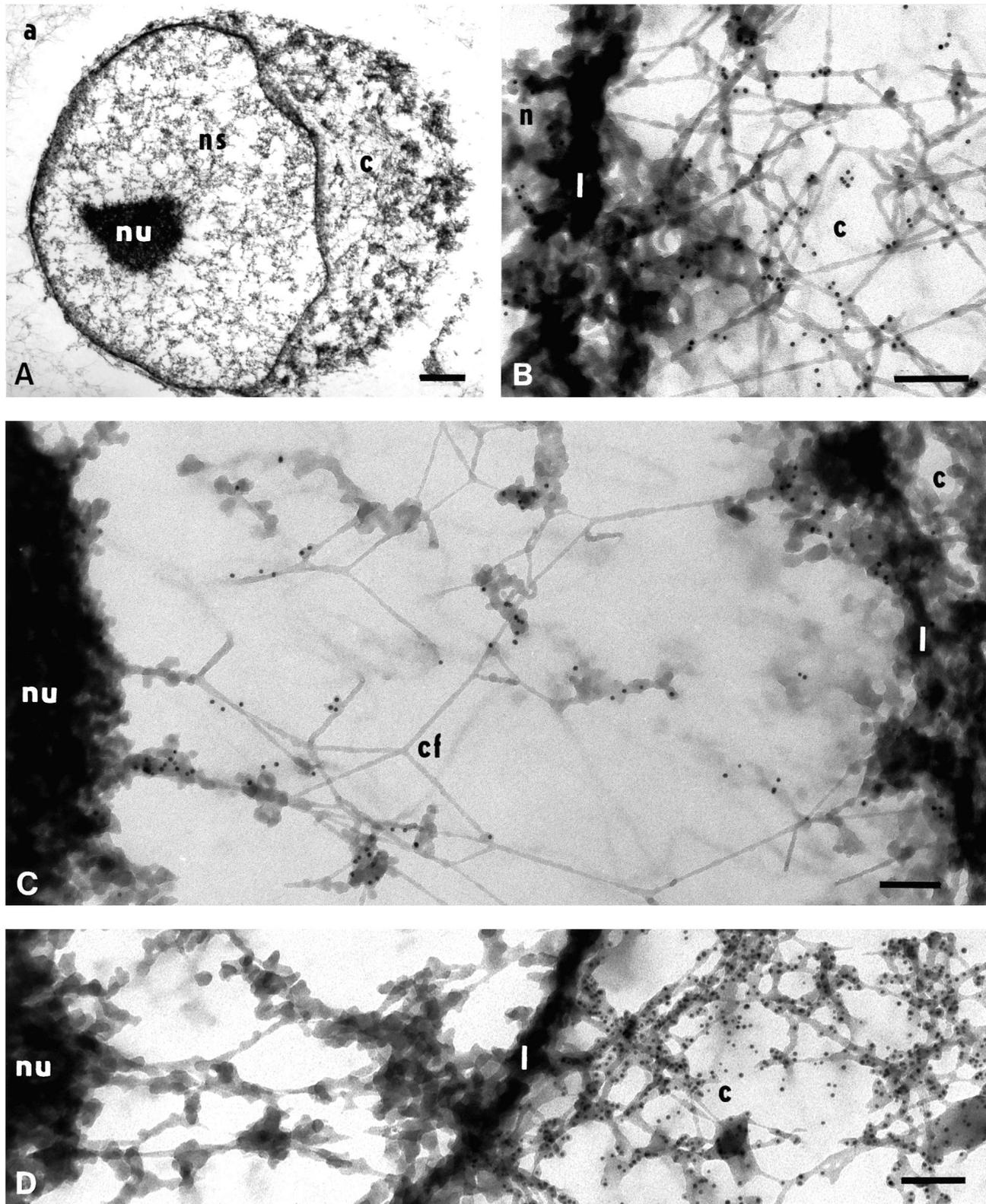


Fig. 4. Visualization of an internal nucleoskeleton in HeLa cells from which ~90% chromatin had been removed. Encapsulated cells were permeabilized, treated with nucleases, chromatin eluted and 500 nm resin-less sections prepared. In B-D, samples were immunolabelled with 5 nm gold particles. a, agarose; c, cytoplasm; nu, nucleolus; ns, nucleoskeleton; n, nucleus; l, lamina; cf, core filaments. Bars: A and B-D, 1 and 0.1 μ m, respectively. (A) Low-power view. (B) Cytoplasmic region; pan-intermediate-filament antibody (i.e. TIB-131). (C) Nuclear region; pan-intermediate-filament antibody; 95% chromatin was removed in this preparation, so more core filaments are visible. (D) Nuclear and cytoplasmic regions; anti-vimentin antibody.

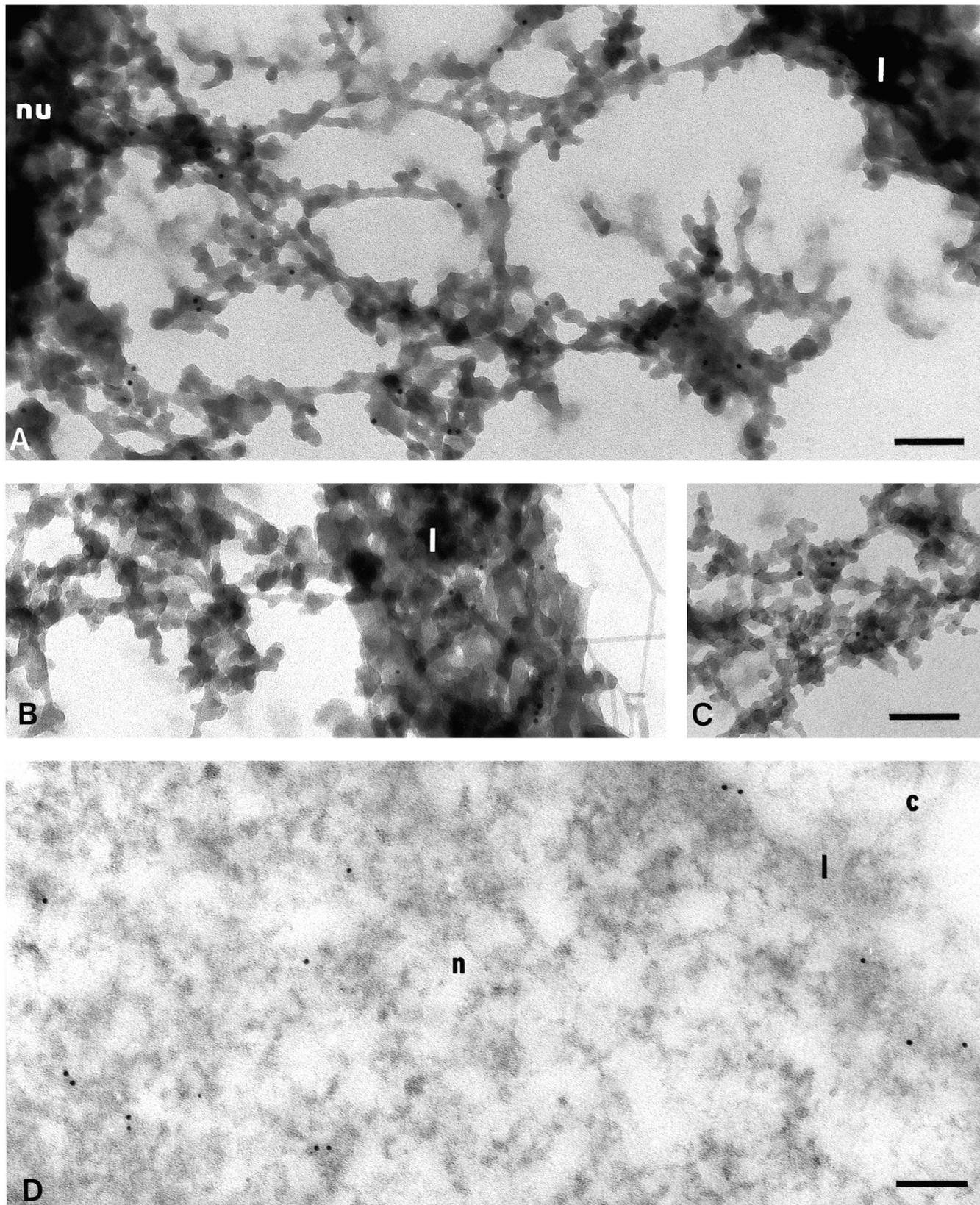


Fig. 5. Lamins detected in nuclear regions of HeLa cells by immunogold labelling in (A-C) resinless sections from which ~90% chromatin has been removed or (D) a conventional thin section prepared without removing any chromatin from cells synchronized in G₂ phase. nu, nucleolus; l, lamina; n, nucleus; c, cytoplasm. Bars: 0.1 μ m. (A) Anti-lamin A (clone 133A2); both lamina and internal skeleton are labelled. (B) Anti-lamin B2 (clone LN43); usually only the lamina is labelled. (C) Anti-lamin B2 (clone LN43); labelling of internal skeleton seen in 10% sections. (D) Anti-lamin A (clone 133A2); vimentin fibrils in the cytoplasm are unlabelled, but both lamina and internal nuclear regions are labelled.

revealed internal 'speckles' in HeLa and HEp-2 cells (Figs 2, 4). Electron microscopy using thick (resinless) sections showed that the specific anti-lamin A antibody, and a pan-intermediate-filament antibody, labelled knobs and nodes on the skeleton (Figs 4, 5), whilst an anti-lamin B2 antibody reacted weakly with foci on the skeleton in a minority of cells (Fig. 5C). None of the antibodies labelled core filaments strongly, but a similar pattern of strong labelling at nodes and weak labelling of intervening core filaments was found when cytoplasmic intermediate filaments were labelled with anti-

vimentin. In addition, electron microscopy using (conventional) thin sections from which no chromatin was removed showed that there was as much lamin A in the interior as at the periphery (Fig. 5D). Therefore, internal lamins could be immunolabelled using different antibodies, techniques and cell types.

Such internal labelling might arise for trivial reasons. For example, the lamina breaks down during mitosis to re-form during anaphase when lamin-containing vesicles coat the segregated mass of chromosomes and fuse into an enclosing

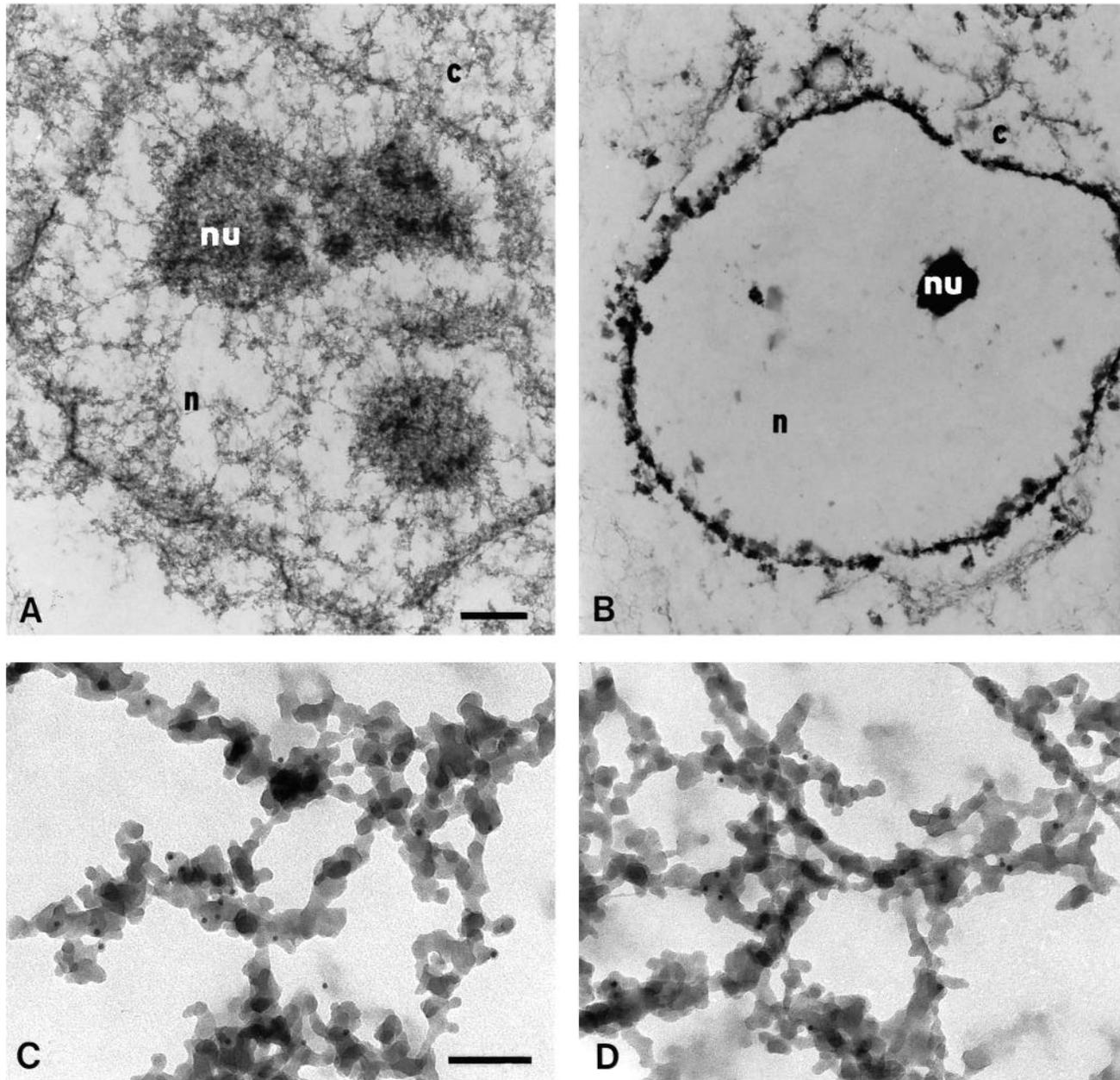


Fig. 6. Characterization of the diffuse nucleoskeleton in HeLa cells. (A,B) The effects of treatment with DNase I or RNase A. Encapsulated cells were permeabilized, treated with *EcoRI* and *HaeIII*, ~90% chromatin eluted, re-treated with (A) DNase or (B) RNase and re-subjected to electrophoresis before resinless sections were prepared. DNase and RNase remove >98% chromatin and >95% nascent RNA, respectively (Jackson and Cook, 1988); DNase does not alter the morphology of the diffuse skeleton but RNase removes it. c, cytoplasm; n, nucleus; nu, nucleolus. Bar, 0.5 μ m. (C,D) The nuclear region immunolabelled using antibodies against C1/C2 (C) and A hnRNPs (D) (resinless sections from which ~90% chromatin removed). In both cases the internal nucleoskeleton is labelled. Bar, 0.1 μ m.

membrane (Gerace and Burke, 1988; Dingwall and Laskey, 1992). Vesicles inside the mass could fuse into an internal 'lamina' that would then be no more than a result of the imperfect mechanism for generating an enclosing membrane. We would expect such an internal lamina to redistribute to the periphery with time, but the density of the skeleton and the intensity of immunolabelling remain roughly constant throughout the cell cycle (not shown; Hozák et al., 1994, have studied the morphology of the skeleton throughout the cycle). Stereo electron micrographs also provide no hint that the internal skeleton has been stripped from the periphery during sample preparation (Hozák et al., 1993); moreover, and decisively, internal lamins can be detected in sections derived from G₂ cells that have neither been permeabilized nor depleted of chromatin (Fig. 5D).

Relationship to other studies

Our results are consistent with observations of a distribution only at the periphery, if the dense chromatin generally prevents antibody access to the interior. Then the examples of internal lamins cited in the Introduction result not from an aberrant location but from an over-concentration at a normal site.

Lamins are frequently associated with an internal 'matrix' (e.g. Capco et al., 1982; Staufenbiel and Deppert, 1982; Fey et al., 1984) which, like the diffuse nucleoskeleton described here (Fig. 6B-D), contains ribonucleoproteins and, perhaps, RNA (e.g. Long et al., 1979; Fey et al., 1986). It is usually assumed that this matrix cannot contain any lamins, and this may be the reason that attempts to isolate it and determine its structure have been so unsuccessful (reviewed by Jack and Eggert, 1992); however, this failure is easily explicable if the assumption is incorrect. Intermediate filaments would then play a role in integrating both cytoplasmic and nuclear space (Lazarides, 1980). As lamins also bind DNA and/or chromatin (e.g. Shoemann and Traub, 1990; Burke, 1990; Glass and Gerace, 1990; Höger et al., 1991; Yuan et al., 1991; Glass et al., 1993) and have been implicated in replication (e.g. Jenkins et al., 1993; Moir et al., 1994), our results provide a physical basis for additional lamin functions within nuclei (e.g. Traub and Shoeman, 1994).

Our results are consistent with the following model. A branched network of 'core' intermediate filaments ramifies throughout the nucleus. Even in chromatin-depleted samples, much of this network remains covered with polymerases, nascent and maturing RNA, as well as RNPs; in S-phase cells it is also associated with dense replication 'factories'. These elements combine to form a diffuse nucleoskeleton that is probably a 'tensegrity' structure like that popularized by Buckminster Fuller (Ingber, 1993), in and on which nuclear functions occur; then, depolymerization of one element in this structure (e.g. RNA) would collapse the whole. The core filaments of this diffuse nucleoskeleton, like their cytoplasmic counterparts, react weakly with a pan-intermediate-filament antibody and so might contain novel members of the intermediate-filament family; however nodes, 'knobs' and clumps on the nuclear network are clearly labelled by anti-lamin A and, to a lesser extent, by anti-lamin B and so probably mark complex lamin-containing structures. Therefore we must now investigate whether specific protein sequences or post-translational modifications like prenylation (see Kitten and Nigg, 1991; Lutz et al., 1992; Hennekes and Nigg, 1994) determine

if a lamin molecule is found at the nuclear periphery, a filament or a node.

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