The EMBO Journal Vol.2 No.3 pp.361–367, 1983

A monoclonal antibody which recognises each of the nuclear lamin polypeptides in mammalian cells

Brian Burke*, **John Tooze and Graham Warren**

European Molecular Biology Laboratory, Postfach 10.2209, Meyerhofstr. 1, D-6900 Heidelberg, FRG

Communicated by L.Philipson
Received on 20 December 1982

A monoclonal IgM has been characterised which recognises the nuclear lamins in all mammalian cells tested. In immunoblotting experiments using both one- and two-dimensional gels it recognises lamins A, B and C. The common antigenic determinant lies on a proteolytic fragment of 46 000 daltons which can be generated from each lamin polypeptide by treatment with chymotrypsin. In immunofluorescence experiments on whole cells and thin frozen sections, the antibody labelled only the nuclear envelope and not the nuclear interior. During mitosis, labelling was found dispersed throughout the cell cytoplasm. By immunoelectron microscopy using the antibody and protein A-gold, only the nucleoplasmic side of the nuclear envelope (the nuclear lamina) was labelled, but there was no labelling of the nuclear pores.

Key words: nuclear lamins/monoclonal antibody/immunofluorescence/immunoblotting/immunoelectron microscopy/peptide mapping

Introduction

The nuclear envelope of eukaryotic cells comprises a double membrane sac, studded with nuclear pores, and lined on the inner (nuclear) face by a layer (usually ~15–80 nm thick) of fibrous material termed the nuclear lamina (reviewed by Franke *et al.*, 1981). The outer face of the double membrane sac is continuous with the endoplasmic reticulum and is often coated with ribosomes. The inner membrane, however, shows no morphologically distinct characteristics other than its association with the fibrous lamina which is presumed to play a predominantly structural role. The nuclear pores, which penetrate both the inner and outer membranes, appear as a ring of proteinaceous subunits (diameter 85 nm) with 8-fold radial symmetry. These are closely associated with the lamina and it is possible to isolate morphologically intact pores attached to the lamina by extracting nuclear envelopes with Triton X-100 (Aaronson and Blobel, 1975).

The nuclear envelope has not yet been thoroughly characterised biochemically. When the proteins of intact rat liver nuclear envelopes or isolated pore complex-lamina fractions from interphase cells are analysed by SDS-gel electrophoresis a complicated pattern is resolved. In both types of preparation, however, there are three major protein components with mol. wts. of 70 000, 67 000 and 60 000 daltons. Since these are associated with the lamina-pore complex, they have been termed lamins A, B and C (Gerace and Blobel, 1980). Lamins A and C appear to be related proteins since they exhibit strong immunological cross-reactivity. Peptide maps obtained from these two proteins also show striking similarities (Gerace and Blobel, 1981). Indeed it has been suggested by Shelton *et al.* (1980, 1980b) that lamin C *in vivo* is derived by proteolytic cleavage of lamin A. Lamin B also appears to be related to lamin A and C, but the evidence is not conclusive (Gerace *et al.*, 1978; Shelton *et al.*, 1980b). Using a monoclonal antibody that recognizes each of the nuclear lamins, we show that lamin B is indeed related to lamins A and C. The antibody arose fortuitously during the preparation of monoclonal antibodies to cellular smooth membranes (Burke *et al.*, 1982).

*To whom reprint requests should be sent.

Results

Immunofluorescence microscopy

Using procedures already described (Burke *et al.*, 1982) we have isolated a hybrid cell line (41CC4) secreting a monoclonal antibody which labelled the nuclei of cultured cells (Figure 1A). Metabolic labelling with [^{14}C]leucine and subsequent analysis of the secretory products on SDS-polyacrylamide gels showed that the monoclonal antibody was an IgM (results not shown).

Immunofluorescence microscopy was performed on a variety of primary cultures and permanent cell lines. The antibody labelled the nuclei of mammalian (rat, mouse, human, marsupial, porcine, dog) but not avian cell types. Cells of other vertebrates have so far not been tested.

By focussing through the labelled nuclei it appeared that the antibody reacted with a component of the nuclear envelope. This interpretation was supported by labelling cryosections of mammalian cells. The nuclear envelopes appeared as brightly staining rings and there was no labelling of the nuclear interior (Figure 1C). The labelling of the nuclear envelope was not however uniform but punctate as shown in Figure 1A and particularly in Figure 1D where the microscope was focussed on the surface of the nuclear envelope nearest to the coverslip.

Immunoblotting studies

To determine which component of the nuclear envelope was recognised by the monoclonal antibody, purified nuclear envelopes were fractioned on one-dimensional SDS-gels and electrophoretically transferred onto nitrocellulose filters. After incubation with the appropriate antibodies and the peroxidase substrates, three polypeptides were labelled which were identified as lamins A, B and C (Figure 2). This identification was confirmed by two-dimensional gel electrophoresis (Figure 3) of rat liver nuclear envelopes. After blotting and incubation with antibody, only the three lamin polypeptides were labelled. On two-dimensional gels, lamin B adopts a characteristically more acidic position than either lamin A or lamin C (Gerace and Blobel, 1980; Shelton *et al.*, 1980b). This rules out the possibility that the labelled polypeptide seen on our one-dimensional gels at the position of lamin B is merely a proteolytic fragment of lamin A. Previous reports have shown that at least two of these three polypeptides are immunologically related (Gerace *et al.*, 1978). Apparently, this monoclonal antibody recognises an antigenic determinant common to each of the lamins.

Earlier studies with polyclonal antisera (Gerace *et al.*, 1978;

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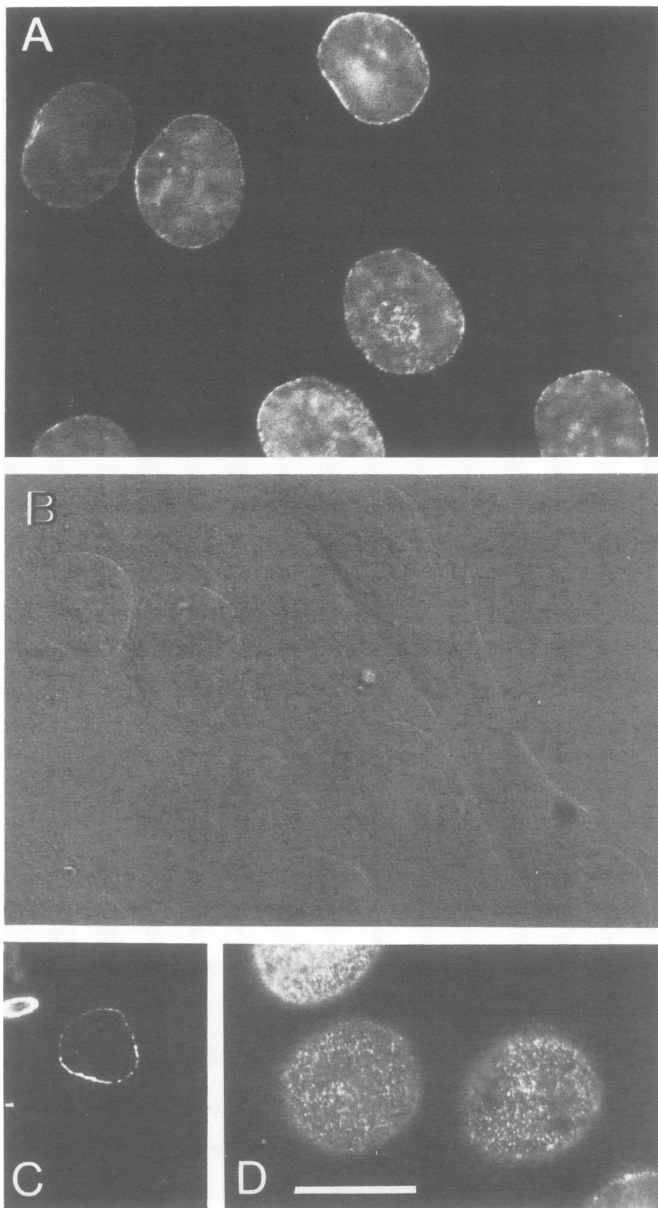

Fig. 1. Indirect immunofluorescence staining of tissue culture cells using 41CC4. (A) NRK cells grown on a glass coverslip. (B) The same field photographed using Nomarski optics. (C) Thin frozen section of mouse L cells. Note that there is no apparent labelling of the nuclear interior. (D) NRK cells grown on a glass coverslip. In this photograph the microscope is focussed on the lower surface of the nucleus nearest to the coverslip, highlighting the punctate pattern of labelling seen with this antibody. Each field is shown at the same magnification. Bar = 20 μm.

Ely *et al.*, 1978) have indicated that the three lamins are distributed evenly upon the inner face of the nuclear envelope. It is possible, therefore, that the punctate immunofluorescence staining pattern which we have observed on interphase nuclei arises by aggregation of the monoclonal antibody (a problem often encountered with IgMs) or because the antigenic determinant recognized by the monoclonal antibody may only be exposed on a small percentage of the lamin molecules.

One-dimensional peptide analysis

Each of the lamin polypeptides was excised from a 10% polyacrylamide SDS-gel, transferred to the sample slot of a

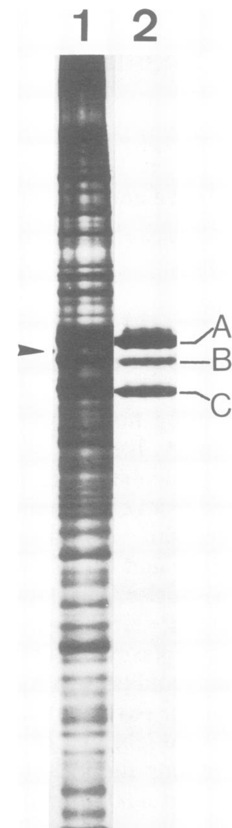

Fig. 2. Detection of nuclear envelope polypeptides by immunoblotting. 16 μg of crude rat liver nuclear envelopes were fractionated on a 10% SDS-polyacrylamide gel and the polypeptides revealed by silver staining (lane 1). A second, identical gel track was blotted onto a nitrocellulose filter and labelled with 41CC4 (lane 2) as described in Materials and methods. Labelling appeared to be confined to the three lamin polypeptides. Note, however, the relatively low level of labelling of lamin B. The arrowhead gives the position of the 68 000 mol. wt. marker.

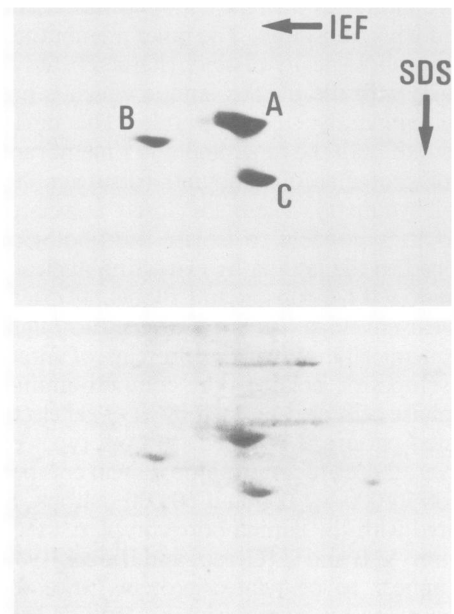

Fig. 3. Immunoblot of a two-dimensional gel of rat liver nuclear envelopes using 41CC4 (top). After blotting the gel on to nitrocellulose, the residual protein in the gel was visualized by silver staining (bottom). It is clear that only the three lamin spots label with the monoclonal antibody.

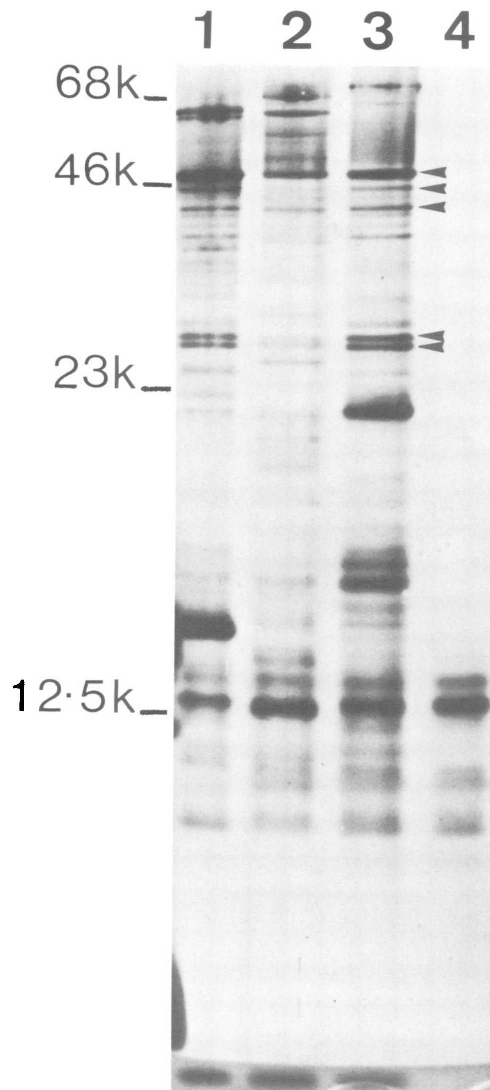

Fig. 4. One-dimensional peptide analysis of each of the three lamins was performed according to the method of Cleveland *et al.* (1977). Chymotryptic fragments were separated on a 15% SDS-polyacrylamide gel and were visualized by silver staining. **Lane 1**, lamin C; **lane 2**, lamin B; **lane 3**, lamin A; **lane 4**, chymotrypsin alone. There is a striking similarity between lamins A and C in the 23 000 – 46 000 mol. wt. range, while several fragments are common to all three lamins (arrowheads). Mol. wts. are shown on the left.

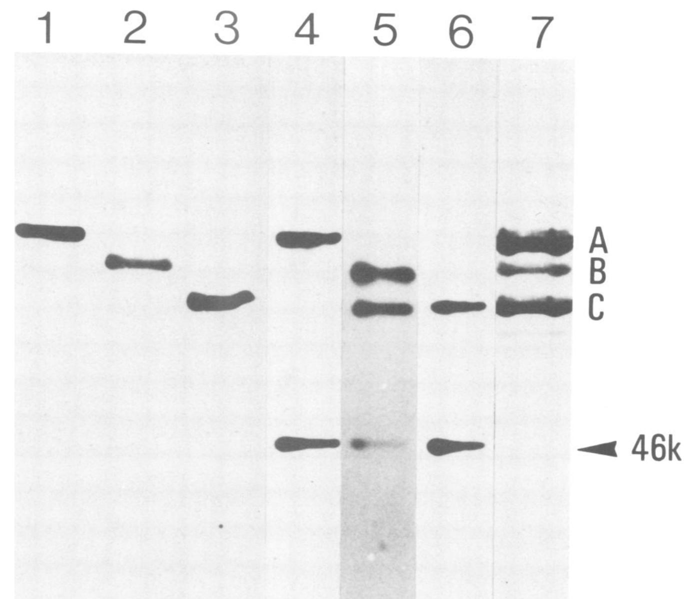

Fig. 5. Immunoblot of the chymotryptic fragments of the three lamins separated on a 10% SDS-polyacrylamide gel. The digestions were carried out as described by Cleveland *et al.* (1977) using 20 μl of 5 μg/ml chymotrypsin. **Lanes 1 – 3** show immunolabelling of undigested lamins A – C, respectively. **Lanes 4 – 6** show immunolabelling of the chymotryptic fragments of lamins A – C, respectively. Note the labelled fragment of mol. wt. 46 000 which is common to each of the lamins. This corresponds to the largest polypeptide marked with an arrowhead in the previous figure. **Lane 7** was loaded with 16 μg of undigested rat liver nuclear envelopes.

second gel and digested *in situ* with chymotrypsin as described by Cleveland *et al.* (1977). After electrophoresis and silver staining, many of the fragments from each of the lamins had the same mobility and colour (Figure 4). Lamins A and C yielded more fragments in common with each other than with lamin B. At least 10 major bands appeared to be shared by lamins A and C while only five bands were common to all three lamins. It should be noted that the colour produced by silver staining may not be the same for different polypeptides, presumably a reflection of their different amino acid compositions. This provides a second criterion in determining whether co-migrating polypeptides are identical. These results are in agreement with previous studies and are in accordance with the known immunological cross-reactivity of the lamins (Gerace *et al.*, 1978). To determine which fragments contained the common antigenic site, the proteolytic fragments were transferred to a nitrocellulose filter and probed with the monoclonal antibody (Figure 5). In addition to the labelling of the three lamins, a common proteolytic fragment of mol. wt. 46 000 was also labelled. Fragments of lower mol. wt. were not labelled, presumably because either the antigenic site had been destroyed or the sensivitity of labelling was insufficient. In the case of lamin B the 46 000-dalton fragment was labelled only weakly when compared with those derived from the other two lamins. However, as shown in the silver-stained gel (Figure 4), this lamin B fragment is present in a much smaller amount. It is possible that this 46 000-dalton fragment, seen consistently in digests of lamin B, could actually arise from a proteolytic fragment of lamin A which comigrates with lamin B on SDS-gels. We consider this unlikely, however, since on our two-dimensional gels there is no evidence of any other immunoreactive protein of the same mol. wt. as lamin B. In addition to this 46 000-dalton fragment, lamin B also shows a second labelled fragment of just less than 60 000 daltons (slightly smaller than lamin C). Taken as a whole, these limited-digest studies show that lamin B cannot be a proteolytic fragment of lamin A, nor can lamin C be a fragment of lamin B.

Mitotic cells

Figure 6 shows a series of immunofluorescence micrographs of mitotic normal rat kidney (NRK) cells stained with the monoclonal antibody. Series similar to this have been published previously (Gerace *et al.*, 1978; Ely *et al.*, 1978) and show the typical dissolution of the nuclear envelope during prophase and its reformation during telophase. However, in contrast to previous investigations, our antibody gives a punctate labelling of the cytoplasm of mitotic cells. This surprised us since Gerace and Blobel (1980) have shown quite unequivocally that lamins A and C become completely solu-

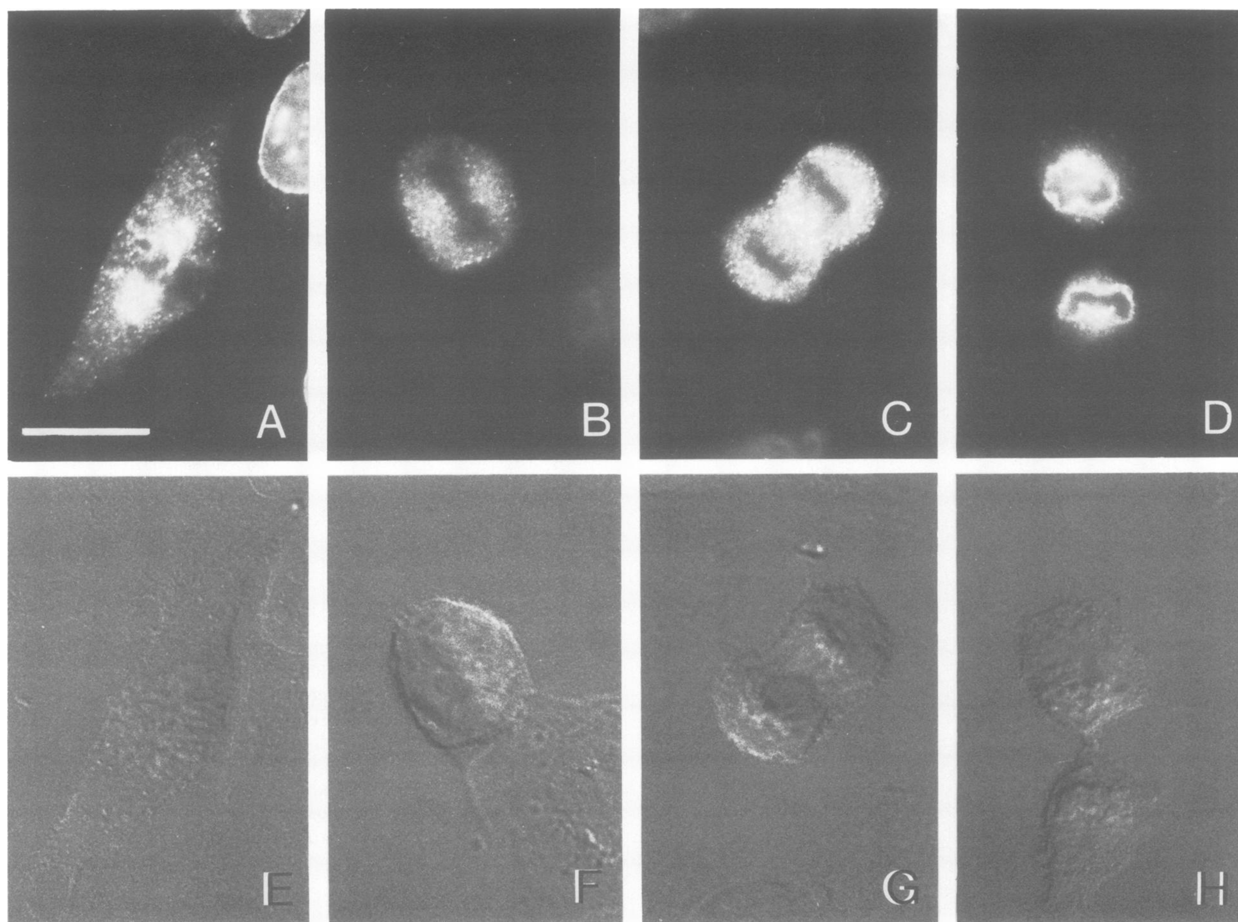

Fig. 6. Indirect immunofluorescent staining of mitotic NRK cells using 41CC4. **(A)** Prophase showing dissolution of the nuclear envelope. **(B)** Metaphase, **(C)** anaphase, **(D)** telophase, showing two new nuclear envelopes reforming about the contours of the chromatids. **(E)**, **(F)**, **(G)** and **(H)** are of the same fields photographed using Nomarski optics. Bar = 20 μm.

ble during mitosis and their antisera give diffuse labelling of the cytoplasm under our fixation conditions. One explanation for this difference could be that the bright points in the cytoplasm are lamin B which Gerace and Blobel (1980) have suggested remains membrane-associated during mitosis and which our antibody recognises. The alternative is that our monoclonal antibody may aggregate during labelling and so give rise to an artefactual punctate appearance. At present we have no way of deciding between these two possibilities.

Electron microscopy

We have used the monoclonal antibody together with protein A conjugated with large (12 nm) or small (6 nm) colloidal gold particles (Faulk and Taylor, 1971) to determine the distribution of the three lamins in preparations of nuclear envelope isolated from rat liver cells as described in Materials and methods. In separate experiments, the nuclear envelope, fixed in glutaraldehyde for 30 min, was either incubated in a 100-fold dilution of the antibody for 60 min at room temperature, or overnight at 4°C before being exposed first to rabbit anti-mouse IgM and then to protein A-gold, post fixed in OsO_4 and embedded in Epon. In a third type of experiment the nuclear envelope was exposed to Triton X-100 to remove the membrane before fixation and incubation with antibody. We had previously demonstrated by immunofluorescence microscopy that the antigenic determinant recognized by 41CC4 survived fixation by glutaraldehyde (data not shown).

Following 60 min exposure to antibody, gold particles were seen on the inner surface of the nuclear envelope associated with the layer of fibrillar and amorphous material (see Figure 7A). The ratio of gold particles on this inner surface of the nuclear envelope to those on the outer face, which is recognisable by the adherent ribosomes, was 7.5:1 (249:33). Only micrographs of sections of nuclear envelopes which were well preserved, had a clearly recognisable morphology and had been transversely sectioned were counted. The 7.5:1 ratio clearly indicates the specificity of the labelling by the antibody and protein A-gold. After overnight incubation at 4°C the ribosomes were no longer present on the outer face of the envelope but the membrane, often swollen, and the inner lamina containing lamins were still identifiable. Only the latter was labelled (see Figure 7B). In sections of controls which were not incubated with the specific antibody there were very few gold particles to be seen anywhere and they were not preferentially distributed on the inner surface of the envelopes.

Although labelling with this monoclonal antibody is quite specific (Figures 7A and 7C), its extent is much less than that usually achieved with polyclonal antibodies in the protein A-gold procedure. Our experience, and that of colleagues working with other antibodies, suggests that the limited extent of labelling may, unless it can be overcome, restrict the exploitation of some monoclonal antibodies for electron

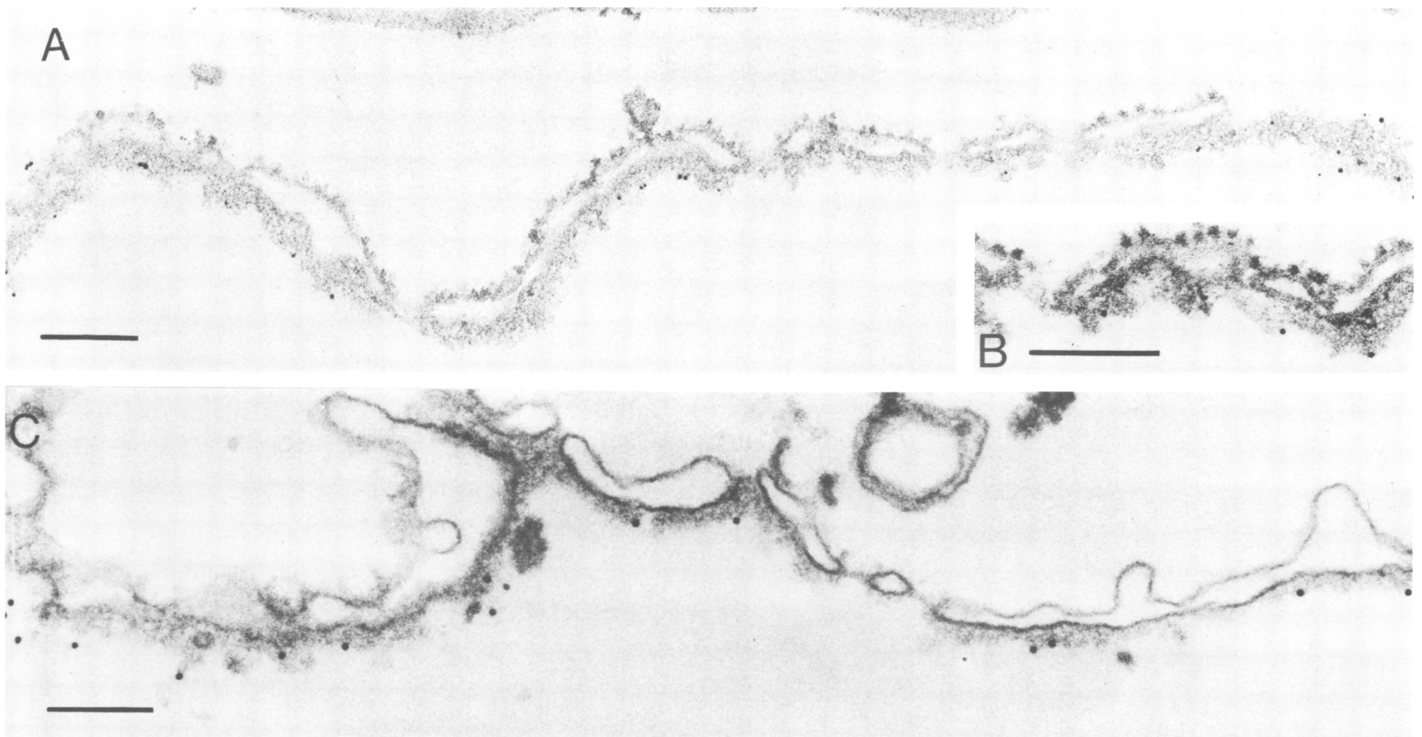

Fig. 7. Immunoelectron micrographs of isolated rat liver nuclear envelopes labelled with 41CC4 and protein A conjugated to colloidal gold. **(A)** and **(B)**, nuclear envelopes after a 60 min incubation at room temperature with the monoclonal antibody and labelling with small (6 nm) protein A-gold. Ribosomes are clearly visible on the outer face, gold labelling being confined to the inner surface. **(C)** Nuclear envelopes after overnight incubation with the monoclonal antibody and labelling with large (12 nm) protein A-gold. Ribosomes have been lost from the outer surface and the membranes have become distended. However, gold labelling is still confined to the fibrillar material on the inner surface. Bars = 200 nm.

microscopy immunocytochemistry.

Following the removal of the nuclear membrane with Triton X-100 all that remains of the nuclear envelope is the apparently structureless layer containing lamins and the nuclear pores. Triton extraction enhances, to a noticeable extent, the labelling of the lamin layer (see Figure 8A), presumably by increasing the accessibility of the lamin molecules to the IgM antibody. Nuclear pores were seen in grazing sections of the nuclear envelope both before and after Triton X-100 extraction. We have never seen gold particles concentrated in or around the nuclear pores, transversely or longitudinally sectioned, either before or after Triton extraction (Figures 7 and 8). This leads us to conclude that in rat liver nuclei the determinant recognised in the three lamins is unlikely to be present or exposed in the nuclear pores but is localised in the amorphous and fibrillar material on the inner face of the nuclear envelope. This is at variance with recent results by Krohne *et al.* (1981) and Stick and Krohne (1982) which clearly demonstrate that a major 68 000-dalton protein found in *Xenopus laevis* oocytes is located in both the fibrous lamina and in the pore complexes themselves. These authors believe that this protein plays a predominantly structural role. However, its relationship to the lamins has yet to be clarified.

Discussion

Our monoclonal IgM antibody, directed against the three nuclear lamins, was detected quite fortuitously during attempts to obtain antibodies against cellular smooth membranes. The appearance of this antibody surprised us in view of the purity of the smooth membrane preparation which was used as antigen (Howell and Palade, 1982) and the difficulties which other workers have encountered when raising polyclonal antisera against the lamins. It is possible that the mouse used as a source of spleen cells was autoimmune although antibodies against the nuclear membrane were not noticed in the mouse serum 2 weeks prior to cell fusion.

Using this monoclonal antibody we have been able to confirm previous reports that all three of the nuclear lamins, at least in mammalian cells, are immunologically related. It is clear, however, from both our one-dimensional peptide analysis and from the results of others that the major similarities are between lamins A and C. Both of these are believed to be peripheral membrane proteins which become completely soluble during mitosis. Lamin B, on the other hand, is believed to remain membrane associated during mitosis being solubilised only in non-ionic detergents. Thus, lamin B must differ from the two others at least in the possession of a hydrophobic domain with which it may interact with the inner nuclear membrane. Certainly the one-dimensional peptide map generated from lamin B differs in many features from those of lamins A and C. One must, of course, be cautious when using a monoclonal antibody as a tool in determining the relationship between different proteins since one is looking at only a single epitope (Nigg *et al.*, 1982). It is well documented that apparently completely unrelated proteins may contain a common epitope recognized by a monoclonal antibody, an extreme example being a cell surface antigen, Thy 1, and an intermediate filament protein (Dulbecco *et al.*, 1981). In the case of the three lamins, however, there is at least some overlap in the one-dimensional peptide maps which may be obtained from each of them. Furthermore, the epitope recognized by this monoclonal antibody resides on a chymotryptic fragment common to each of the lamins as

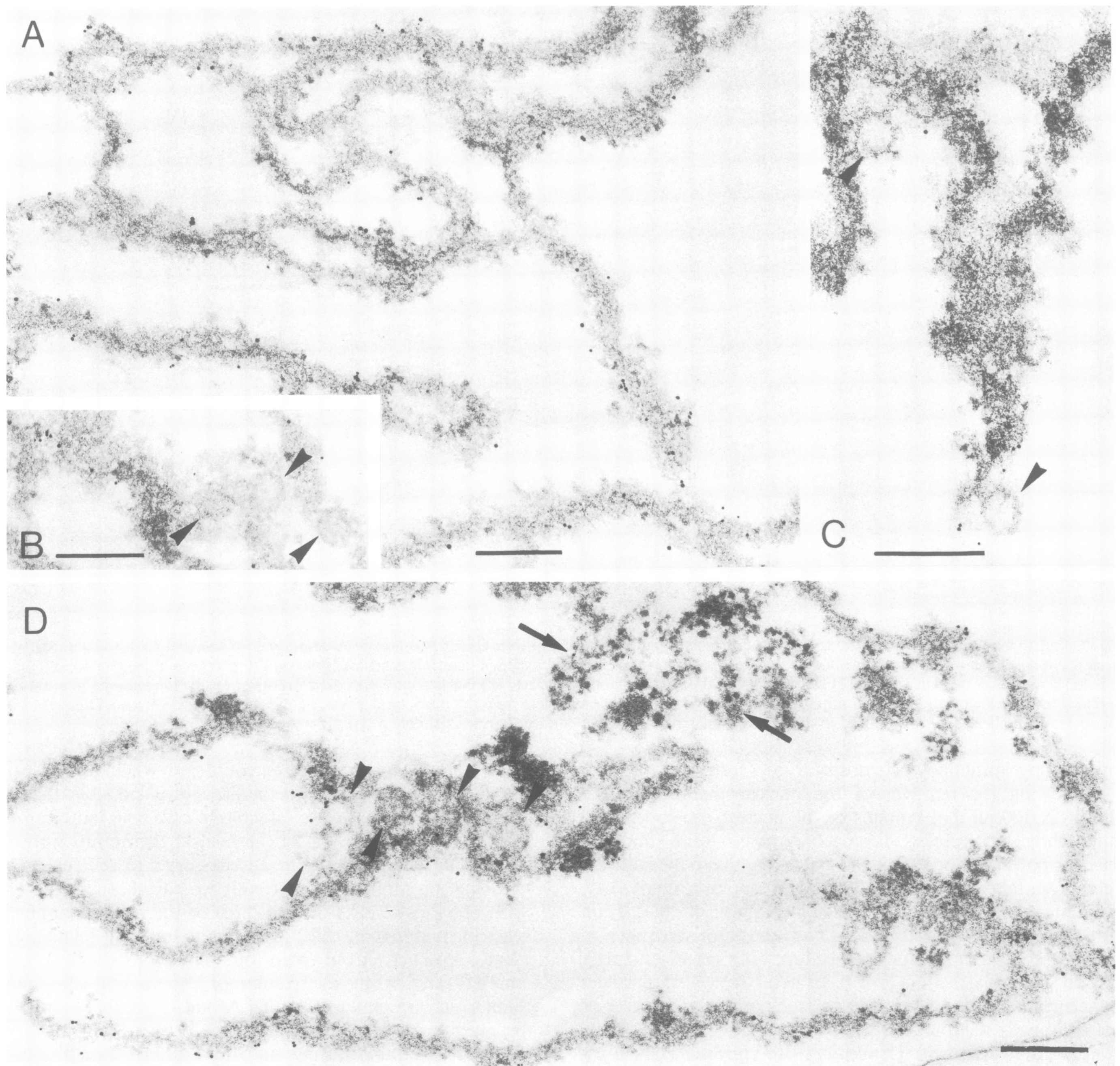

Fig. 8. Immunoelectron micrographs of Triton X-100 extracted rat liver nuclear envelopes labelled for 1 h at room temperature with the monoclonal antibody and then with rabbit anti-mouse IgM and small (6 nm) protein A-gold. Note in **(A)** the increased labelling of the fibrillar material in these Triton-extracted nuclei when compared with the intact membranes shown in Figure 7A. **(B – D)** show several nuclear pores cut tangentially (arrowheads). In none of these figures is there any significant antibody labelling of these structures. In **(D)** some residual chromatin is also apparent (arrows) and appears unlabelled. Bars = 200 nm.

judged by both mol. wt. and colour on silver-stained gels. It is therefore reasonable to conclude that the three lamin polypeptides are related by more than just chance possession of a single epitope. This, then, fully justifies the collective name given to these three related proteins. Such a similarity may reflect a common function, although at present what such a function might be remains a matter of speculation. An intriguing possibility, however, is that it might be required for the interaction of these essentially karyoskeletal proteins with chromatin in the nuclear interior. Certainly there is convincing data showing that transcription of genes takes place in close association with the nuclear lamina or 'cage' (Jackson *et al.*, 1981; Cook *et al.*, 1982).

Finally, using immunoelectron microscopy, we have provided evidence that the lamins appear to be confined to the nuclear fibrous lamina which is located exclusively at the periphery of the nucleus. Since the nuclear pore complexes were not labelled with the antibody and protein A-gold, we conclude that the antigenic site recognised is not exposed in these structures, and probably that none of the three lamins form part of the nuclear pores.

Materials and methods

Cells

NRK cells were grown in minimum essential medium (MEM) containing 10% (v/v) foetal calf serum (FCS) plus antibodies. Mouse L cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FCS. All cells were grown at 37°C in a humidified CO_2 incubator (95% air, 5% CO_2 v/v).

Preparation of the monoclonal antibody

Since this antibody was obtained fortuitously during the preparation of monoclonal antibodies to cellular smooth membranes the procedures have been described elsewhere (Burke *et al.*, 1982).

Immunofluorescence microscopy

NRK cells grown on glass coverslips were fixed and labelled with the monoclonal antibody using the procedures described by Ash *et al.* (1977). The second antibody was an affinity-purified rabbit anti-mouse IgG (which cross-reacted with mouse IgM) conjugated to rhodamine which was kindly supplied by D.Louvard of EMBL, Heidelberg. Frozen thin sections of mouse L cells were prepared as described by Tokuyasu (1973), attached to glass slides and then processed for microscopy in the same manner as the cells. Specimens were examined under epi-illumination using a Zeiss photomicroscope III fitted with the appropriate filter set for rhodamine and with a x63 oil immersion lens.

Isolation of rat liver nuclei

Rat liver nuclei were isolated as described by Blobel and Potter (1966) and were used to prepare nuclear envelopes and nuclear pore complex-lamina fractions according to the methods of Dwyer and Blobel (1976) and Aaronson and Blobel (1975). For preparation of nuclear envelopes for electron microscopy, nuclei were first allowed to adhere to polylysine-coated 6 cm Falcon tissue culture dishes and then lysed and washed *in situ*. Envelopes fixed to a solid support greatly simplified later treatment with antibodies and protein A conjugated with colloidal gold (see below).

Immunoblotting

The blotting procedure was carried out using a modification of the method described by Burnette (1981). After transfer of protein on to the nitrocellulose filter, all remaining protein binding sites were blocked by incubation in phosphate buffered saline (PBS) containing 10% (v/v) new born calf serum and 0.2% (w/v) Triton X-100. All further incubations with antibodies and subsequent washes were carried out at room temperature in this solution. The filter was incubated for 2 h with the monoclonal antibody ($\sim 20\ \mu g/ml$) followed by three 15 min washes. This was followed by a 1 h incubation in affinity-purified rabbit anti-mouse IgG (10 $\mu g/ml$), three 15 min washes and then a 1 h incubation in peroxidase-conjugated sheep anti-rabbit IgG (Institut Pasteur, Paris, 1:1000 dilution). Finally, after three further 15 min washes, the filter was rinsed in PBS and the antibody-labelled proteins visualized by immersing the filter for ~ 30 s in 50 mM Tris-HCl pH 7.6 containing 5 mg/ml diaminobenzidine tetrahydrochloride (Sigma) and 0.01% (v/v) H_2O_2. The reaction was stopped by rinsing the filter in PBS. In some experiments, incubation with the intermediate rabbit anti-mouse IgG was omitted and instead the monoclonal antibody was labelled directly with peroxidase-linked sheep anti-mouse IgG (Institut Pasteur). The sensitivity, however, was reduced.

One-dimensional peptide analysis of lamins

One-dimensional peptide maps of each of the three lamins, separated on 10 or 15% polyacrylamide SDS-gels (Maizel, 1969) were carried out as described by Cleveland *et al.* (1977) using chymotrypsin. $100-400\ \mu g$ of total rat liver nuclear envelopes were fractionated on each gel track and the excised lamin bands were digested with 20 μl of 5 $\mu g/ml$ chymotrypsin. Gels were either silver stained as described by Ansorge (1983) or blotted on to nitrocellulose followed by labelling with monoclonal antibody and peroxidase-linked second antibody.

Electron microscopy

Rat liver nuclear envelopes, either before or after extraction with Triton X-100 and attachment to Falcon plastic Petri dishes, were fixed for 30 min at room temperature in 1% glutaraldehyde in 100 mM PIPES buffer pH 7.0 containing 5% (w/v) sucrose. They were then washed twice in 100 mM PIPES, pH 7.0 containing 10% (w/v) sucrose, followed by a 10 min incubation in PBS containing 50 mM NH_4Cl and then three washes in PBS. Next, the fixed nuclear envelopes were incubated with the monoclonal antibody in PBS containing 0.2% (w/v) gelatin, either for 60 min at room temperature or overnight at 4°C. They were then washed three times in PBS and further incubated for 1 h at room temperature in rabbit anti-mouse IgM (a gift from D.Louvard, EMBL). Finally, following three washes in PBS, they were incubated for 1 h at room temperature with protein A, conjugated either to small (~ 6 nm) or large ($\sim 10-12$ nm) colloidal gold (Faulk and Taylor, 1971), in PBS containing 0.2% gelatin. Following the protein A-gold labelling, they were washed in PBS, fixed again in glutaraldehyde (above), post-fixed in OsO_4 (Karnovsky, 1978) and dehydrated. The labelled nuclei were then removed from the plastic surface in propylene oxide and embedded in epon. Thin sections were cut and stained with 2% uranyl acetate and lead citrate before being examined in a Philips 400 electron microscope. Controls were prepared in the above way except that incubation with PBS replaced incubation with the monoclonal antibody.

Acknowledgements

We thank Georg Krohne and Werner Franke for critical reading of the manuscript and running the two-dimensional gels; Gareth Griffiths and Paul Webster for help with the immunoelectron microscopy; Alix Cockcroft and Ruth Giovanelli for excellent technical assistance. J.T. is greatly indebted to A.Ohlsen, G.Griffiths, F.Booy and other electron microscopists at EMBL for their generosity and patience whilst reintroducing him to the electron microscope. B.B. was supported by a long-term EMBO Fellowship.

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