THE MAJOR 67 000 MOLECULAR WEIGHT PROTEIN OF THE CLAM OOCYTE NUCLEAR ENVELOPE IS LAMIN-LIKE

GERD G. MAUL AND FRANK A. BAGLIA
The Wistar Institute of Anatomy and Biology, Philadelphia, PA 19104, U.S.A.

DONALD D. NEWMEYER
Department of Radiation Biology and Biophysics, University of Rochester Medical Center, Rochester, NY 14642, U.S.A.

BETSY M. OHLSSON-WILHELM
Department of Microbiology, University of Rochester Medical Center, Rochester, NY 14642, U.S.A.

SUMMARY

Nuclear envelopes of somatic cells have at least two different major proteins in the 60–70(×10³) molecular weight range (lamins A(C) and B) that seem to be involved in chromatin attachment. In contrast, nuclear envelopes from clam germinal vesicles have only a single major protein of the same size class (~67×10³ Mᵣ) and have no chromatin attached to them. The data presented in this report show that this 67×10³ Mᵣ clam protein shares a variety of physical properties with lamins A(C) and B, derived from rat liver nuclei. These properties include similar size, although different isoelectric points; phosphorylated forms; strong tendencies to cross-link by disulphide bonds; presence of carbohydrates, demonstrated by direct incorporation of mannose and labelling with borohydride; and shared epitopes, demonstrated using both monoclonal and polyclonal antibodies. Taken together, these observations identify the clam 67×10³ Mᵣ protein, the major structural protein of a nuclear envelope that lacks attached chromatin, as being lamin-like and demonstrate that it is more closely related to lamin A(C) than to lamin B.

INTRODUCTION

The nuclear envelope (NE) is characterized by two parallel membranes interrupted by the pore complex and a fibrous lamina (for reviews see Maul, 1977; Franke, 1974). This fibrous lamina conveys extraordinary strength to the NE and may function in the attachment of chromatin to the inner NE. There are, however, nuclei that do not have chromatin attached to them and are characterized by a very high pore complex density. Examples of such nuclei that have been isolated and partially characterized are those from amphibian oocytes (Krohne, Dabauvalle & Franke, 1981; Stick & Krohne, 1982; Krohne et al. 1982) and those from oocytes of the surf clam Spisula solidissima (Maul, 1980; Maul & Avdalovic, 1980).

The majority of proteins in NEs from both of these types of nuclei are in the 60–70(×10³) Mᵣ size class. The three from rat liver nuclei have been designated lamins A, B and C (Gerace & Blobel, 1980), where lamin C shares many peptides with lamin A (Gerace & Blobel, 1982; Kaufmann, Gibson & Shaper, 1983; Lam & Kasper, 1979; Shelton & Cochrane, 1978) and may be a lamin A derivative. Only a single polypeptide is seen in this size range (67×10³) in clam and amphibian oocyte NEs.
Because of the high density of pore complexes in this type of nucleus, it has been postulated that this polypeptide is the major pore complex protein in *Xenopus* oocytes (Stick & Krohne, 1982; Krohne, Franke & Scheer, 1978; Krohne et al. 1982). However, the similarly sized rat lamins cannot be demonstrated to be part of the pore complex in somatic cells by ultrastructural immunohistochemical means (Gerace, Blum & Blobel, 1978; Kaufmann et al. 1983; Burke, Tooze & Warren, 1983) and are viewed as part of the fibrous lamina between the pore complexes. We therefore wondered if the major NE protein of the oocyte might instead have a lamin-like structural role in the fibrous network, holding the densely packed pore complexes together, rather than in the pore complex itself. If this were true it would suggest that the second lamin, characteristic of somatic NEs and absent from the NE of the germinal vesicle (GV), might then be involved in chromatin attachment.

We present evidence here that the major NE 67 × 10^3 M_r polypeptide of the clam GV has several physical properties in common with the rat lamins, specifically lamins A/C.

**MATERIALS AND METHODS**

**Isolation of clam NE proteins**

The isolation of nuclei from mature eggs of *S. solidissima* was performed according to established procedures (Maul, 1980), modified by the inclusion of 0.5 mM-iodoacetamide in all isolation buffers. Nuclear subfractions were separated on discontinuous sucrose gradient (Maul & Avdalovic, 1980). The NEs recovered at the 35% to 50% sucrose interface fraction were used fresh or after treatment with 1 M-NaCl and 1% Triton X-100, yielding a fibrous lamina pore complex (FLPC) fraction comparable with that of rat liver nuclei (Dwyer & Blobel, 1976). Rat liver nuclei were isolated (Blobel & Potter, 1966), and rat liver NEs were prepared by a modification of the method described by Kaufmann, Coffey & Shaper (1981). The detergent step was omitted and the NEs were banded on a step gradient after the 1-6M-NaCl extraction. After a 20-min centrifugation at 41 500 g, NEs were collected as a broad band between 35% and 45% sucrose. All isolation buffers contained protease inhibitors, either phenylmethylsulphonyl fluoride (PMSF) or the chloride form (PMSC). When FLPC was required, the NEs were extracted with 1% Triton X-100 and centrifuged (10 min at 41 500 g) onto a cushion of 66% sucrose to facilitate resuspension.

**Monoclonal antibodies against rat nuclear subfraction**

Mice were immunized with a chromatin-containing nuclear subfraction prepared from purified rat liver nuclei. Spleen cells from two mice were fused with P3X63Ag8.653 mouse myeloma cells and the hybrids were screened by ELISA for secretion of antibodies to the immunogen. Monoclonal antibody-producing cells were then screened by immunofluorescence for antibodies reactive with antigens at the nuclear periphery, and then characterized using immune blotting. These procedures are described in detail by Newmeyer (1983).

Clam nuclei were tested for reactivity with monoclonal antibodies by indirect immunofluorescence. Isolated clam GV were absorbed to glass coverslips and fixed in 90% (v/v) ethanol. GV were then incubated for 1 h at 37°C in phosphate-buffered saline (PBS; 140 mM-NaCl, 2.7 mM-KCl, 8.1 mM-Na_2HPO_4, 1.5 mM-KH_2PO_4, pH 7.2) containing 3% bovine serum albumin (BSA) and 20% foetal bovine serum (FBS/PBS), and for 30 min at 37°C with monoclonal antibody, diluted in the same buffer with Tween-20 added to 0.05%. Coverslips were washed extensively in PBS containing 0.05% Tween-20 and incubated for 30 min at 37°C with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG) (affinity-purified; Boehringer-Mannheim) diluted 1:250 (4 μg/ml antibody) in BSA/Tween-20/PBS. Monoclonal and fluorescein conjugated antibodies were centrifuged at 80 000 g for 30 min to remove aggregates, before use. After another series of washes, coverslips were mounted upside-down on glass slides over 50% glycerol/0.25 M-Tris·HCl (pH 8.5). Photography was done with a Zeiss research microscope with mercury lamp and
filters optimized for fluorescein fluorescence. Kodak Tri-X film was used at ASA 400 with exposures of 10–60 s. Cells incubated without monoclonal antibody, with normal mouse serum or with irrelevant myeloma protein were used as controls.

**Sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE)**

SDS/PAGE was performed using 7 % to 18 % (w/v) acrylamide gradient gels. The discontinuous buffer system of Laemmli (1970) was used with a Hoefer Scientific slab gel apparatus. The samples were solubilized for 1 h at room temperature in 2 % SDS, 25 mM-dithiothreitol and 20 % (v/v) glycerol in 80 mM-Tris-HCl (pH 6.8) and then heated in boiling water for 3 min. After electrophoresis, the gels were fixed overnight in acetic acid/methanol/water and then stained with Coomassie Blue. Lamins were separated by isoelectric focusing as described by O'Farrell, Goodman & O'Farrell (1977). Unfixed rat and clam NE were extracted with 4 M-urea and 1 mM-dithiothreitol and centrifuged in a Beckman Airfuge for 10 min. The supernatant was brought to 9.5 M by adding solid urea before preparation for isoelectric focusing (O'Farrell et al. 1977). For the second dimension, SDS/10 % polyacrylamide gels were used.

**Production of antibodies to clam NE antigens**

Rabbits were injected subdermally with fresh NE or FLPC in 40 % sucrose in Freund’s adjuvant. Animals were injected a total of three times at 7-day intervals, and bled 1 week after the third injection. Sera were stored in 0.1 % NaN₃ at 5 °C or maintained frozen at −70 °C. Antisera were used for antigen identification on nitrocellulose blots.

**Identification of antigens on nitrocellulose blots**

Polyacrylamide gels (7 % to 18 %) were washed briefly in blotting buffer and transferred to nitrocellulose sheets overnight at 250 mA, and stored in the same solution (Southern, 1975). Gels were stained with Coomassie Blue to check the quality of protein separation. All proteins seem to transfer, as judged by reaction of antibodies against total NEs. Before reaction with the antibodies, all binding sites of the nitrocellulose were covered by overnight incubation with 20 % bovine serum, 3 % BSA and 0.05 % Tween-20 in PBS without MgCl₂ or CaCl₂, in the cold (wash buffer). The respective antisera were added to this solution resulting in a dilution of 1:25 or 1:100, or in the case of monoclonal antibodies, culture supernatants were diluted 1:10 to 1:20. After 3 h, the nitrocellulose blots were washed three times in PBS over a 30-min period. Then, wash buffer was added to the plastic bags for 10 min before adding biotinylated goat anti-rabbit antibodies for 30 min at the manufacturer’s (Vector Inc., Burlingame, CA) recommended concentration. After a 30-min wash with PBS, avidin/peroxidase in 1 % (v/v) call serum in PBS was added for 1 h followed by three washes over 30 min, and 0.1 % 3,3’-diaminobenzidine (DAB) was added.

**Identification of NE glycoproteins**

To label glycoproteins directly, mouse L cells grown in MEM with 10 % calf serum in T-30 Falcon flasks were incubated with [³C]mannose. One day after plating, the medium was replaced, and 24 h later 25 μCi/ml of [³C]mannose (59 mCi/mmol; Amersham) was added. Incorporation proceeded for 24 h, after which the cells were thoroughly washed with PBS. Trypsin was added for 30 s, removed, and washed off with medium and serum. The cells were then washed five times in PBS with 0.5 mM-PMSC. After a brief hypotonic treatment (1 min in 1 mM-PIPES, pH 7.2, 0.1 mM-MgCl₂, 0.5 mM-PMSC, mixed 9:1 with the cell pellet in PBS), the cells were homogenized with a tight-fitting Dounce homogenizer until all cells were broken. Sucrose (40 %) was added to make the homogenate 7 % sucrose. Nuclei were sedimented through 1 M-sucrose for 10 min at 3000 g in a refrigerated JEC DPR 6000 centrifuge. NEs were then isolated by digestion with DNase and RNase followed by extraction with 1.6 M-NaCl (Kaufmann et al. 1981), without first removing the enzymes. NE were then collected from a step gradient of 30 %, 35 % and 40 % sucrose as a wide band after 20 min at 83 000 g and fixed in 66 % ethanol. To separate proteins cross-linked by disulphide bonds, samples were electrophoresed according to Laemmli (1970), unreduced in the first dimension in slab gels and then re-electrophoresed after reduction in the gel strip using sample buffer with 25 mM-dithiothreitol. This is referred to as diagonal electrophoresis (see also Maul & Avdalovic, 1980; Shelton et al. 1980; Lebkowski & Laemmli, 1982). Dried gels were exposed for 2 weeks before development of the film (Laskey & Mills, 1975).
Glycoprotein determination in clam proteins was done using an indirect method, since direct incorporation is not possible because of the low metabolic activity of the eggs. This method relies on the oxidation of sugar residues to generate aldehydic functions and on a final reduction with tritiated sodium borohydride. Ethanol-precipitated clam NEs and rat FLPC were labelled with $[^3H]$borohydride (van Lenten & Ashwell, 1971). Pellets were resuspended in 50 μl of PBS with 5% Nonidet P-40 (pH 6.5). Galactose oxidase (Sigma Chemical Co., St Louis, MO) was reconstituted by adding 1 ml of PBS with 40% glycerol to 450 units in the original vial. Six microlitres of a 1:10 dilution of the galactose oxidase stock solution were added to the 50 μl suspensions of clam NE and rat FLPC; this was followed by a 1-h incubation at 37°C. The enzyme was then removed by centrifugation in an Eppendorf tabletop centrifuge and enzyme residues were inactivated by 3-min boiling in a water bath. $[^3H]$borohydride (New England Nuclear, Boston, MA) (246 mCi/mol) was added (0.02 mCi/50 μl) and allowed to react for 30 min at room temperature. Both samples were then diluted to 0.4 ml with PBS and the FLPC was pelleted. The pellet was resuspended in 66% ethanol and washed several times. Proteins separated by SDS/PAGE and labelled with $[^3H]$borohydride were fixed and stained with Coomassie Blue, washed with several changes of water over 2 h, incubated in 1 M-sodium salicylate (pH 6.0) for 30 min and exposed to flash-sensitized Kodak film (Laskey & Mills, 1975).

RESULTS

It is not practical to obtain somatic tissue and germ-line tissue from either rat or clam. Rat oocytes are few in number and the only promising somatic tissue of the clam, the hepatopancreas, is filled with proteases. We have therefore compared NEs from the clam germinal vesicle with those from rat liver nuclei.

Rat and clam FLPC fractions were obtained by similar techniques, with the exception that no nucleases were used in the preparation of clam NEs. Chromatin, like that attached to the NE in somatic cells, which must be released by treatment with nuclease, is absent from the clam preparation, because clam GV contain condensed chromosomes only. Fig. 1 shows the FLPC fractions from clam GV and rat somatic cells. In both preparations, the nuclear pore complex appears as the dominant structure separated by variable amounts of interspersed material. The pore complexes in the clam GV preparation are recognized as closely spaced densities (Fig. 1A), whereas they are substantially further apart in the FLPC from rat somatic nuclei (Fig. 1B).

Differences in isoelectric points

To compare the isoelectric point of the single dominant $67 \times 10^3 M_r$ protein of the clam NE with those of the three rat lamins, FLPC from rat and clam were mixed before solubilizing. The isoelectric point for the clam protein is 6.3, intermediate between those of the rat lamins A and C, and the more acidic rat lamin B (Fig. 2). Thus, the determination of the isoelectric point did not reveal a close affinity of the clam $67 \times 10^3 M_r$ protein to either rat lamins A and C or to rat lamin B. All of these proteins seem to show a train of charge isomers of roughly the same length.

Cross-linking of the $67 \times 10^3 M_r$ clam protein by disulphide bonds

Several investigators have noted the tendency of lamins to cross-link by disulphide bonding (Maul & Avdalovic, 1980; Kaufmann et al. 1983; Lam & Kasper, 1979; Shelton & Cochran, 1978) during the isolation procedure (presumably due to air.
Clam GV envelope protein is lamin-like

Fig. 1. A. In the clam FLPC from GV, pore complexes are connected by very short areas of fibrous lamina. ×12 000. B. In the rat FLPC from somatic cells, pore complexes are also the dominant structure but are not as closely spaced as in clam FLPC. ×15 000.

oxidation). It is apparent that this property is shared by the 67×10³ Mr clam protein. Fig. 3, lane 1 demonstrates that oxidation of clam NE by chloramine T as part of our iodination procedure, followed by extraction with 1% SDS under non-reducing conditions and subsequent electrophoresis of the supernatant, results in one major protein band of approximately 190×10³ Mr. If that band is excised and incubated in 1% SDS containing 25 mM-dithiothreitol before separation on an SDS/polyacrylamide gel, it co-migrates with the reduced lamin (Fig. 3, lane 2). Lane 3 shows the reduced SDS extract and lane 4 the pellet of the unreduced SDS-extracted clam
NE. It thus appears that a large portion of the clam $67 \times 10^3 M_r$ protein is cross-linked to a trimer, suggesting that the protein must be positioned in a way that allows a selective cross-linking of sulphhydryl groups. Incomplete reduction before SDS/PAGE analysis may also account for the presence of enough protein in the $180 \times 10^3$ to $200 \times 10^3 M_r$ range to allow its recognition by antibodies in an immunoblotting assay (see below).

The clam $67 \times 10^3 M_r$ protein shares epitopes with rat somatic lamins

Antibodies have traditionally been used to ascertain whether or not two protein species are related to one another structurally. Demonstration of shared epitopes indicates at least some structural homology and may also indicate functional homology, depending on the specificity of the antibody employed.

We have utilized four antibodies in these studies. Two are polyclonal antisera, one raised against total clam GV NE and the other against total clam GV FLPC. The other two are monoclonal antibodies raised against a chromatin-containing nuclear subtraction prepared from purified rat liver nuclei (Newmeyer, 1983). All four of these antibodies recognize some epitopes that occur on at least one of the rat somatic lamins.

Fig. 2. Two-dimensional gel electrophoresis of a urea extract from rat and clam NE preparations. A pellet of undissolved material remains upon 5-min centrifugation at 100000$g$ in a Beckman airfuge. The major clam NE protein ($67 \times 10^3$) migrates between the more basic rat lamins A and C and the more acidic rat lamin B.
Clam GV envelope protein is lamin-like

Fig. 3. Separation of 1% SDS/chloramine-T-oxidized clam NE extract: lane 1, unreduced; lane 2, major band cut out and reduced; lane 3, total extract reduced; lane 4, pellet reduced; lane 5, molecular weight markers (Mr×10⁻³). The 67×10³ Mr protein migrates as a trimer of ~190×10³.

The antigens that we have tested are shown as Coomassie Blue-stained SDS/polyacrylamide gels in Fig. 4A. Lane 1 shows total protein from clam GV; lane 2, total protein from clam GV NE; lane 3, total protein from somatic rat liver nuclei; and lane 4, total protein from rat liver NEs.

Polyclonal antiserum to total clam GV NE (Fig. 4c)
In this and all following immunological analyses, the same sequence of antigens as described above was probed. This antiserum reacted with a variety of proteins when tested with the clam NE (Fig. 4c, lane 2), as expected. In addition, this serum detected two bands in the rat NE (lane 3), the large molecular weight polypeptide corresponding to lamin B, and the other lower molecular weight (~50×10³) cross-reactive protein, possibly the lamin B breakdown product observed by several investigators (Stick & Krohne, 1982; Krohne et al. 1982; Gerace & Blobel, 1980; Gerace et al. 1982), despite the presence of proteinase inhibitors. In the nuclear preparation, an additional cross-reactive protein with an apparent molecular weight of 38×10³ is seen. Blots of two-dimensional gels confirmed that the rat lamin B was recognized by the anti-clam NE antibodies (not shown but see Fig. 6a).

Polyclonal antiserum to total clam GV FLPC
Antibodies were prepared against total clam FLPC, as the clam FLPC is equivalent to the antigen used by Stick & Krohne (1982) in their studies on the Xenopus protein.
Fig. 4. Immunochemical comparison of clam and rat nuclei and NEs. A. Coomassie Blue-stained gel. Lane 1, clam GV; lane 2, clam NE; lane 3, rat NE; lane 4, rat nuclei. The same sequence is used for the nitrocellulose blots. B. Antibodies against total clam FLPC were incubated with clam and rat preparations. A large number of proteins are recognized by antibodies raised to FLPC in the clam GV preparation (lane 1). This antibody also recognizes some proteins migrating somewhat faster than the rat lamins (lanes 3 and 4). C. Antibodies against total clam NE react with most clam NE proteins in addition to the major clam NE protein ($67 \times 10^3 \, M_r$). Many more proteins in the 60 to $70 \times 10^3 \, M_r$ protein range react with the antibodies to total clam NE than with anti-clam FLPC antibodies. Only two bands are cross-reactive with the rat NE, corresponding to lamin B and its breakdown product (lane 3). In the rat nucleus, only one band reacts in the lamin B range and one with an apparent molecular weight of $38 \times 10^3$ (lane 4). D. Monoclonal antibodies from clone J9 react with lamins A and C of the rat NE (lane 3) and two faster migrating bands (breakdown products of lamins A and C). These are not present in the rat nuclear preparation (lane 4). There are three reactive bands present in the rat nuclear preparation. The middle one is usually very faint. These antibodies cross-react with the clam NE protein of comparable molecular weight (lane 2) and the corresponding one in the clam nucleus (lane 1). They also cross-react with a $190 \times 10^3 \, M_r$ protein (lane 2). E. Monoclonal antibodies from clone J16 react with all three rat lamins (lane 3), but react strongly with only one protein in the rat nuclear preparation. One major band reacts in the area of the lamin breakdown products. A small circle denotes the position of the clam $67 \times 10^3 \, M_r$ NE protein; a bracket, lamin B; and horizontal lines, lamins A and C as well as their breakdown products.
The $67 \times 10^3 \, M_r$ clam NE protein emerged as the dominant antigen in the clam NE (Fig. 4B, lane 2) after incubation with total anti-clam FLPC antibody. However, most clam GV proteins (lane 1), except for the soluble major $47 \times 10^3 \, M_r$ protein, also reacted. A group of three proteins cross-reacted weakly in the rat nuclear fractions. This test showed that a multitude of clam proteins, in addition to the dominant $67 \times 10^3 \, M_r$ protein, elicited antibodies, a finding in sharp contrast to that reported by Stick & Krohne (1982) for the *Xenopus* immunogen.

**Monoclonal antibody J9**

Polyspecific antibodies against the $67 \times 10^3 \, M_r$, clam protein cross-react with lamins A and C (Baglia & Maul, 1983) but not with rat lamin B (not shown). We used monoclonal antibodies raised against rat antigen to determine whether the rat lamin antibodies cross-react with the clam $67 \times 10^3 \, M_r$ protein and if so, which of these antibodies did. The monoclonal antibody from clone J9, which reacts with rat lamins A and C (Fig. 4D, lane 3) as demonstrated by two-dimensional PAGE (Newmeyer, 1983), recognizes the clam $67 \times 10^3 \, M_r$, NE protein (Fig. 4D, lane 2). In addition, it recognizes a $190 \times 10^3 \, M_r$, clam protein. This $190 \times 10^3 \, M_r$, antigen could be the incompletely reduced trimer of the $67 \times 10^3 \, M_r$, protein. Note the appearance of a similar high molecular weight band in the rat NE (lane 3). Two bands in the lower molecular weight region of the rat NE (Fig. 4D, lane 3) also react. They migrate below the heavy band in the $50 \times 10^3 \, M_r$ region (see the Coomassie Blue-stained rat NE, Fig. 4A, lane 3) and may be breakdown products of lamin A and C. They are not found in total rat nuclei and may be generated during NE isolation. Rat nuclei show three reactive bands (Fig. 4D, lane 4), equivalent to the bands seen in the two-dimensional gel (Fig. 2) of the A(C) lamins. Lamin A was reduced in size during our NE isolation procedure despite the presence of a protease inhibitor (PMSF). Use of this antibody allows us to define one of the epitopes shared between somatic lamin A(C) and the clam $67 \times 10^3 \, M_r$, protein.

**Monoclonal antibody J16**

Monoclonal antibodies derived from clone J16 reacted as expected with the three rat lamins (Fig. 4E, lane 3) as well as with two antigens from the nuclear preparation (Fig. 4E, lane 4), which run at a similar position, although one of the bands stained only faintly (in this particular preparation). Only a very weak reaction at the $190 \times 10^3 \, M_r$ position is seen in the clam preparation. Thus, the three rat lamins have a common determinant that is not present on the clam $67 \times 10^3 \, M_r$, protein.

**The clam $67 \times 10^3 \, M_r$, protein is present mostly in the NE**

Immunofluorescence assays of a variety of cells treated with the monoclonal antibodies directed against rat nuclear antigens that react with lamins A, B and C (J16) and lamins A and C (J9) localizes them predominantly on the NE of several tissue-culture cell types (Newmeyer, 1983). Though both antibodies could be detected
Fig. 5. Immunofluorescent staining of bovine heart artery cells. a. Monoclonal antibody J9 stains the nuclear periphery and some intranuclear material. ×1000. b. Monoclonal antibody J16 reacts only with the nuclear periphery of bovine heart artery cells. ×1000. Immunofluorescent staining of isolated clam GV. c. Monoclonal antibody J9, which reacts with rat lamins A and C and with the clam lamin, labels antigens in the NE, the chromosomes and faintly throughout the nuclear interior. ×1600. d. Monoclonal antibody J16 reacts with all three rat lamins but with no clam protein and it does not label clam GV structures above the background level. ×1600.

at the nuclear periphery of the bovine heart artery endothelial cells in Fig. 5, J9 seemed to stain some intranuclear material (Fig. 5A), whereas J16 was exclusively localized at the NE (Fig. 5B). When isolated clam oocyte nuclei were probed with these two monoclonal antibodies, only J9 reacted with those nuclei (Fig. 5C), decorating the NE prominently and reacting slightly with the chromosomes and the nuclear interior. J16 did not react with the clam nuclei, as might be predicted from the blotting experiment (Fig. 5D). The antigen recognized by J16 is therefore not prominent in the clam GV.
Demonstration of carbohydrate moieties

If the mammalian lamins react with lectins as suggested recently (Guinivan, Noonan & Noonan, 1980), they should contain carbohydrates. The clam 67×10^3 M₉ protein, if lamin-like as indicated by antigenic relatedness, should then also react like a glycoprotein. To test whether the lamins are glycoproteins, mouse L cells were grown in the presence of \[^{14}C\]mannose for 24 h before isolating nuclei and the NEs. Many glycoproteins were found in the insoluble nuclear residue, but only faint bands appeared over a general background in the area of the lamins in autoradiograms of one-dimensional SDS/polyacrylamide gels. Diagonal electrophoresis was therefore used to separate the lamins, since minor cytoplasmic contamination of the nuclear preparation from tissue-culture cells could have resulted in considerable contamination after removal of the bulk of the nuclear proteins by extraction with 1·6 M NaCl (see also Lebkowski & Laemmli, 1982). The lamin-enriching effect of extraction with 1% SDS in the absence of reducing agents as shown for the clam NE (Fig. 3) was sufficient to permit positive identification.

Immunoblotting techniques were then used to mark the expected location of lamins A, C and lamin B. The Coomassie Blue-stained gel shows the general distribution of the unreduced rat NE proteins (Fig. 6A). There is substantial staining of proteins that barely enter the separating gel, and three bands migrate to the predicted location of the lamins (arrows). The slowest moving lamin (or lamin B in our preparations) is present in reduced amounts. Probing nitrocellulose blots of this material with clam anti-NE antibodies reveals reaction product at the top of the gel and a single line where lamin B and its breakdown product migrate (Fig. 6C). If the reduced gel strip is re-electrophoresed, blotted and probed, two spots at the same height can be identified (Fig. 6A). If the same is done with antibodies reacting with lamins A and C, two spots of unequal height appear (Fig. 6D), or if the unreduced gel is probed (Fig. 6E), two lines appear representing lamins A and C. Thus, the part of lamin B that had been cross-linked by disulphide bonds migrated off the diagonal, indicating that the material at the top of the separating gel is lamin B (see also Kaufmann et al. 1983).

If diagonal electrophoresis is carried out with a NE fraction from mouse L cells prelabelled with \[^{14}C\]mannose, the same spots can be seen after autoradiography of the dried gel. Fig. 6F shows the stained gel, arrows pointing left identify lamins A and C, and the arrowhead pointing down indicates the location of lamin B. Fig. 6F represents the autoradiograph, with the same labelling. The immunological cross-reactivity of a protein migrating in the same position after diagonal electrophoresis (i.e. off the diagonal) as that demonstrated autoradiographically is taken as evidence that these proteins are lamin B. Lamins A and C as well as B incorporate mannose and are thus glycoproteins.

Because direct incorporation experiments to test whether the clam 67×10^3 M₉ protein is a glycoprotein are not possible with fully mature clam eggs, an indirect approach was used by labelling isolated FLPC with \[^{3}H\]borohydride to reduce the aldehydic functions generated on carbohydrate moieties by galactose oxidase (see
Materials and Methods). The autoradiographic demonstration of \[^{3}H\]borohydride indicates the presence of glycoproteins (Fig. 7). Thus, the clam $67 \times 10^3 M_r$ protein has carbohydrate groups, as do the rat lamins. The rat gp190 (Gerace et al. 1982) was
Clam GV envelope protein is lamin-like

Fig. 7. Autoradiograph of clam (lane 1) and rat (lane 2) FLPC after reduction of the modified sugar residues with [3H]borohydrate. The lamins of both clam and rat are labelled. The marker on the right indicates where BSA migrates (68×10^3 M_r). The circle denotes the clam 67×10^3 M_r protein; and the lines in lane 2, the rat lamin.

not apparent. This may be due to removal of the bulk of gp190, if detergents and high-salt extraction are used for the isolation of the FLPC.

Fig. 6. Localization of lamins A, B, C on nitrocellulose blots after diagonal electrophoresis, by immunoperoxidase staining and demonstration of carbohydrate incorporation into the lamins. A. Rat NEs separated without reduction. Arrowheads point to lamins A, B and C; Coomassie Blue-stained. B. The unreduced proteins were reduced in the gel and re-electrophoresed before probing with total clam NE antibody. The left-hand one (arrowhead) is removed from the diagonal and represents lamin B, which was cross-linked by disulphide bonds. The right-hand one (arrow) is the lamin B monomer. C. The cross-linked lamin B (left), the monomer (middle band) and a lamin B breakdown product (right). D. The same blots as in B but incubated with antibodies that cross-react with rat lamins A and C. Both lamins are seen only on the diagonal. E. Reaction product in unreduced gel using the same antibodies as in D. F. Coomassie Blue-stained pattern in mouse L-cell NE proteins after incorporation of [14C]mannose for 24 h and diagonal electrophoresis. The arrowhead points to where lamin B migrates; the arrows point to lamins A and C. G. Autoradiograph of F.
DISCUSSION

We have previously reported (Maul & Avdalovic, 1980) that NEs from *S. solidissima* oocytes contain two dominant proteins, one of which has an apparent molecular weight of $67 \times 10^3$. We speculated that this protein was the equivalent of the rat lamin B, because it was attached to the NE at the late meiotic prophase in a way similar to the membrane attachment of rat lamin B in mitotic tissue-culture cells (Gerace & Blobel, 1980). Others have suggested that the single major protein characteristic of *Xenopus* oocyte GV of similar molecular weight is a pore complex protein (Stick & Krohne, 1982; Krohne et al. 1978, 1982). If both this $68 \times 10^3$ protein and its counterpart from clam (the $67 \times 10^3$ clam NE protein) are lamins they should not be pore complex proteins, as shown by Gerace et al. (1978, 1982) for rat lamins.

A comparison of the major GV NE protein with the major proteins (lamins) of the rat liver NE was therefore undertaken to determine whether or not the presumptive pore protein (Stick & Krohne, 1982; Krohne et al. 1978, 1982) has additional properties in common with rat lamins.

The isoelectric point of the clam protein is intermediate between that of rat lamins A and C and that of rat lamin B. This difference in isoelectric point may indicate that the clam $67 \times 10^3$ protein is different from the rat lamins or might represent an evolutionary change of the same protein. There may be tissue differences or the mitotic form of the lamins might always be more acidic due to increased phosphorylation, as observed by Gerace & Blobel (1980). Though the NE is still intact in the clam oocyte, these cells are arrested in meiosis at late prophase when the chromosomes are condensed. That these cells are very nearly mitotic is indicated by the fact that GV breakdown occurs within 10 min after sperm penetration of the GV, yielding a mitotic cell. Assuming that phosphorylation has resulted in an acidic shift (Gerace & Blobel, 1980), the pI is closer to that of the rat lamins A and C in the less phosphorylated state of a non-mitotic cell.

Guinivan et al. (1980) suggested that the lamins may be glycoproteins. They found that iodine-labelled lectins bound to proteins co-migrating with the lamins. If the lamins of somatic cells contain carbohydrate groups, then the clam $67 \times 10^3$ protein, if it is a lamin-like protein, should also contain them. We therefore first verified that the somatic lamins contain carbohydrate moieties, by direct incorporation of mannose into mouse L-cell lamins. We then used an indirect demonstration of carbohydrates on the clam protein, since the egg is not synthetically very active after it is shed. Our evidence suggests that labelled borohydrate is associated with both the clam and the rat protein and, therefore, that both the clam protein and the rat lamins contain carbohydrate groups.

The clam protein then has the following properties in common with the rat lamins. It can be phosphorylated *in vitro* (Maul & Avdalovic, 1980; Gerace & Blobel, 1980; Lam & Kasper, 1979), it contains carbohydrate groups and has a comparable molecular mass. It also cross-links by disulphide bonds (see also Maul & Avdalovic, 1980; Lebkowski & Laemmli, 1982; Kaufmann et al. 1983; Shelton & Cochran, 1978). This cross-linking was induced but occurs in a non-random fashion, resulting
Clam GV envelope protein is lamin-like

in trimers. Polyspecific antibodies against the clam \(67 \times 10^3 M\), protein cross-react with rat lamin A and C (Maul & Baglia, 1983), and monoclonal antibodies against rat lamin AC cross-react with the clam protein, demonstrating that these proteins are immunologically related. Others (Gerace & Blobel, 1980, 1982; Krohne et al. 1978) have also demonstrated that antibodies against rat lamins cross-react with nuclei from an evolutionary very divergent group of animals. Finally, the localization operationally defined by the isolation procedure and by immunofluorescent localization is the same, namely the NE. These data support a lamin-like function for the clam \(67 \times 10^3 M\), protein rather than a role in the nuclear pore complex.

The *Xenopus* oocyte NE, like that of the surf clam, contains a major protein in the size range of the rat lamins. The *Xenopus* NE protein was considered the major structural protein of the nuclear pore complex by Krohne and colleagues (Stick & Krohne, 1982; Krohne et al. 1978, 1982) and reported to be different from any of the lamins (Krohne et al. 1981). In their more recent report, immunohistological evidence was presented (Stick & Krohne, 1982) for the localization of the \(68 \times 10^3 M\), protein on the pore complex and the pore inter-connecting material. None of the rat lamins has been localized on the pore complex (Gerace et al. 1978, 1982; Gerace & Blobel, 1982), nor do antibodies against purified chicken lamin A cross-react with *Xenopus* GV pore complexes (Stick & Krohne, 1982). The antibody used in their study that localized on the pore complex was raised against the total karyoskeletal residue of extracted *Xenopus* oocyte nuclei. When we prepared polyclonal antibodies to a similar fraction from clam oocytes, we also found a predominant reaction with the clam \(67 \times 10^3 M\), protein but, in addition, we observed reactivity with many other nuclear antigens. If antibodies against total karyoskeletal residues of *Xenopus* GV recognize the same major antigen from *Xenopus* RBC (lamin A) and oocyte GV (lamin-like protein) but label the pore complex, one may have to consider minor contaminating antigens responsible for pore-complex binding antibodies. Such an explanation would support the idea that pore complexes, like ribosomes, may consist of a complex set of minor proteins (Maul & Baglia, 1983; Aaronson, 1982).

Antibodies that detect lamin B only were initially difficult to prepare, particularly in rabbits (Gerace et al. 1978). It was therefore surprising to find a blotting reaction specific for rat lamin B in the rat NE when probed with antibodies directed against total native clam NE. This finding suggests that at least some protein equivalent to rat liver lamin B must be present in clam nuclei and NEs. The antigen must be exposed in such a way that antibodies elicited by total NE recognize it more readily in NE than the major clam lamin. Clam NE has pore complexes at a much higher density than rat liver NE, but contains considerably less lamin B. Thus, it is unlikely that lamin B is a major pore complex protein, though it may still be present in the pore-connecting material or on the membrane, both of which are strongly reduced in amount relative to somatic cells with lower pore complex frequency. The same conclusion can be drawn from the results of Stick & Krohne (1982). Most recently, Stick & Schwarz (1983) reported that a lamin is present during specific stages of meiosis in oocytes. We predict that this lamin is the protein previously shown to be the major pore complex protein of *Xenopus* oocytes, by Stick & Krohne (1982).
The relatedness of all three lamins was initially supported by the finding that antibodies against each of them cross-react with all the other lamins (Gerace et al. 1978) and the observation that major internal homologies are revealed in peptide mapping (Shaper et al. 1979). Lately, however, lamin B has been reported to differ substantially from lamin A and lamin C (Kaufmann et al. 1983; Gerace & Blobel, 1982), where lamin C is considered to be a degradation product of lamin A (Shelton & Cochran, 1978). Kaufmann et al. (1983) found no evidence of relatedness between lamin B and lamin A using two-dimensionally separated peptides, and antibodies elicited by the lamins do not cross-react (Gerace et al. 1982; Gerace & Blobel, 1982). It is interesting in this respect that the monoclonal antibody J16 reacts with all three lamins, demonstrating the existence of at least one common antigenic site (see also Burke et al. 1983), which is not shared by the clam 67×10^3 Mr protein. However, monoclonal antibody J9 does define an epitope, present on lamins A(C) but missing from lamin B, which does occur on the clam 67×10^3 Mr protein.

The expert technical assistance of Marie Gileno is acknowledged. This work was supported by grants GM-21615 from the National Institute of General Medicine and CA-10815 from the National Cancer Institute.

REFERENCES


Clam GV envelope protein is lamin-like


(Received 13 September 1983—Accepted 23 September 1983)