

## Behaviour of the Germ Cell Specific Lamin Through Mammalian Spermatogenesis as Probed with Monoclonal Antibodies\*

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**ABSTRACT.** We had earlier identified a 60 kDa nuclear lamin protein (lamin<sub>g</sub>) unique to the germ cells of rat testis which was subsequently shown to be antigenically conserved in germ cells of grasshopper, rooster, frog and plants. We have now obtained eight monoclonal antibodies in mouse against this lamin<sub>g</sub> antigen. While all the eight Mabs reacted with lamin<sub>g</sub> antigen in an immunoblot analysis, only three Mabs (*A*<sub>11</sub>*C*<sub>7</sub>, *A*<sub>11</sub>*D*<sub>4</sub>, *C*<sub>1</sub>*F*<sub>7</sub>) showed strong reactivity in the immunofluorescence analysis of the germ cells. The Mabs *A*<sub>11</sub>*C*<sub>7</sub> and *A*<sub>11</sub>*D*<sub>4</sub> showed a slight cross-reactivity with rat liver lamin B. Indirect immunofluorescence analysis of pre-meiotic, meiotic and post-meiotic germ cells with Mabs have shown that while the lamin<sub>g</sub> is localized in the lamina structures of spermatogonia and round spermatids, it is localized to the phase dense regions of pachytene spermatocytes which is in conformity with our previous observations using rabbit polyclonal antibodies. The localization of the antigen in the germ cells was also confirmed by immunohistochemical staining of the thin sections of seminiferous tubules. By immunostaining the surface spread pachytene spermatocytes, the antigen was further localized to the telomeric ends of the paired homologous chromosomes. Using anti-somatic lamin B antibodies, we have also demonstrated the absence of somatic lamins in meiotic and post-meiotic germ cells. The lamina structure of pre-meiotic spermatogonial nucleus contains both somatic lamin B and lamin<sub>g</sub> as evidenced by immunofluorescence studies with two differently fluorochrome labelled anti-lamin B and anti-lamin<sub>g</sub> antibodies. The selective retention of lamin<sub>g</sub> in the pachytene spermatocytes is probably essential for anchoring the telomeric ends of the paired chromosomes to the inner nuclear membrane.

The nuclear lamina is an important ubiquitous proteinaceous structure present in eukaryotic cells adjacent to the inner nuclear membrane (15). In addition to providing mechanical stability to the shape of the nucleus, the lamina structure also provides attachment sites for chromatin in the interphase nucleus. In the vertebrates, this lamina structure is composed of three major polypeptides, namely lamins A, B and C, which have a molecular mass in the range of 60 to 80 kilodaltons. Recently, Kaufmann (13) has reported two more acidic lamins, D and E, in the rat liver. Furthermore, lamin B which was originally thought to be a single polypeptide is now shown to be made up of a major component lamin *B*<sub>1</sub> and a minor one lamin *B*<sub>2</sub> (17).

The nuclear lamina structure is dynamic in nature. For example, during cell division, at the metaphase, the lamins undergo hyperphosphorylation. Consequently, the lamina structure is depolymerized to yield individual lamina polypeptides. During this process, lamins A

and C have been shown to be present in the cytoplasm, while lamin B is associated with the membrane vesicles (8). The depolymerization of the lamina structure is believed to be essential for the condensation of the chromosomes. Following the mitotic division, the lamina structure is reformed at the telophase interval (7). The disassembly of the lamina structure is also observed at the onset of meiosis (26). Such a disassembly process is again thought to be necessary to facilitate chromosome condensation. By using specific antibodies to somatic lamins, Stick and Schwarz (26) observed that there are no remnants of the lamina structure in the meiotic prophase pachytene nucleus. Furthermore, they could not detect any lamina structure in the haploid round spermatids using the same antibodies. However, we have recently shown the presence of a germ cell specific lamin which is expressed only in the germ cells of various eukaryotic species (1, 27). We have termed this lamin as lamin<sub>g</sub> and shown it to be retained in the pachytene spermatocyte nucleus even though the lamina structure has disassembled. This lamin<sub>g</sub> also becomes the sole component of the lamina structure of the haploid round sper-

\* This paper is dedicated to the memory of Dr. L. Sudhakar.

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matid nucleus. The rabbit polyclonal antibodies against the lamin<sub>g</sub> which was used in most of these studies showed very little cross-reactivity with the somatic lamins A, B and C of rat (28). These observations prompted us to generate specific monoclonal antibodies against lamin<sub>g</sub> in order to study the epitopes that are exclusively present in lamin<sub>g</sub>, but not in other somatic lamins. Moreover, these monoclonal antibodies would be valuable tools for studies on the expression of this antigen during the spermatogenic process. We have been successful in generating a set of monoclonal antibodies against lamin<sub>g</sub> in mouse and we describe here our studies on the nature and the extent of cross-reactivity these monoclonal antibodies exhibit with somatic lamins. We are also describing the results of the immunolocalization of this antigen in pre-meiotic, meiotic prophase and post-meiotic germ cells using these monoclonal antibodies.

#### MATERIALS AND METHODS

**Materials.** All the biochemicals used, unless specified were from Sigma Chemical Company, St. Louis, MO, U.S.A. FITC labelled goat anti-rabbit IgG, from Cappel Laboratories Inc., Cochranville, PA, U.S.A., HRP-conjugated goat anti-rabbit IgG, rabbit anti-mouse IgG and FITC labelled goat anti-mouse IgG were purchased from Bangalore Genei, Bangalore, India. Nitrocellulose sheets were obtained from Schleicher & Schuell, Dassel, Germany.

All plasticware used for raising monoclonal antibodies were purchased from Becton and Dickinson Labware. Polyethylene glycol 3500, Nystatin, glutamine, hypoxanthine, aminopterin, thymidine and p-nitrophenyl phosphate were obtained from Sigma Chemical Company, U.S.A. Iscove's modified Dulbecco's medium (IMDM) was purchased from Gibco Laboratories, U.S.A. Fetal calf serum was the product of SERA labs, U.K. Pencillin, streptomycin sulfate and gentamycin were obtained from Alembic Chemicals, Sarabhai Chemicals and Pharmaceutical company of India, Bombay, respectively. All other reagents used were of analytical reagent grade.

**Animals.** Male albino rats of Wistar strain were used in all the studies. Splenocytes from immunized BALB/c mice were used for establishing hybridoma.

**Purification of nuclei.** Rats were sacrificed by cervical dislocation, testes removed and decapsulated. Livers were perfused with ice-cold physiological saline before they were excised. The tissues were homogenized in 9 volumes of buffer A (10 mM Tris-HCl, pH 7.5/25 mM KCl/5 mM MgCl<sub>2</sub>/1 mM CaCl<sub>2</sub>) containing 0.34 M sucrose in a Teflon Potter-Elvehjem homogenizer. Testicular tissue was homogenized by hand while livers were homogenized with a motor-driven pestle. Nuclei were isolated from the homogenate as described by Rao *et al.* (22).

**Preparation of sonication-resistant spermatid nuclei.** Sonication-resistant nuclei from rat testes represent nuclei

from elongating and elongated spermatids. They were isolated from the testes of 60 day old rats as described by Singh and Rao (24). Briefly, testes were homogenized in buffer B (10 mM Tris-HCl, pH 7.4/0.1 mM phenyl methyl sulfonyl fluoride /0.1% Triton X-100) containing 0.34 M sucrose, filtered through cheese cloth and centrifuged at 1,000 × g for 10 minutes. The pellet was suspended in buffer B and sonicated for 12 bursts at setting 6 for 15 seconds (each duration) with 45 seconds intervals in a Branson (Model B-30) sonifier. The sonicate was then layered on a 10 ml cushion of 1.5 M sucrose in buffer B and centrifuged at 1,000 × g for 30 minutes. The purity of the pelleted sonication resistant nuclei was checked under a microscope.

**Preparation of liver nuclear lamina and the DNase 1 and high salt resistant fraction of sonication resistant nuclei.** Nuclear lamina fraction was isolated from purified rat liver nuclei essentially according to the method of Dwyer and Blobel (5). The sonication resistant nuclei of rat testes were also subjected to a similar procedure as described by Sudhakar *et al.* (28) to obtain in DNase 1 and high salt resistant fraction.

**SDS-PAGE, Electroelution and Immunoblotting.** Analysis of the polypeptides was done on SDS 5–15% gradient polyacrylamide gels essentially according to the method of Laemmli (16). The polypeptides were visualized by staining with 0.1% coomassie brilliant blue. In some cases, the gel was stained with AgNO<sub>3</sub> as described by Merrill *et al.* (19).

For electroelution of lamin<sub>g</sub>, the polypeptides of the sonication resistant nuclei were separated on a gradient gel as described above and stained with KCl as described by Hager and Burgess (10). The 60 kDa polypeptide bands, reacting with polyclonal anti-lamin<sub>g</sub> antibodies in a parallel immunoblot, were excised out from several lanes. They were then placed in the large well of the sample cup of the ISCO sample concentrator (Model 1750). The sample cup and the inner compartment buffer composition was 0.057 M Tris-acetate, pH 8.6/0.002 M EDTA/0.001% SDS. The composition of the electrode buffer was 0.1 M Tris-acetate, pH 8.6/0.002 M EDTA/0.001% SDS. Electroelution was carried out for 12 hr. at 4°C following which all the fractions of the sample cup were collected and lyophilized. The residue was dissolved in double distilled water, dialyzed against 1% acetic acid and relyophilized.

For immunoblotting studies, proteins were transferred electrophoretically onto a nitrocellulose sheet according to the method of Towbin *et al.* (29) for 2 hr. at 1 Amp current using a Hoeffer Transphor electrophoresis unit. On the nitrocellulose sheet lamin<sub>g</sub> antigen was detected by using HRP-conjugated goat anti-rabbit IgG (for polyclonal antibodies) or HRP-conjugated rabbit anti-mouse IgG (for monoclonal antibodies) as secondary antibodies at a dilution ranging from 1 : 500 to 1 : 2000.

**Generation of monoclonal antibodies.** For immunization of BALB/c mice, the lamin<sub>g</sub> bands were cut out from a coomassie blue stained gel of sonication resistant nuclear associated proteins. A gel piece containing approximately 10 μg of lamin<sub>g</sub> was inserted subdermally on the dorsolateral side of

the torso of each mouse through an incision which was sutured later. The mice were subjected to this protocol three times at 21 day intervals. Test serum was obtained from the orbital plexus 9th day after the third administration. Three days before the scheduled fusion, mice were injected intraperitoneally with approximately 20  $\mu\text{g}$  of the electroeluted antigen in saline. Splenocytes were fused with Sp2/O mouse myeloma cells to generate hybridoma using standard procedures (32). The screening procedures used at various stages of generating monoclonal antibodies included ELISA (11), Dot-binding assay (12), solid phase radioimmuno assay (11) and finally the immunoblotting technique. One month after fusion, subcloning was carried out by the method of limiting dilution to obtain monoclonals.

**Immunofluorescence microscopy.** Indirect immunofluorescence studies on purified nuclear preparations were done according to the method of Mura and Stollar (20) with minor modifications. Freshly prepared nuclei were washed with TKM (50 mM Tris-HCl, pH 7.5/25 mM KCl/5 mM  $\text{MgCl}_2$ ) containing 0.2% Triton X-100. The nuclear pellet was resuspended in TKM and filtered through 4 layers of cheese cloth. A small aliquot of the suspension was spread on a clean microscopic slide. After air drying, nuclei were fixed by adding a drop of fixative (freshly prepared 4% paraformaldehyde in 0.1 M sucrose). The slides were kept aside for 5 min, and were washed 3–4 times with TKM in a petri dish. Approximately, 200  $\mu\text{l}$  of the culture supernatant containing monoclonal antibodies was placed on the nuclear spread and incubated at room temperature for 1 hr. The slides were subsequently washed several times with TKM. A 1 : 100 dilution of FITC labelled goat anti-mouse IgG was then spread on the slide and incubated for 1 hr at room temperature. The excess secondary antibody was washed with TKM. The nuclear spread was then air dried and mounted with 50% glycerol in TKM and viewed under a Zeiss Axioplan microscope fitted with epifluorescence attachment. Photographs were taken with either ORWO NP27/ILFORD ASA 400 or KONICA ASA 400 colour films.

**Immunohistochemical staining of seminiferous tubules.** Testes from a 30 day old rat was excised and immediately immersed in Bouin's fixative. After 2 minutes, they were cut transversely into 3–4 mm pieces and immersed in the fixative for 8 hours. After washing and dehydration, paraffin blocks were made. Sections of 3–4  $\mu\text{m}$  were then cut with the help of a microtome. After fixing these sections on a slide, they were further dewaxed in xylene for 3 minutes with two changes. The sections were then rehydrated by passing them through graded alcohol series including two changes in absolute ethanol (3 minutes each), 2 changes in 95% ethanol and 2 changes in 70% ethanol. After rinsing the sections in double distilled water, the sections were treated with 0.1% Triton X 100 in phosphate buffer saline (PBS) 2 times. Endogenous peroxidase activity was blocked by incubating the specimen with a solution containing 4 parts of methanol and 1 part of 3%  $\text{H}_2\text{O}_2$  for 20 minutes. The slide was then rinsed with PBS. Subsequently, sections were incubated with 1% normal goat serum

in PBS for 10 minutes to ensure thorough blocking.

The sections were then separately incubated with either Mab  $A_{11}D_4$  (neat supernatant) or normal mouse serum (1 : 1000 diluted in PBS) overnight at 4°C.

After washing the sections 3 times with PBS (for 5 minutes each) they were incubated with rabbit anti-mouse peroxidase conjugate diluted 1 : 50 in PBS for 1 hour and were washed similar manner as mentioned earlier. The sections were then stained with Diaminobenzidine in PBS (6 mg DAB in 12 ml of PBS). To this, 20  $\mu\text{l}$  of 30%  $\text{H}_2\text{O}_2$  was added. The sections were then washed in PBS and mounted in 50% glycerol medium. Colour photographs of sections were taken using Kodak ASA 100 colour film using Zeiss Axioplan microscope. Alternatively, cryostat sections of rat testes were taken, fixed with ice cold acetone, incubated with Mab  $A_{11}D_4$  and later with FITC-labelled rabbit anti-mouse IgG. Photographs were taken using a 400 ASA black and white film.

**Double antibody staining of spermatogonial nuclei.** Nuclei from 10 day old rats were isolated in the same manner as mentioned earlier. A moderately thick suspension was smeared on a clean glass slide and fixed with 4% paraformaldehyde. The nuclei were incubated first with anti liver lamin B antibody and later with monoclonal anti lamin $_{\text{g}}$ - $A_{11}D_4$  with washes in between. Each incubation step was carried out for a period of 1 hr. Subsequently, after washing off excess antibody, the nuclei were incubated with rhodamine labelled goat anti rabbit IgG and later with FITC labelled goat anti mouse IgG. After thorough washing, the slide was air dried, mounted with 50% glycerol in PBS and viewed under the fluorescence microscope. Colour photographs were taken of the phase contrast as well as the fluorescence pattern using different filters. The film used was Konica ASA 400 colour film.

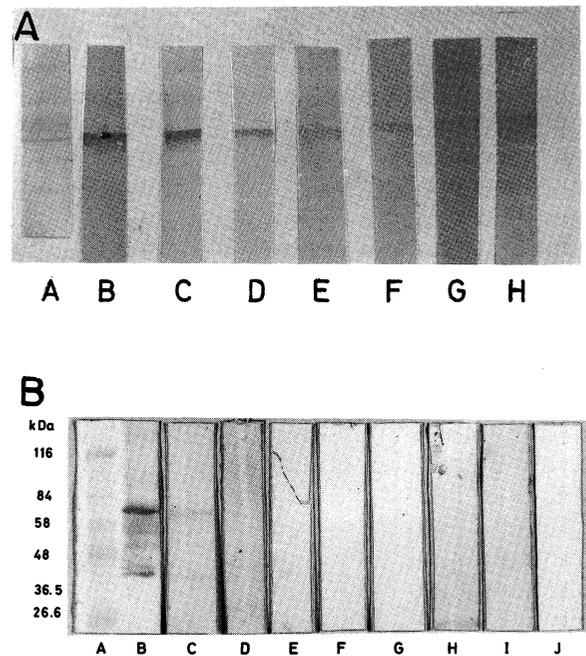
**Immunolocalization in surface spread pachytene spermatocytes.** The seminiferous tubules from the testes of 40 day old rats were thoroughly minced in PBS and incubated on ice for 10 minutes. The supernatant was decanted and centrifuged at 3,500  $\times$  g for 10 minutes. After two washes with ice cold PBS, the cells were suspended in a small volume of PBS to give a medium thick suspension. A 100  $\mu\text{l}$  aliquot was placed on the top of a drop of 0.5% sodium chloride, on a piece of parafilm. After 1 minute, the spread nuclei were picked up on a clean microscopic slide and left to air dry for 5 minutes. Later, the specimen was fixed with 4% paraformaldehyde in 0.1 M sucrose for 30 minutes. Subsequently, the slides were washed gently 3–4 times, with PBS. The nuclear spread was now treated with anti-lamin $_{\text{g}}$  monoclonal antibodies and subsequently with FITC-labelled goat anti-mouse IgG as described above. After taking photographs of the phase contrast and the fluorescence pattern, the slide was processed for silver staining to visualize the synaptonemal complexes (9). A drop of  $\text{AgNO}_3$  solution (1 g/ml in  $\text{H}_2\text{O}$ ) was placed on the nuclear spread and incubated overnight at 60°C in a humidified chamber. The slide was then rinsed briefly in double distilled water and air dried. After mounting with 50% glycerol, the same nucleus was relocated using pre-recorded coordinates and photo-

graphed.

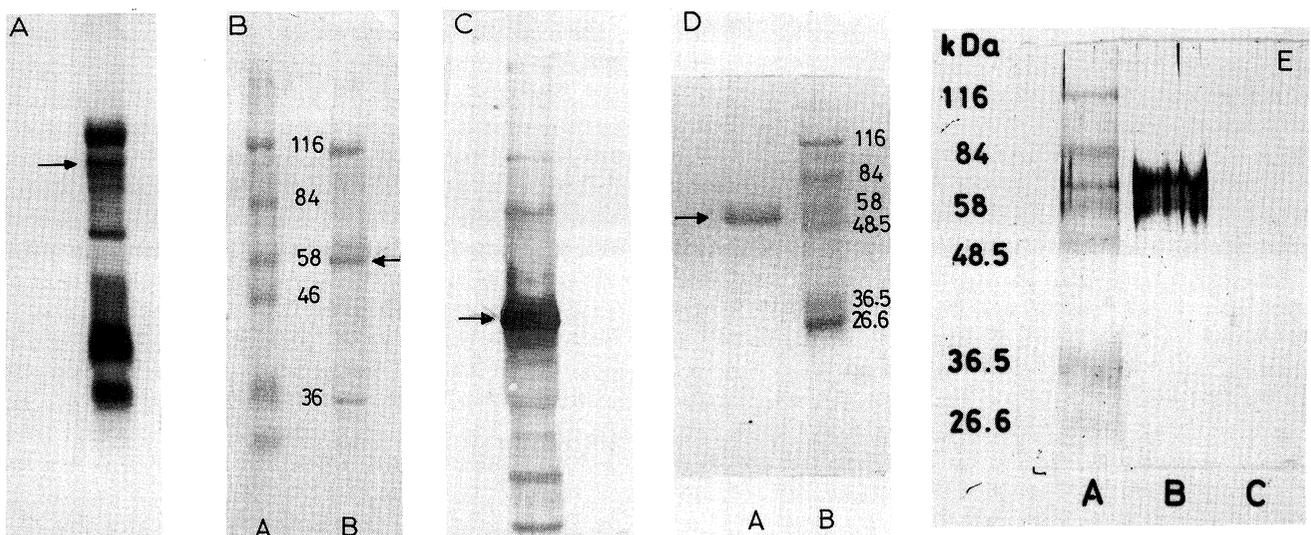
**Analytical procedures.** Protein estimations were done either by the method of Lowry *et al.* (18) or turbidometry (21).

## RESULTS

**Monoclonal antibodies against lamin<sub>g</sub> and their cross-reactivity with somatic lamins.** A previous communication from our laboratory had shown that the lamin<sub>g</sub>, which is the sole component of the lamina structure of round spermatids, persists through spermiogenesis process and is even present in the epididymal sperm nucleus (28). Lamin<sub>g</sub> was a component of the DNase 1 and high salt resistant fraction of sonication resistant nuclei (representing elongating and elongated spermatid nuclei). Although we used the same lamina isolation procedure to obtain this fraction, we have called it as the 'DNase 1 and high salt resistant fraction' since it is not clear as to what type of structure the lamina is transformed into, in the condensed nucleus of the sperm. The SDS-PAGE pattern of the polypeptides associated with this fraction isolated from sonication resistant nuclei is shown in Fig. 1A. We used the DNase 1 and high salt resistant fraction of sonication resistant nuclei as the source of the lamin<sub>g</sub> antigen for raising monoclonal antibodies for two reasons (1). The sonication resistant nuclei can be isolated as a pure fraction from total testicular nuclei and (2) one can get enough starting material to run several gels wherefrom the polypeptide can be iso-



**Fig. 2.** Immunoblot analysis of lamin<sub>g</sub> (A) and liver lamina proteins (B) with mouse monoclonal anti lamin<sub>g</sub> antibodies. Lanes A, B, C, D, E, F, G and H in panel A and lanes C, D, E, F, G, H, I and J in panel B represent immunoblots with Mabs A<sub>11</sub>C<sub>7</sub>, A<sub>11</sub>D<sub>4</sub>, C<sub>1</sub>F<sub>7</sub>, C<sub>1</sub>G<sub>8</sub>, C<sub>8</sub>B<sub>3</sub>, C<sub>8</sub>G<sub>5</sub>, A<sub>2</sub>C<sub>2</sub>, D<sub>9</sub>E<sub>6</sub> respectively. Lane A in panel B is molecular weight markers and Lane B is an immunoblot with rabbit polyclonal anti mouse lamin B antibodies.



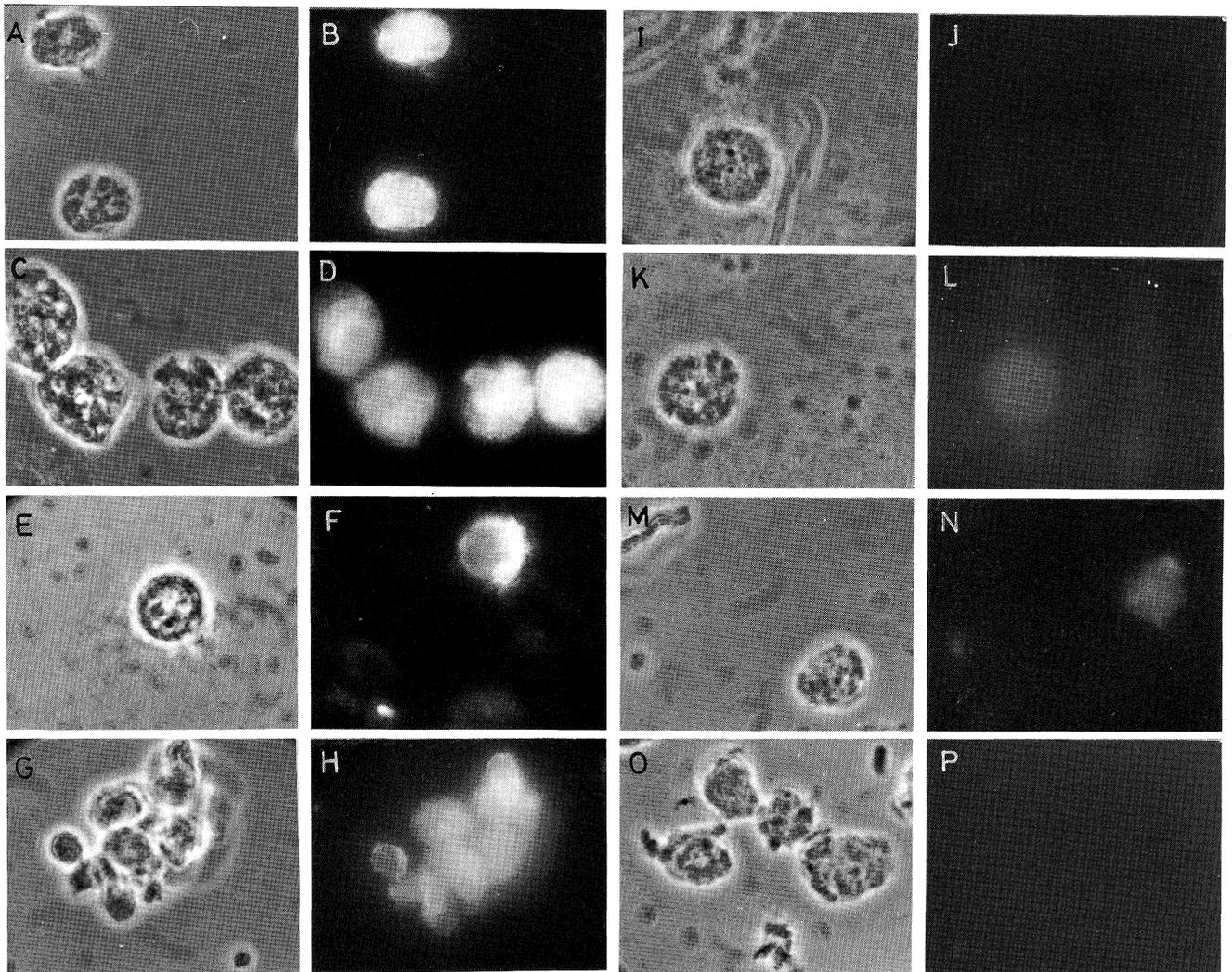
**Fig. 1.** Generation of mouse polyclonal antibodies against lamin<sub>g</sub>. (A) SDS-polyacrylamide gel electrophoretic pattern of sonication resistant nuclei of rat testes. The arrow represents the 60 kDa lamin<sub>g</sub> band which was cut out and used for raising antibodies in mouse. (B) Immunoblot analysis of round spermatid lamina proteins with rabbit polyclonal antibodies (Sudhakar and Rao, 1991) Lane A, molecular weight markers; Lane B, immunoblot. (C) Immunoblot analysis of sonication resistant nuclear proteins with mouse polyclonal anti lamin<sub>g</sub> antibodies. (D) Silver stained pattern of electroeluted lamin<sub>g</sub> (Lane A), Lane B, molecular weight markers. (E) Immunoblot analysis of purified lamin<sub>g</sub> and liver lamina proteins with mouse polyclonal antibodies. Lane A, molecular weight markers; Lane B, electroeluted lamin<sub>g</sub>; C, liver lamina proteins.

lated by electroelution after staining with KCl.

We have tried several protocols for immunizing the mouse and found that subdermal placement of the gel band gave the best immune response. Fig. 1C shows the results of an immunoblot analysis of mouse polyclonal anti-lamin<sub>g</sub> antibodies in comparison with rabbit polyclonal anti-lamin<sub>g</sub> antibodies, shown in Fig. 1B, which clearly indicates the efficiency of the immunization protocol we have used. The specificity of antigen recognition of the mouse polyclonal antibodies was then compared between (a) purified lamin<sub>g</sub> (isolated by electroelution from KCl stained bands, the purity of which is shown in Fig. 1D) and (b) liver lamina proteins. The immunoblot pattern shown in Fig. 1E clearly shows that the antibodies are highly reactive with lamin<sub>g</sub>. Two very faint bands lighted up with the liver lamina proteins.

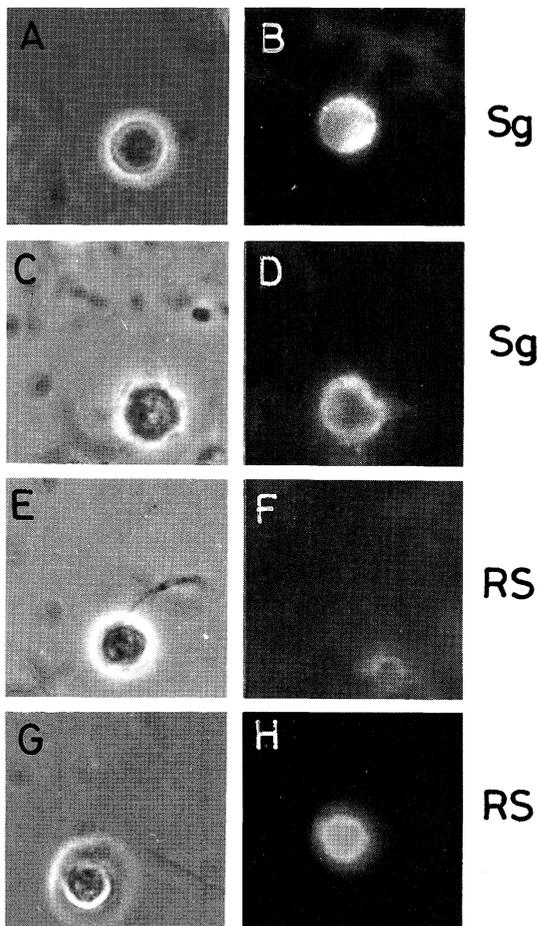
The upper band corresponds to lamin B while the bottom band may be either lamin C or a partially degraded lamin B itself. It may be worth mentioning here that even the rabbit anti-lamin<sub>g</sub> polyclonal antibodies also showed a very faint cross reactivity with liver lamin B (27). In fact, we had inferred that lamin<sub>g</sub> may be a variant form of lamin B based on the iodinated tryptic map analysis.

After confirming the presence of anti-lamin<sub>g</sub> antibodies in the immunized mouse serum, we then proceeded to construct hybridoma cell lines and generate specific monoclonal antibodies. Eight monoclonal antibodies were obtained after various screening procedures and each of them was then finally checked for its reactivity with lamin<sub>g</sub> by immunoblotting. The results of such an analysis is shown in Fig. 2A. It is clear that all the



**Fig. 3A.** Indirect immunofluorescence analysis of pachytene spermatocyte nuclei with anti lamin<sub>g</sub> Mabs, *AB*(A<sub>11</sub>C<sub>7</sub>), *CD*(A<sub>11</sub>D<sub>4</sub>), *EF*(C<sub>1</sub>F<sub>7</sub>), *GH*(C<sub>1</sub>G<sub>8</sub>), *IJ*(C<sub>8</sub>B<sub>3</sub>), *KL*(C<sub>3</sub>C<sub>3</sub>), *MN*(A<sub>2</sub>C<sub>2</sub>) and *OP*(D<sub>9</sub>E<sub>6</sub>). ACEGIKMO represent phase contrast micrographs while BDFHJLNP represent the fluorescence pattern, ×1,000.

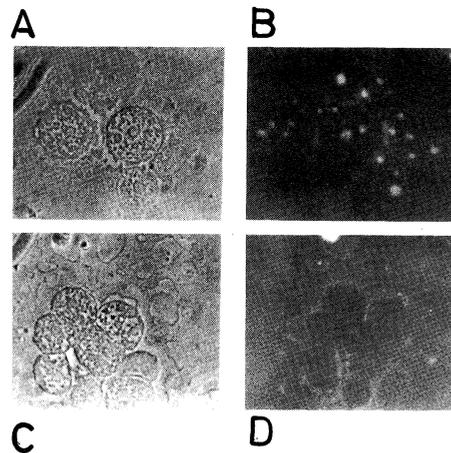
eight monoclonal antibodies reacted specifically with the lamin<sub>g</sub> polypeptide, among with *A*<sub>11</sub>*D*<sub>4</sub>, *C*<sub>1</sub>*F*<sub>7</sub> and *C*<sub>1</sub>*G*<sub>8</sub> showed strong reactions. The differences in the intensity of reaction observed in the immunoblot analysis may reflect differences in the affinities of the monoclonal antibodies with the antigen. Occasionally, there was also a reaction with a band just below the 60 kDa lamin<sub>g</sub> band. Since this was not reproducible in all the immunoblots, it may probably represent partially degraded lamin<sub>g</sub>. All the monoclonal antibodies were then checked for their cross-reactivity with the liver lamina proteins. The results of these immunoblotting studies are shown in Fig. 2B. As a positive control, we have used rabbit anti-mouse lamin B polyclonal antibodies (courtesy of Dr. Veena Parnaik, CCMB, Hyderabad) which is shown in lane B. Among the monoclonals, only *A*<sub>11</sub>*D*<sub>4</sub> and *A*<sub>11</sub>*C*<sub>7</sub> showed a very faint reaction with somatic lamin B. It has been observed that the strongest epitope in lamin B



**Fig. 3B.** Indirect immunofluorescence analysis of spermatogonia and round spermatid nuclei with *A*<sub>11</sub>*C*<sub>7</sub> (A, B, E and F) and *A*<sub>11</sub>*D*<sub>4</sub> (C, D, G and H) anti lamin<sub>g</sub> Mabs. A, C, E and G represent phase contrast micrographs while B, D, F and H represent the fluorescence pattern,  $\times 1,000$ .

lies in a 46 kDa chymotryptic fragment of lamin B (3). Since the two monoclonals *A*<sub>11</sub>*D*<sub>4</sub> and *A*<sub>11</sub>*C*<sub>7</sub> showed only a faint reaction with lamin B, it is possible that this epitope is extensively modified in lamin<sub>g</sub>. The observation that the other six monoclonals did not react with lamin B at all, indicates probably that they might recognize unique epitope(s) in lamin<sub>g</sub> which are absent in lamin B.

*Immunofluorescence studies on the localization pattern of lamin<sub>g</sub> in germ cells using Mabs.* Earlier, we had made a detailed study on the localization of lamin<sub>g</sub> in spermatogonia, spermatocytes and round spermatids by the indirect immunofluorescence technique using rabbit polyclonal antibodies. Now, since we have obtained 8 different monoclonals recognizing the lamin<sub>g</sub> antigen, we have repeated the indirect immunofluorescence studies with each of the monoclonals to get more insight on the pattern of localization of this antigen in the spermatogenic cells. Fig. 3A shows the fluorescence pattern obtained with pachytene spermatocyte nuclei. Among the 8 Mabs, *A*<sub>11</sub>*C*<sub>7</sub>, *A*<sub>11</sub>*D*<sub>4</sub> and *C*<sub>1</sub>*G*<sub>8</sub> showed the strongest fluorescence pattern. The fluorescence pattern is very similar to the ones we have reported earlier using polyclonal antibodies in that it is patchy in appearance with a discontinuous fluorescence pattern around the nuclear periphery. Furthermore, the strongest fluorescence within the nucleus was localized to the phase dense regions. The fluorescence pattern observed with Mabs *C*<sub>1</sub>*F*<sub>7</sub> and *A*<sub>2</sub>*C*<sub>2</sub> was much less intense as compared to the ones observed with *A*<sub>11</sub>*C*<sub>7</sub>, *A*<sub>11</sub>*D*<sub>4</sub> and *C*<sub>1</sub>*G*<sub>8</sub>. On the other hand, there was negligible fluorescence observed with Mabs *C*<sub>8</sub>*B*<sub>3</sub>, *C*<sub>8</sub>*C*<sub>5</sub> and *D*<sub>9</sub>*E*<sub>6</sub>. It is interesting to note here that although all the 8 Mabs showed reaction with the lamin<sub>g</sub> antigen in the immunoblot analysis



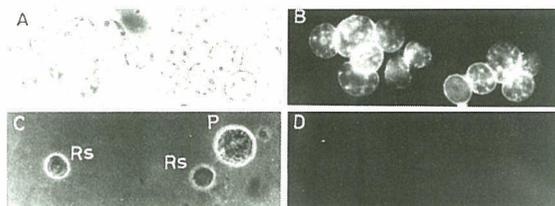
**Fig. 4.** Indirect immunofluorescence pattern of liver nuclei with anti lamin<sub>g</sub> Mabs, *A*<sub>11</sub>*C*<sub>7</sub> (A, B) and *A*<sub>11</sub>*D*<sub>4</sub> (C, D). A and C represent phase contrast micrographs while B and D represent the fluorescence pattern,  $\times 1,000$ .

(Fig. 2A), only 5 Mabs showed significant fluorescence in the pachytene nucleus. These results, therefore, suggest that the two sets of Mabs may be recognizing two different regions in the protein. The epitope(s) recognized by Mabs *C<sub>8</sub>B<sub>3</sub>*, *C<sub>8</sub>C<sub>5</sub>* and *D<sub>9</sub>E<sub>6</sub>* may not be accessible in lamin<sub>g</sub> in the lamina structure while it is accessible in the immunoblot analysis.

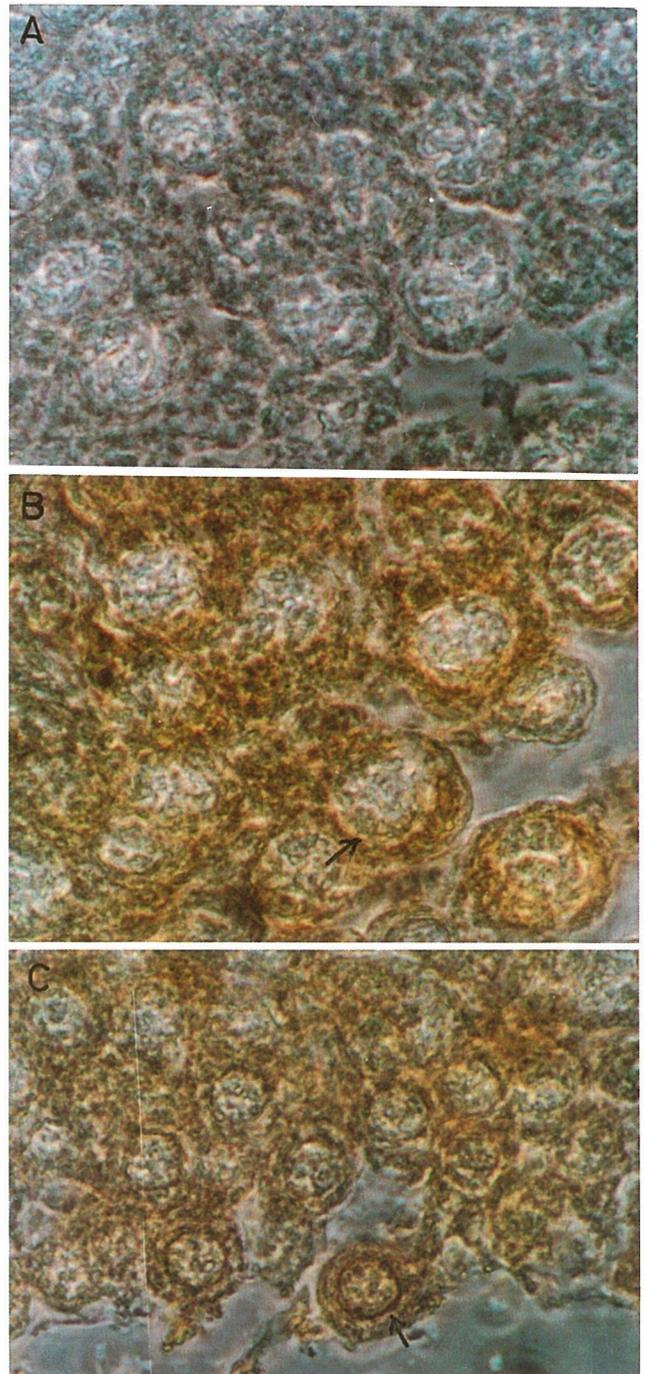
Among the 8 Mabs, *A<sub>11</sub>D<sub>4</sub>* and *A<sub>11</sub>C<sub>7</sub>* which showed strongest fluorescence in pachytene spermatocyte nucleus were then used to screen the pre-meiotic spermatogonial nuclei and post-meiotic round spermatid nuclei. As can be seen from the immunofluorescence pattern shown in Fig. 3B, it is clear that both the Mabs decorated the nuclear periphery characteristic of the laminar fluorescence. The other Mabs which were negative with respect to fluorescence in the pachytene nucleus (*D<sub>9</sub>E<sub>6</sub>*, *C<sub>8</sub>B<sub>3</sub>*, *C<sub>8</sub>C<sub>5</sub>*) were also negative in spermatogonial and round spermatid nuclei (data not shown). While both the Mabs exhibited comparable reactivities with the spermatogonial nuclei the immunofluorescence intensity was much stronger in round spermatid nuclei with the Mab *A<sub>11</sub>D<sub>4</sub>* as compared to the Mab *A<sub>11</sub>C<sub>7</sub>*. A closer look at the fluorescence pattern of pachytene nuclei with these two Mabs also reveal that the intensity with *A<sub>11</sub>D<sub>4</sub>* is a little stronger than *A<sub>11</sub>C<sub>7</sub>*. It may be pertinent to mention here that the Mab *A<sub>11</sub>D<sub>4</sub>* also exhibited a stronger reaction than Mab *A<sub>11</sub>C<sub>7</sub>* with the lamin<sub>g</sub> even in the immunoblot. These observations suggest that the Mabs *A<sub>11</sub>C<sub>7</sub>* and *A<sub>11</sub>D<sub>4</sub>* may be recognising different epitopes in lamin<sub>g</sub>.

Since Mabs *A<sub>11</sub>D<sub>4</sub>* and *A<sub>11</sub>C<sub>7</sub>* showed a faint cross-reactivity with somatic lamin B in the immunoblot analysis (Fig. 2B), we were curious to see whether they can immunostain the liver nuclei. The results shown in Fig. 4 indicate that both the Mabs yielded only faint laminar fluorescence, in contrast to the strong laminar fluorescence observed in the positive control using anti-lamin B antibodies (Fig. 5).

All the immunofluorescent studies described above and in our earlier reports (27, 28) were carried out on isolated testicular nuclei. In order to confirm that these



**Fig. 5.** Indirect immunofluorescence pattern of liver nuclei (A and B) and germ cells (C and D) with rabbit polyclonal anti lamin B antibodies. A and C represent phase contrast micrographs while B and D represent the fluorescence pattern. P, pachytene; Rs, round spermatids. ×1,000.



**Fig. 6.** Immunohistochemical localization of lamin<sub>g</sub> in thin sections of seminiferous tubules. A section treated with normal mouse serum (1 : 1000 dilution in PBS). B and C sections treated with Mab *A<sub>11</sub>D<sub>4</sub>*. Subsequently, the sections were processed for peroxidase staining as described in Methods. D represents a thin section stained with FITC secondary antibodies. A, B and C; ×1,000 magnification and D, ×400 magnification. Shaded arrows point to round spermatids. Blank arrows point to pachytene nuclei.

**Table I.** CHARACTERISATION OF MONOCLONAL ANTIBODIES AGAINST THE GERM CELL SPECIFIC LAMIN.

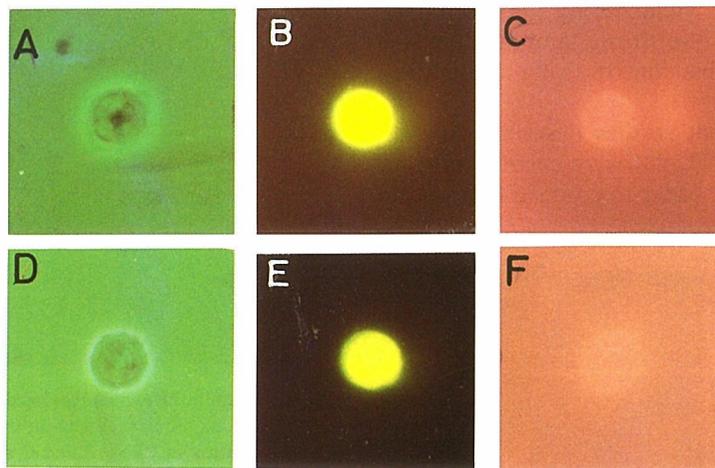
Monoclonal Antibody	Isotype	Reactivity with $L_g$ in immunoblot	Reactivity with LLB in immunoblot	Fluorescence in Nuclei			
				liver	Sg	Pac	Rs
$A_{11}C_7$	$IgG_3$	*	*	Faint dotted	*	Patchy	*
$A_{11}D_4$	$IgG_{2b}$	**	Faint	Faint laminar	Laminar *	Patchy **	Laminar **
$C_1F_7$	$IgG_{2b}$	**	—	—	—	Patchy *	—
$C_1G_8$	—	**	—	—	—	Patchy **	—
$C_8B_3$	$IgG_1$	*	—	—	—	—	—
$C_8C_5$	$IgG_1$	*	—	—	—	—	—
$A_2C_2$	$IgG_{2b}$	*	—	—	—	Patchy	—
$D_9E_6$	$IgG_1$	*	—	—	—	—	—

S<sub>g</sub>: spermatogonia. pac: pachytene; R.S.: Round spermatids; \*\* Strong. \* Weak.

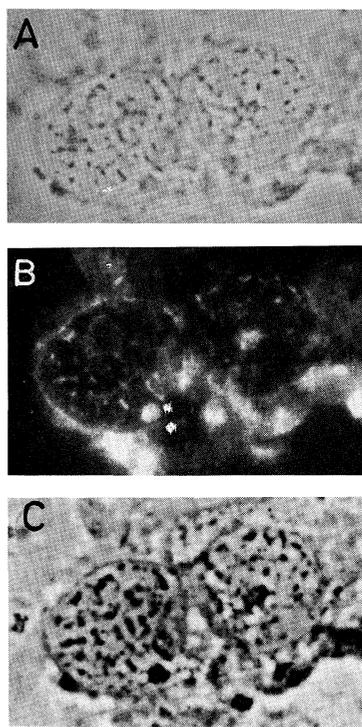
results are not an artefact, we have also carried out localization of the lamin<sub>g</sub> antigen in thin sections using Mab  $A_{11}D_4$ . These results are presented in Fig. 6. It is clear that the antigen shows a laminar localization in both pachytene spermatocyte (Figs. 6B and 6D) and round spermatid (Fig. 6C) nuclei, confirming the observations made with isolated nuclei.

As mentioned in the Introduction, Stick and Schwarz (26) could not detect lamina proteins either in pachytene spermatocytes or in round spermatids. We had argued in our previous paper that the lack of detection of the lamina structure in their studies was due to the use of antibodies against somatic lamin B. In order to make

sure that there are no experimental artefacts in our studies using both the polyclonal antibodies (27) and monoclonal antibodies (present study), we have carried out indirect immunofluorescence studies of both liver and germ cell nuclei with polyclonal anti-mouse lamin B antibodies. These results presented in Fig. 5 clearly show that while these antibodies exhibited strong laminar fluorescence in liver nuclei, the same antibodies did not decorate nuclei either from pachytene spermatocytes or round spermatids. The punctate kind of fluorescence of the lamina structure has also been observed by Burke *et al.* (3). Thus, these results confirm our earlier conclusion that a germ cell specific lamin which exhibits



**Fig. 7.** Colocalization of lamin B and lamin<sub>g</sub> in spermatogonia. Spermatogonial nuclei were doubly stained with rabbit polyclonal anti lamin B antibodies and anti lamin<sub>g</sub> Mab  $A_{11}D_4$ . The lamin<sub>g</sub> and lamin B antigens were detected by FITC labelled anti rabbit IgG in goat and rhodamine labelled anti rabbit IgG in goat respectively. A, B, C and D, E, F represent two different spermatogonial nuclei. A and D represent the phase contrast micrographs. B and E represent the fluorescence pattern of FITC conjugated secondary antibody while C and F represent the fluorescence pattern of rhodamine conjugated secondary antibody.



**Fig. 8.** Localization of lamin<sub>g</sub> antigen in surface spread spermatocytes. Surface spread spermatocytes were prepared as described in Methods. The microscope slide was first treated with Mab A<sub>11</sub>D<sub>4</sub> and FITC-conjugated secondary antibody and visualized for the fluorescence pattern (B). Subsequently the spread was processed for AgNO<sub>3</sub> staining to visualize the S.Cs (C) A, phase contrast micrograph. The arrow in the phase contrast micrograph indicates two phase dense regions corresponding to the two telomeric ends of the chromosomes as is evident from the Ag-stained pattern in (C). The arrow in C points to the dense body which is also immunostained with Mab A<sub>11</sub>D<sub>4</sub>.

very little cross-reactivity with somatic lamin B is present in meiotic and post-meiotic germ cells.

A summary of the observations made with all the 8 Mabs with respect to the fluorescence pattern and the immunoblot analysis of lamin<sub>g</sub> antigen is presented in Table I. The main conclusions from these studies agree very well with those drawn from studies done with polyclonal antibodies made in our previous study. Namely, (a) lamin<sub>g</sub> antigen is a part of the lamina structure in the pre-meiotic germ cells, (b) As the germ cells enter meiotic prophase, the lamin<sub>g</sub> antigen is retained in the pachytene nucleus even though the lamina structure has disassembled and (c) following meiotic division, the lamina structure is reformed using lamin<sub>g</sub> as the only lamina component.

*Co-localization of lamin B and lamin<sub>g</sub> in the spermatogonial cells.* In the model proposed by us in our previous paper (27), we had speculated that both the somatic lamins and lamin<sub>g</sub> coexist in the nuclei of pre-meiotic spermatogonial cells.

Upon entry into the meiotic prophase, only lamin<sub>g</sub> is retained and the same is relocalized into the lamina structure in round spermatids. However, we had not provided evidence for the coexistence of the lamin B and lamin<sub>g</sub> within the same nucleus. Now since we have shown in this paper that anti-lamin B antibodies do not react with lamin<sub>g</sub> (Fig. 5) and anti-lamin<sub>g</sub> antibodies have negligible cross-reactivity with lamin B (Fig. 2B and Fig. 4), we have carried out double antibody labelling of spermatogonial nuclei. For this purpose, nuclei were isolated from the tests of 12 day old rats (containing predominantly spermatogonia and a few preleptotene spermatocytes) and then subjected to double antibody labelling as mentioned in Methods. The lamin B was followed by Rhodamine labelled anti rabbit IgG in goat and lamin<sub>g</sub> by FITC labelled anti-mouse IgG in goat as secondary antibodies. The immunofluorescence pattern shown in Fig. 7 shows that both lamin B and lamin<sub>g</sub> coexist within the same spermatogonial nucleus. A more confirmatory evidence for this colocalization needs to be obtained by immunoelectron-microscopic studies.

*Localization of lamin<sub>g</sub> in surface spread pachytene spermatocytes.* An event concomitant to the disassembly process of the nuclear lamina structure during meiotic prophase is the appearance of synaptonemal complexes (SC). The lamin<sub>g</sub> antigen was coisolated with synaptonemal complexes, from rat pachytene spermatocytes (27). However, the fluorescence pattern in the pachytene nucleus did not indicate that it is localized to either lateral elements or central elements of SCs. Both the immunofluorescence and preliminary immunoelectron microscopic studies showed it to be localized at the phase dense regions within the nucleus. These regions were speculated to correspond to the attachment plaque regions of the telomeric ends of the chromosomes through which they are attached to the nuclear membrane. To gain a further insight into the localization of lamin<sub>g</sub> antigen, we have carried out immunolocalization studies in surface spread pachytene spermatocytes. For this purpose, testicular germ cells were given a mild hypotonic treatment (0.5% NaCl) and then fixed with 4% paraformaldehyde. The spread was first reacted with A<sub>11</sub>D<sub>4</sub> Mab and subsequently with FITC labelled goat anti-mouse IgG. The fluorescence pattern was recorded and then the same slide was processed for silver staining to visualize the SCs. A similar technique has been used by Dresser and Moses (4). Fig. 8 shows the results of such an experiment. Again, the fluorescence is observed at phase dense regions (Fig. 8B) in addition to a discontinuous fluorescence along the periphery of the nucleus. From a comparison of the phase contrast micrograph (Fig. 8A) with that of the silver stained micrograph (Fig. 8C), it is very clear that the phase dense regions correspond to the telomeric ends of the paired homolo-

gous chromosomes. The fluorescence is also observed at the "dense body" region. The significance of these results is discussed below.

### DISCUSSION

The present investigation is a continuation of our studies on the immunological characterization of the germ cell specific lamin<sub>g</sub>. In our previous study using rabbit polyclonal antibodies, we had made several interesting observations (28). They were (a) lamin<sub>g</sub> is retained in the pachytene spermatocytes even though the lamina structure is believed to be absent in these meiotic prophase cells (26), (b) the lamina structure of haploid gametes is made up solely of this lamin<sub>g</sub> and (c) lamin<sub>g</sub> is related but not identical to somatic lamin B. To gain further insight into the nature of lamin<sub>g</sub> protein, we have produced and characterized 8 monoclonal antibodies. Although all these monoclonal antibodies reacted strongly with lamin<sub>g</sub> in an immunoblot analysis, based on their reactivity with the antigen in immunofluorescence studies, they can be classified into 3 groups: (a) those showing a strong fluorescence (*A*<sub>11</sub>*D*<sub>4</sub>, *A*<sub>11</sub>*C*<sub>7</sub>, *C*<sub>1</sub>*G*<sub>8</sub>); (b) those showing a medium intensity of fluorescence (*C*<sub>1</sub>*F*<sub>7</sub>, *A*<sub>2</sub>*C*<sub>2</sub>); and (c) those showing no fluorescence at all in the germ cells (*C*<sub>8</sub>*B*<sub>3</sub>, *C*<sub>8</sub>*C*<sub>5</sub>, *D*<sub>9</sub>*E*<sub>6</sub>). It is interesting to note that the monoclonal antibodies showing a strong fluorescence with germ cells, showed a very faint reaction with the laminar structure of liver nuclei (Fig. 4) and also with lamin B in an immunoblot analysis (Fig. 2B). These observations, in addition to strengthening our above conclusion (c), also suggest that there is only a small degree of homology in the epitope being recognized by these monoclonal antibodies in lamin<sub>g</sub> and lamin B. The epitopes recognised by the other two sets of monoclonal antibodies (b and c) may be unique to lamin<sub>g</sub> which are absent in lamin B. The different levels of fluorescence generated by the three sets of monoclonal antibodies may reflect the differential accessibility of the respective epitopes in germ cells.

The lack of cross-reactivity, or, in some cases a low level of cross-reactivity of the anti-lamin<sub>g</sub> monoclonal antibodies with lamin B is rather intriguing, particularly considering the fact that somatic lamins A, B and C are immunologically related as reported by Raymond and Gagnon (23). These workers have obtained 2 monoclonal antibodies (34B6 and 55B3) which reacted with all the three lamins A, B and C while another (36C2) reacted only with lamins A and C. One of the 2 monoclonal antibodies 31B5 reacted specifically with lamin B only. Using an IgM monoclonal antibody, Burke *et al.* (3) had earlier shown that it reacts with all the three somatic lamins and this epitope is localized to a 46 kDa chymotryptic fragment generated from each of the lam-

ins. In addition to their immunological relatedness, the lamins also share antigenic determinants with intermediate filament proteins (23). It may be pertinent to point out here, that even the rabbit anti-lamin<sub>g</sub> polyclonal antibodies reacted very faintly with lamin B (27). Conversely, the experiments described here with anti somatic lamin B antibodies revealed that they do not light up germ cells in immunofluorescence studies (Fig. 5). Therefore, lamin<sub>g</sub> stands out distinctly among the lamina proteins and it becomes very crucial to identify and characterize the epitopes recognized by the anti-lamin<sub>g</sub> monoclonal antibodies that we have generated. These studies are now in progress in our laboratory.

An important observation made in this study is the colocalization of lamin B and lamin<sub>g</sub> in spermatogonial cells, which supports the model proposed by us (27). Although both the somatic lamin B and lamin<sub>g</sub> are present in the premeiotic germ cells, only lamin<sub>g</sub> is selectively retained in the pachytene nucleus. Therefore, we believe that lamin<sub>g</sub> must have some unique properties that are absent in lamin B in order to facilitate the organization of the meiotic chromosomes. Interestingly, lamin B is constitutively expressed in all the tissues while lamins A/C are acquired only upon tissue maturation (25). Recently, we have shown that lamin<sub>g</sub> is evolutionarily conserved in the germ cells of many eukaryotic cells (28). This antigen, however was not observed in somatic cells of these organisms, stressing the functional importance of lamin<sub>g</sub> in the germ cells. It is very essential, therefore, to compare the sequence organization and molecular properties of lamin<sub>g</sub> and lamin B. For this purpose we are presently characterizing the cDNA clone for lamin<sub>g</sub> which should provide valuable information to answer many of the questions raised above.

Another series of experiments described in the present study are concerned with the localization of lamin<sub>g</sub> in pachytene spermatocytes which included both isolated nuclei as well as thin sections of seminiferous tubules period. The results obtained with monoclonal antibodies confirm our earlier conclusions, namely it is localized in the phase dense regions of the pachytene spermatocyte nucleus. Our results on the double staining of surface spread pachytene spermatocytes with FITC and silver have clearly shown that the phase dense regions correspond to the telomeric ends of the meiotic chromosomes. A question that arises at this juncture is why lamin<sub>g</sub> is retained in the pachytene nucleus when there is no lamina structure. However, there is one important difference in the organization of nuclear structure between a mitotic cell and a meiotic cell particularly in mammals. Although the lamina structure is disassembled in both the cases, the nuclear membrane is retained in the meiotic cell nucleus but not in the mitotic cell. Both ends of the synaptonemal complex appear to be in contact with the inner nuclear membrane in many organ-

isms (31). Therefore, we believe that the retention of lamin<sub>g</sub> is necessary to facilitate the attachment of the telomeric ends of the chromosomes to the inner nuclear membrane. In fact, lamin B is believed to provide the anchorage sites for the surrounding lipid bilayer membrane (7). The regions of attachment of the telomeric ends of the chromosomes to the nuclear membrane have been termed cytologically as "attachment plaques" or "chromosomal bouquets". Recently, Klein *et al.* (14) have shown that RAP1 (repressor activator protein 1) is localized similarly to the telomeric ends of meiotic chromosomes in *Saccharomyces cerevisiae* using similar technique. The functional significance of the localization of RAP1 at telomeric ends, however, is not known. In addition to the staining of the telomeric ends of the chromosomes, we have also observed a faint discontinuous fluorescence pattern around the nuclear periphery (Fig. 8) which was also evident in the immunohistochemical staining of thin sections of seminiferous tubules (Fig. 6B and 6D). Therefore we believe that there are remnants of a lamina structure in the meiotic prophase nucleus attached to the inner nuclear membrane. Recently Vester *et al.* (1993) have also observed using a broad specificity antibody, lamina structure in rat spermatocytes involving a lamin protein that is closely related to lamin B which is in conformity with our findings.

Recently we have also come across a paper by Furukawa and Hotta (6) describing the isolation and characterization of a cDNA clone for a germ cell specific lamin in mouse. They have termed it as lamin B3 which arises out of alternate splicing from lamin B2. It remains to be seen whether the lamin<sub>g</sub> that we have described in the rat is the same protein as lamin B3 of mouse.

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