elevated, and then their activity, along with the cytoplasmic free calcium concentration, starts to decline owing to a gradual depletion of the intracellular calcium pool(s), until the cytoplasmic free calcium concentration reaches control values.

In measurements of ^{45}Ca uptake, the sole source of labeled calcium is extracellular; hence, the increase in Ca^{2+}-ATPase activity results in the expulsion of ^{45}Ca, which is not compensated since ^{45}Ca is not present in the intracellular calcium pool(s), and in a rapid decline in cellular ^{45}Ca values.

In the presence of a low, subphysiological concentration of calcium (5 μM), the proportionate increase in calcium uptake produced by T_3 is about the same as that in the presence of 1 mM extracellular calcium, but the increase in calcium uptake in absolute values is minute so that cytoplasmic free calcium concentration remains below the threshold level and there occurs no release of calcium from the intracellular pool(s) and no activation of Ca^{2+}-ATPase. Thus, no significant change in cytoplasmic free calcium concentration (measured with the quin 2 probe) is apparent, and the elevated values of ^{45}Ca concentration in the T_3-treated cells are sustained.[3]

Further support for this biphasic effect of T_3 on cellular calcium concentration in the rat thymocyte comes from the studies with the β-adrenergic antagonist alprenolol, in which alprenolol blocked in a similar fashion the effects of T_3 on thymocyte ^{45}Ca uptake and on cytoplasmic free calcium concentration (Figure 3).

Registry No. T_3, 6893-02-3; Ca, 7440-70-2; alprenolol, 13655-52-2.

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Lamin B Shares a Number of Distinct Epitopes with Lamins A and C and with Intermediate Filament Proteins†

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ABSTRACT: Four monoclonal antibodies raised against rat liver nuclear lamins and an anti-intermediate filament antibody [Pruss, R. M., Mirsky, R., & Raff, M. C. (1981) *Cell (Cambridge, Mass.) 27*, 419–428] have been used to identify epitopes shared by lamin B with lamins A and C, and with intermediate filament proteins. The antibodies defined two major antigenic regions on the three lamins which were both homologous with mouse epidermal keratins as well as hamster vimentin and desmin. Three distinguishable epitopes shared by lamin B with lamins A and C were identified by competition studies between pairs of antibodies and by reaction against *N*-chlorosuccinimide and cyanogen bromide cleavage fragments. These results support the hypothesis that lamin B, despite important biochemical differences with lamins A and C, shares with them some of the structural characteristics typical of intermediate filament proteins.

The peripheral lamina is an important structural component of nuclei: located between chromatin and inner nuclear membrane, it forms a continuous structure with the pore complexes which can be isolated biochemically while still maintaining the original shape of the nucleus [for recent reviews, see Berezney (1984), Gerace et al. (1984), and Krohne and Benavente (1986)]. The exact molecular details of the interaction between elements of the lamina and pore complexes as well as between lamina and chromatin or nuclear membrane are not well understood. Peripheral lamina appears to be exclusively composed of polypeptides called lamins, varying in number from 1 to 3 according to species and cell type (Krohne & Benavente, 1986).

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Lamins A, B, and C of vertebrate somatic cells have been most extensively characterized. In rat liver (Kaufmann et al., 1983), lamins A and C of 69 and 62 kilodaltons (kDa),[1] respectively, have the same isoelectric point variants (6.8–7.2) and generate nearly identical complete tryptic digests. The cDNA sequence of lamin C in human cells (McKeon et al., 1986; Fisher et al., 1986) is identical with that of lamin A (which has an extension of 98 amino acids at the carboxy terminus) except for the last 6 amino acid residues at the carboxy terminus of lamin C. Lamin B in rat liver is a 67-kDa polypeptide with an isoelectric point of 5.7, and its complete tryptic digest is different from that of lamins A and C (Kaufmann et al., 1983). Lamin B also differs from lamins A and C with respect to the properties of its attachment to the inner nuclear membrane both in interphase and during mitosis (Gerace & Blobel, 1980; Lebel & Raymond, 1984; Burke & Gerace, 1986), and lamin B shows a larger increase in its level of phosphorylation at the onset of cell division (Ottaviano & Gerace, 1985). Similarities between lamin B and lamins A and C do, however, exist: their amino acid composition is similar (Kaufmann et al., 1983), and all three polypeptides share at least one epitope as evidenced by the cross-reactivity of a number of monoclonal antibodies (Burke et al., 1983; Krohne et al., 1984; Newmeyer & Ohlson-Wilhelm, 1985).

The recent finding of important sequence homologies between lamins A and C (no sequence of lamin B has been published to date) and intermediate filament proteins (McKeon et al., 1986; Fisher et al., 1986) and also the ability of isolated lamins to form filaments or paracrystalline structures in vitro (Aebi et al., 1986; Goldman et al., 1986) are the first indications of the mechanism by which lamin polypeptides interact to form the insoluble lamina. In this study, we have used monoclonal antibodies to show that lamin B shares sequence homologies with intermediate filament proteins [a preliminary report of this work using an anti-intermediate filament antibody (Pruss et al., 1981) has been previously presented (Lebel & Raymond, 1987)] and that lamin B also shares with lamins A and C at least three distinguishable epitopes.

Materials and Methods

Preparation of Nuclear Matrix and Cytoskeleton. Rat liver nuclear matrix was prepared as described (Lebel & Raymond, 1984), and lyophilized aliquots of alkylated proteins were stored at −80 °C. The only modification to the original protocol involved pretreatment of DNase with 0.25 times its weight of soybean trypsin inhibitor for two 15-min periods at 4 °C, each followed by centrifugation of insoluble material, and addition to the DNase of phenylmethanesulfonyl fluoride at a final concentration of 2 mM. This treatment was found to eliminate the chymotryptic activity contaminating the DNase. Rat liver nuclear matrix prepared by using this protocol, i.e., in the absence of reducing or alkylating agents, was very similar to preparations of peripheral lamina [compare Lebel and Raymond (1984) with Kaufmann and Shaper (1984)] and contained very little internal matrix proteins. Baby hamster kidney cell cytoskeleton was prepared essentially as described for epithelial cells (Franke et al., 1979). The final cytoskeletal preparation was dissolved in guanidine hydrochloride, alkylated, and dialyzed as described for the nuclear matrix preparation (Lebel & Raymond, 1984). The dialyzed suspension was sonicated with a tip sonifier for three 5-s periods to reduce the molecular weight of the DNA still present, and aliquots were lyophilized and stored at −80 °C. Newborn mouse epidermal keratins prepared as described (Schweizer & Goerttler, 1980) were kindly provided by Thérèse Ouellet of this institute.

Immunization and Preparation of Monoclonal Antibodies. Female BALB/c mice (15–25 g body weight) received five intraperitoneal injections of 500 μg [protein was estimated by the method of Lowry (Lowry et al., 1951) using bovine serum albumin as a standard] of rat liver nuclear matrix proteins at 2-week intervals. The first injection was in the presence of complete Freund's adjuvant and the last four injections with incomplete adjuvant. Several months later, animals were challenged with intraperitoneal injections of matrix proteins (100 μg per injection; in the case of hybridomas 31B5, 34B6, and 36C2) or of electroeluted lamins A, B, and C (75 μg total for each injection, in the case of hybridoma 55B3) suspended in phosphate-buffered saline, on 3 consecutive days. On the following day, the spleen was removed, and cell fusion with the nonimmunoglobulin producing P3/X63-Ag8.653 mouse myeloma cells using poly(ethylene glycol) 4000 was performed as described (Raymond & Suh, 1986). Positive wells were identified by an enzyme-linked immunosorbent assay (ELISA) against nuclear matrix proteins (described below), and cells were cloned by a single-cell transfer technique (Gagnon & Raymond, 1985). Ascites fluid was prepared by injection of hybridoma cells in pristane-primed BALB/c mice as described (Brodeur et al., 1984). The anti-intermediate filament antibody (aIF; Pruss et al., 1981) was prepared by ammonium sulfate precipitation from culture supernatant of hybridoma obtained from the American Type Culture Collection (TIB 131).

Electrophoresis and Immunoblotting. Methods for polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate either as a first dimension or as a second dimension following isoelectric focusing (O'Farrell, 1975) or nonequilibrium pH gradient electrophoresis (O'Farrell et al., 1977) and methods for Coomassie blue or silver staining of gels were as described (Lebel & Raymond, 1984). Electrophoretic transfer of proteins from gels to nitrocellulose membranes was performed on a Trans-blot cell (Bio-Rad) using 25 mM Tris–192 mM glycine buffer (pH 8.3) at 40 V for 1.5 h at room temperature (Lebel & Raymond, 1987). Nitrocellulose transfers were quenched with 3% casein in 10 mM Tris-HCl, pH 7.4, and 0.15 M NaCl for 1 h at 37 °C and incubated overnight at 4 °C with the antibody at dilutions ranging from 1/5 to 1/20 using either ascites fluid or concentrated culture supernatant. Detection of bound antibody was performed by sequential incubations (2 h each at room temperature) with a biotin-conjugated anti-mouse IgG (heavy and light chain) immunoglobulin and an avidin-conjugated horseradish peroxidase, each diluted at 1/1000. Color reactions were then performed with hydrogen peroxide and chloronaphthol (Hawkes et al., 1982). Alternatively, an ^{125}I-labeled anti-mouse IgG (heavy and light chains) or anti-mouse IgM immunoglobulin was used (2–10 μCi per transfer, 2 h at room temperature), and nitrocellulose transfers were radioautographed at room temperature.

Chemical Cleavage of Proteins. Lamins A, B, and C were individually purified by excising the appropriate spots from two-dimensional gels stained with Coomassie blue (10–30 min) and destained as briefly as possible. Individual spots were stored dry at −20 °C until further use. Cleavage at tryptophan

[1] Abbreviations: aIF, anti-intermediate filament antibody; ELISA, enzyme-linked immunosorbent assay; NCS, *N*-chlorosuccinimide; kDa, kilodalton(s); Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

Table I: Properties of Anti-Lamin Monoclonal Antibodies[a]

antibody	isotype	reaction on lamin A	B	C
31B5	IgM	–	+	–
34B6	IgM	+	+	+
36C2	IgM	+	–	+
55B3	IgM	+	+	+
aIF	IgG	+	+	+

[a] Isotypes were determined by an ELISA test using heavy-chain-specific anti-mouse Ig immunoglobulins. Specificities of the reactions on nuclear lamins were determined on nitrocellulose transfers of rat liver matrix proteins separated by two-dimensional gel electrophoresis.

residues using *N*-chlorosuccinimide (Lischwe & Ochs, 1982) and at methionine residues using cyanogen bromide (Nikodem & Fresco, 1979) was performed on gel pieces exactly as described.

Competition Studies. ELISA was performed on poly(vinyl chloride) microtiter plates coated with nuclear matrix proteins (1 μg/well, in phosphate-buffered saline) and quenched with 2% bovine serum albumin in 10 mM Tris-buffered saline, pH 7.4, containing 0.01% Thimerosal as a preservative. Concentrations of ascites fluid and of concentrated culture supernatant required for saturation of the binding sites available on the plates were determined for each antibody. Competition tests were carried out by prior mixing of pairs of antibodies (each at a saturating concentration) followed by serial dilution from well to well. Dilutions were made in 2% bovine serum albumin containing 0.05% Tween 20, and plates were incubated overnight at 4 °C. Detection of bound antibody was performed as described above using the biotin and avidin conjugates followed by reaction with hydrogen peroxide and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate). Reactions were terminated by the addition of sodium azide at a final concentration of 0.02% (w/v) and optical densities measured at 410 nm.

Results

Recognition of individual lamin molecules by each monoclonal antibody was assayed by incubation with rat liver nuclear matrix samples separated by two-dimensional gel electrophoresis, as previously described for the aIF antibody (Lebel & Raymond, 1987). The results summarized in Table I defined three groups of anti-lamin antibodies: those that reacted against all three lamin polypeptides (34B6, 55B3, and aIF), one antibody specific for lamins A and C (36C2), and one antibody specific for lamin B (31B5). All four antibodies reacted with the peripheral lamina on isolated rat liver nuclei by immunofluorescence (S. Lebel and Y. Raymond, unpublished results) with the same features as those previously published for the aIF antibody (Lebel & Raymond, 1987), indicating the accessibility of their respective epitopes in the fully polymerized lamina.

Further definition of the individual epitopes recognized by the antibodies was obtained by competition studies between pairs of antibodies. Such studies provide qualitative information on the ability of an antibody to bind onto the antigen in the presence of a second antibody. Quantitative studies require purification and labeling of each antibody, and these studies are in progress. As illustrated in Figure 1, the outcome of such experiments can be of two types [see, for example, Mendelson and Bustin (1984)]. Competition between two antibodies for the same or closely located epitopes will result in a binding curve for the mixture of antibodies that is intermediate in value with respect to the individual curves, at saturating antibody concentrations. At antibody concentrations below saturation, the mixture may give additive values. Such a situation is illustrated in Figure 1A for antibodies 34B6 and 55B3. Absence of competition between two antibodies will result in a binding curve for the mixture with higher values at all antibody concentrations, as illustrated in Figure 1B for antibodies 34B6 and aIF. Results from similar experiments for all five antibodies are summarized in Table II.

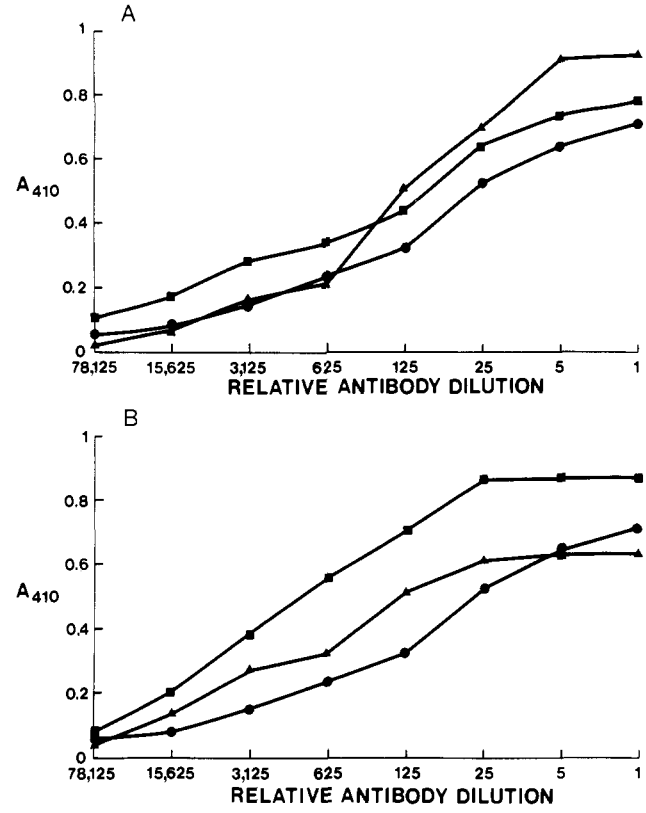

FIGURE 1: Competition studies were performed between pairs of monoclonal antibodies by an ELISA test on nuclear matrix proteins. Panel A shows the binding curves of 34B6 (closed circles), 55B3 (closed triangles), and the mixture of both antibodies (closed squares) as a function of antibody concentration. Panel B shows the binding curves of 34B6 (closed circles), aIF (closed triangles), and the mixture of both (closed squares). A relative antibody dilution of 1 corresponds to a dilution factor of 4.

Table II: Competition Experiments between Pairs of Monoclonal Antibodies[a]

antibodies	55B3	36C2	34B6	31B5
aIF	–	–	–	–
55B3		+	+	+
36C2			+	–
34B6				–

[a] Conditions were as described in the legend to Figure 2. (+) indicates competition.

These results appeared to define two major antigenic regions on the lamin polypeptides. The first region was recognized by the aIF antibody exclusively. A second region was recognized by the 55B3 antibody as well as by the other three antibodies (36C2, 34B6, and 31B5) with which it competed for binding. The latter three antibodies must, however, have been recognizing distinct epitopes: 36C2 was specific for lamins A and C and did not compete (Table II) with the lamin B specific 31B5 antibody; 34B6 was specific for lamins A, B, and C and competed with 36C2 but not with 31B5 (Table II) in which it was different from 55B3. Taken together, these results defined three distinguishable epitopes shared by lamin B with lamins A and C, recognized by the aIF, 55B3, and 34B6 antibodies. The latter two epitopes appeared to be

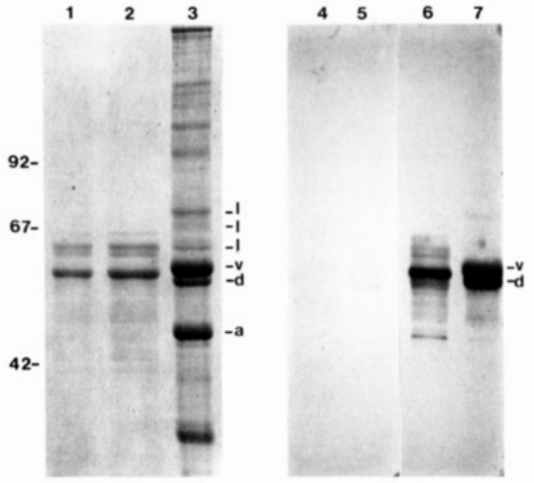

FIGURE 2: Newborn mouse epidermal keratins (lanes 1 and 2, lane 2 received twice the amount of protein of lane 1) and baby hamster kidney cell cytoskeletons (lane 3) were electrophoresed on an 8% polyacrylamide gel and stained with Coomassie blue. Positions of hamster lamins (l), vimentin (v), desmin (d), and actin (a) are indicated. Mouse keratins (lanes 4 and 6, same amount as lane 1) and hamster cytoskeletons (lanes 5 and 7, same amount as lane 3) were transferred onto nitrocellulose and probed with ascites fluid prepared by injecting a mouse with the parent myeloma cells (lanes 4 and 5) and with the aIF antibody (lanes 6 and 7). Bound antibodies were detected by the biotin–avidin peroxidase method.

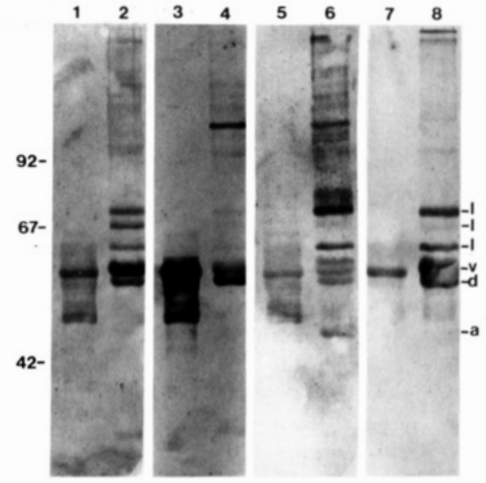

FIGURE 3: Mouse keratins (lanes 1, 3, 5, and 7) and hamster cytoskeletons (lanes 2, 4, 6, and 8) were transferred onto nitrocellulose as described in the legend to Figure 2 and probed with the monoclonal antibodies 31B5 (lanes 1 and 2), 34B6 (lanes 3 and 4), 36C2 (lanes 5 and 6), and 55B3 (lanes 7 and 8). Bound antibodies were detected by an ^{125}I-labeled anti-mouse IgM immunoglobulin followed by radioautography for 16 h using Kodak XAR film. Positions of hamster lamins (l), vimentin (v), desmin (d), and actin (a) are indicated.

located close enough on the lamin polypeptides for their respective antibodies to compete for binding, although this may have involved a relatively large distance if one considers the size of these IgM molecules.

Recent experiments (McKeon et al., 1986; Fisher et al., 1986) have identified regions of extensive homology between lamins A and C of human origin and intermediate filament proteins. We have shown (Lebel & Raymond, 1987) that lamin B as well as lamins A and C from rat liver is recognized by the aIF antibody, the epitope of which is part of the common domain of a large number of intermediate filament proteins (Pruss et al., 1981). It was therefore interesting to test whether the anti-lamin antibodies could react against representatives of some subclasses (Steinert & Parry, 1985) of intermediate filament proteins: samples of newborn mouse epidermal keratins consisting of a highly enriched mixture of keratin polypeptides ranging from 67 to 42 kDa (Schweizer & Goerttler, 1980) and baby hamster kidney cell cytoskeletons characterized by the presence of relatively large amounts of vimentin and desmin (Blose & Bushnell, 1982) were chosen for this purpose.

Coomassie blue stained electrophoretograms of these samples are shown in Figure 2: a number of keratin bands of variable intensity were visible in the 67–42-kDa range in lanes 1 and 2, and the hamster cytoskeletons (Figure 2, lane 3) were characterized by the presence of vimentin, desmin, and actin bands and less prominent lamin bands. The latter copurify with intermediate filament proteins of the cytoskeleton due to their insolubility in detergent and concentrated salt solutions (Dagenais et al., 1985). Figure 2 shows that the aIF antibody reacted against a large number of keratin bands [lane 6; see Cooper et al. (1984)] and against hamster vimentin and desmin (Figure 2, lane 7). Incubation in the presence of an ascites fluid prepared from the parent myeloma cells gave no reaction on similar blots under the same conditions (Figure 2, lanes 4 and 5).

The anti-lamin antibodies were allowed to react against keratins and hamster cytoskeletons, and the results are shown in Figure 3. Three antibodies (31B5, 34B6, and 36C2) reacted against a larger number of keratin bands (Figure 3, lanes 1, 3, and 5, respectively) while 55B3 (Figure 3, lane 7) reacted against the major Coomassie blue stained keratin band (Figure 2, lanes 1 and 2). These antibodies also reacted against hamster vimentin and desmin and, to variable extents, against hamster lamins (Figure 3, lanes 2, 4, 6, and 8).

The pattern of reactivity on hamster lamins separated on a one-dimensional gel (Figure 3) appeared to differ from the reactivity on rat liver lamins as determined after two-dimensional gel electrophoresis (Table I). Figure 3 (lane 2) shows 31B5 reacting against three bands in the area where hamster lamins migrated while it recognized only lamin B in rat liver samples; 34B6 reacted weakly against two hamster lamin bands (Figure 3, lane 4) while it recognized all three rat liver lamins; reactivity of 36C2 was restricted to two lamin bands, on both hamster (Figure 3, lane 6) and rat samples, while 55B3 appeared to react against two hamster (Figure 3, lane 8) and three rat lamin bands. Whether these differences, which would have to be confirmed by two-dimensional electrophoresis of hamster lamins, can be attributed to species differences will have to await further sequencing studies on lamins of rodent origin.

Other cross-reactive material present in the hamster cytoskeletons included an unindentified 100-kDa polypeptide recognized by 34B6 (Figure 3, lane 4) and 36C2 (Figure 3, lane 6). This polypeptide may be related to or identical with a 95-kDa intermediate filament protein present in fibroblasts, epithelial cells, myoblasts, and neuroblastomas from a number of species (Lin, 1981; Lin & Feramisco, 1981). This 95-kDa polypeptide, through present in amounts smaller than vimentin in fibroblasts, gave rise to the same pattern by immunofluorescence using a specific monoclonal antibody (Lin, 1981). The 36C2 antibody also recognized a band comigrating with actin and a band migrating slightly above the vimentin band.

The results of Figure 3 show clearly that lamin B like lamins A and C shares sequence homologies with intermediate filament proteins: four epitopes (aIF, 55B3, 34B6, and 31B5) have been identified with such properties, the last one being unique to lamin B while the first three are shared with lamins A and C.

The possibility that the antibodies were recognizing α-helical conformations on intermediate filament proteins and lamins

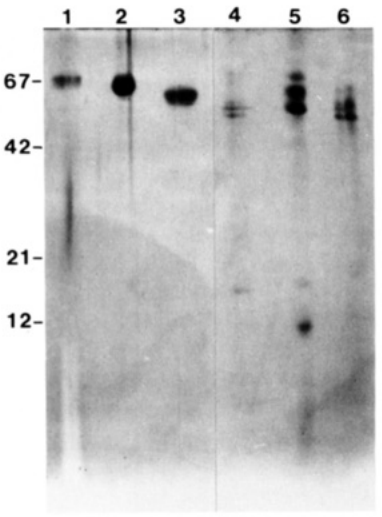

FIGURE 4: Lamins A (lanes 1 and 4), B (lanes 2 and 5), and C (lanes 3 and 6) were purified by excising the appropriate spots from two-dimensional gels and incubated in the absence (lanes 1–3) or presence (lanes 4–6) of *N*-chlorosuccinimide. Gel pieces were then transferred in the wells of a 15% SDS–polyacrylamide gel which was stained with silver nitrate.

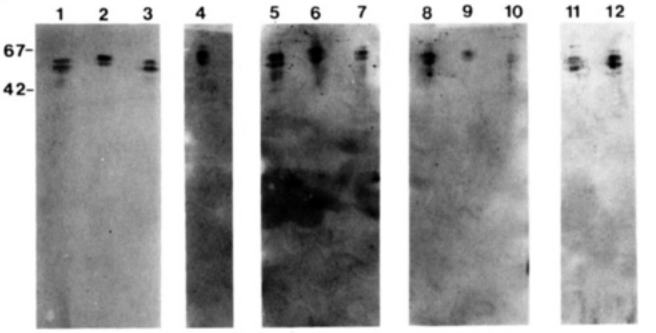

FIGURE 5: Lamins A (lanes 1, 5, 8, and 11), B (lanes 2, 4, 6, and 9), and C (lanes 3, 7, 10, and 12) were hydrolyzed with *N*-chlorosuccinimide and electrophoresed as described in the legend to Figure 4, transferred onto nitrocellulose, and probed with the monoclonal antibodies aIF (lanes 1–3), 31B5 (lane 4), 55B3 (lanes 5–7), 34B6 (lanes 8–10), and 36C2 (lanes 11 and 12). Bound antibodies were detected by the biotin–avidin peroxidase method (lanes 1–3) or by an ^{125}I-labeled anti-mouse IgM immunoglobulin followed by radioautography for 24 h (lane 4) or 4 days (lanes 5–12) using Kodak XRP film. Fragments detected corresponded to the major fragments shown in Figure 4, lanes 4–6.

rather than sequential epitopes shared by these polypeptides was ruled out by the lack of reaction against neurofilament subunits of high and medium molecular weights (recognized by aIF only, data not shown) which possess α-helical domains forming coiled-coil structures.

A more precise localization of the epitopes was attempted by reacting the antibodies against fragments of lamin polypeptides produced by chemical cleavage reactions. The products of *N*-chlorosuccinimide (NCS) hydrolysis at tryptophan residues are shown in Figure 4. Hydrolysis was dependent on the presence of NCS and generated two major fragments with apparent mobilities of 49 kDa (sometimes seen as a closely spaced doublet) and 45 kDa from both lamins A and C. Minor bands were also visible in the lower part of the gel (between 21 and 12 kDa) which differed between lamin A and C hydrolysis products. Hydrolysis of lamin B generated two major fragments with apparent mobilities of 60 kDa (in the form of a doublet, see Figure 5) and 51 kDa and two minor fragments between 21 and 12 kDa. NCS cleavage products were transferred from gels onto nitrocellulose membranes and probed with the monoclonal antibodies, the results of which are shown in Figure 5. The major high molecular mass fragments (49–45 kDa for lamins A and C, 60–51 kDa for lamin B) were recognized by all of the antibodies used in this study on their respective antigens.

The results of the NCS hydrolysis experiments were consistent with the hypothesis that tryptophan residues are similarly located in rat and human lamins A and C. On the human lamins (McKeon et al., 1986; Fisher et al., 1986), the epitope for the aIF antibody (Geisler et al., 1983) was present as a single copy, and it mapped between residues 368 and 387 (it is identical with the glial fibrillary acidic protein in 15 out of the 20 amino acid residues), and tryptophan residues lie in positions 467, 498, 514, and 520. One can predict from these data that NCS cleavage of both human lamins A and C would generate identical amino-terminal 46–52-kDa overlapping fragments reacting positively with the aIF antibody, which is very close to the situation seen with rat liver lamins A and C (Figure 5). One can therefore assume that the 45–49-kDa NCS fragments represented the amino-terminal portions of lamins A and C. This location would be compatible with the location on lamins A and C of major sequence homologies with intermediate filament proteins (McKeon et al., 1986; Fisher et al., 1986).

Antibody-reactive species in the molecular mass range between 40 and 45 kDa, i.e., smaller than the major NCS fragments mentioned above, could be seen in the hydrolysis products of lamin A (Figure 5, lanes 1, 5, and 8), lamin B (Figure 5, lane 6), and lamin C (Figure 5, lanes 3 and 7). The presence of these bands cannot be explained by the positions of tryptophan residues discussed above and may be due to artifactual hydrolysis of aspartyl–prolyl and threonyl–prolyl bonds (Piszkiewicz et al., 1970) under the acidic conditions used during NCS hydrolysis (6 M acetic acid for 80 min at room temperature). Lamins A and C of human origin (McKeon et al., 1986; Fisher et al., 1986) share two threonyl–prolyl bonds (between positions 3 and 4, and 19 and 20) and one aspartyl–prolyl bond (between positions 476 and 477) that are also present and susceptible to acid hydrolysis on lamins A and C from rat liver (Fisher et al., 1986; unpublished experiments). Incubation of large samples of all three lamins under the conditions of NCS hydrolysis, but in the absence of NCS, followed by electrophoresis on an 8% polyacrylamide gel revealed the presence of minor bands of lower molecular weight in all three cases (unpublished experiments). These minor bands by themselves, or after subsequent hydrolysis by NCS, were probably responsible for the less intense antibody reactions mentioned above due to their overlap with the major NCS fragments.

Fragments generated by cyanogen bromide (CNBr) cleavage of rat liver lamins A, B, and C are shown in Figure 6. Hydrolysis was dependent on the presence of CNBr. The minor band seen below the intact lamins after incubation in the absence of CNBr (Figure 6, lanes 1–3) was not due to the presence of a contaminant but may have resulted from acid hydrolysis of susceptible aspartyl–prolyl bonds under the conditions used for hydrolysis. Acid hydrolysis of the aspartyl–prolyl bond between positions 476 and 477 would be expected to give a cleaved product of identical size from both lamins A and C which is what was observed (Figure 6, lanes 1 and 3). Whether the same is true for lamin B (a fragment of similar size as that from lamins A and C is seen in Figure 6, lane 2) will become clear when its complete sequence is published. The pattern generated upon CNBr hydrolysis of lamin C was very similar to that of lamin A which showed a few extra bands in the lower molecular weight region of the

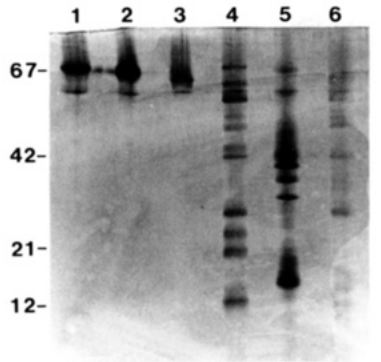

FIGURE 6: Purified (see legend to Figure 4) lamins A (lanes 1 and 4), B (lanes 2 and 5), and C (lanes 3 and 6) were incubated in the absence (lanes 1–3) or presence (lanes 4–6) of cyanogen bromide. Electrophoresis was on a 15% polyacrylamide gel that was stained with silver nitrate.

Table III: Reaction of Monoclonal Antibodies against CNBr Cleavage Products of Lamins A, B, and C[a]

	antibodies			
lamins A/C (kDa)	aIF	55B3	34B6	36C2
61.7	+	+	+	+
60.3	+	+		+
56.2	+	+	+	+
49.0	+/−	+/−	+	
45.7	+/−	+	+	+
39.8				
37.1	+[b]			
25.1				
21.9[b]				
19.5[b]				
13.2[b]				

	antibodies		
lamin B (kDa)	aIF	55B3	31B5
58.2	+		+
44.2	+/−	+	
37.2	+	+	+
34.3	+	+	
30.9			
27.9			
15.9			+
15.5			+

[a] (+) indicates a strong reaction while (+/−) indicates a weak reaction. [b] Fragments present in lamin A but not in lamin C.

gel. The hydrolysis pattern generated from lamin B was completely different from that of lamins A and C, as previously found by others (Gerace & Blobel, 1982). The sizes of the fragments (listed in Table III) produced by hydrolysis of lamins A and C from rats did not correspond to the sizes predicted from the methionine positions in lamins A and C of human origin (McKeon et al., 1986; Fisher et al., 1986). This may be explained by the finding of nine methionine residues in human lamin A (McKeon et al., 1986; Fisher et al., 1986) and five residues in lamin A from rats (Kaufmann et al., 1983) (figures for lamin C are eight and five, respectively). If, however, fragments of identical sizes are recognized by an antibody in the digests from both lamins A and C, one may deduce that these fragments represent the amino-terminal portion of the molecule, as reasoned above for the NCS fragments.

CNBr hydrolysates were transferred onto nitrocellulose membranes and probed with the antibodies, as illustrated in Figure 7 and summarized in Table III. Nitrocellulose blots probed with the 34B6 antibody (Figure 7, lanes 8–10) showed important background reactions that could not be eliminated by the inclusion of 15% fetal calf serum with the casein used for quenching the blots. The pattern of recognition of 34B6

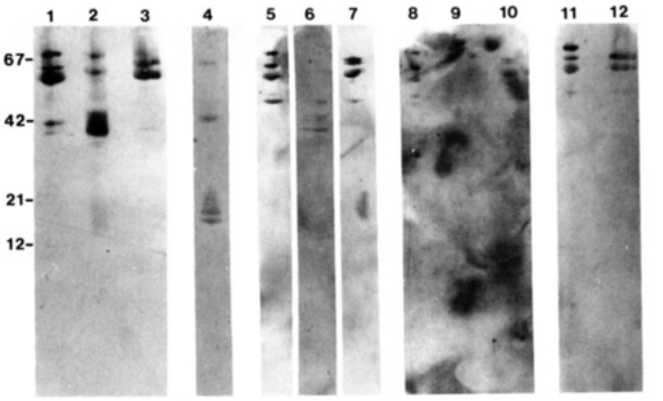

FIGURE 7: Lamins A (lanes 1, 5, 8, and 11), B (lanes 2, 4, 6, and 9), and C (lanes 3, 7, 10, and 12) were hydrolyzed with cyanogen bromide and electrophoresed as described in the legend to Figure 6, transferred onto nitrocellulose, and probed with the monoclonal antibodies aIF (lanes 1–3) 31B5 (lane 4), 55B3 (lanes 5–7), 34B6 (lanes 8–10), and 36C2 (lanes 11 and 12). The second antibody used was ^{125}I-labeled anti-mouse Ig immunoglobulin, and exposure times for radioautography ranged from 3 to 25 days, using Kodak XAR film for lane 6 and Kodak XRP film for the other lanes.

on fragments of lamins A and C was however reproducible, and this, combined with the absence of reaction on other fragments of lamins A and C (compare with Figure 6) or on fragments of lamin B (see below), made these results interpretable as the specific reactivity of 34B6.

The fragments of lamins A and C recognized by the anti-lamin antibodies (55B3, 34B6, and 36C2) were also recognized by the aIF antibody (Figure 7). The aIF antibody also reacted against a 37.1-kDa fragment present only in the hydrolysate from lamin A but not from lamin C, which would assign this fragment to the carboxy-terminal portion of lamin A. All fragments of lamins A and C recognized by 34B6 and 36C2 were recognized also by 55B3, but all three patterns differed from each other in one fragment, thereby reinforcing the results of competition studies (Table II).

Reactivity of three antibodies on CNBr hydrolysates of lamin B is illustrated in Figure 7 and summarized in Table III. We have been unable to detect any reaction of the fourth anti-lamin B antibody (34B6) on such hydrolysates: CNBr hydrolysis may have disturbed the 34B6 epitope on lamin B while leaving it intact on lamins A and C.

Table III shows that the fragments of lamin B recognized by 55B3 and 31B5 were also recognized by aIF, with the notable exception of two small (15.9 and 15.5 kDa) fragments detected by 31B5 exclusively, in agreement with the results of Table II showing absence of competition between 31B5 and aIF. The 51-kDa NCS fragment and the 37.2-kDa CNBr fragment of lamin B probably represent the same portion of the molecule since both are recognized by the same three antibodies (Figures 5 and 7). The 34.3-kDa CNBr fragment recognized by aIF and 55B3 but not by 31B5 may be considered as overlapping with the 37.2-kDa CNBr fragment, but at the exclusion of the 15.5-kDa CNBr fragment recognized by 31B5. Since the latter antibody competed with 55B3 while neither competed with aIF (Table II), the epitopes for 55B3 and aIF must be localized on different portions of the 34.3-kDa CNBr fragment, with the former being closer to the 15.5-kDa CNBr fragment on the intact lamin B molecule. A definitive localization of these epitopes will only be possible when the complete amino acid sequence for lamin B is known.

DISCUSSION

Lamin B differs from lamins A and C in a number of important characteristics: lamin B has a single isoelectric form

of acidic p*I*, it is strongly attached to the inner nuclear membrane, it can be modified by methyl esterification (Chelsky et al., 1987) and by a different level of phosphorylation, and it is constitutively expressed in most cell types studied to date (Krohne & Benavente, 1986; Lebel et al., 1987; Maul et al., 1987; Lehner et al., 1987).

The secondary structure of lamin B is not known, but there is preliminary evidence that it resembles the secondary structure of lamins A and C and, consequently, of intermediate filament proteins. In vitro studies using baby hamster kidney cell cytoskeletons (Goldman et al., 1986) and purified rat liver lamin B (Aebi et al., 1986) have shown that lamin B can form dimers and tetramers and participate in the formation of paracrystalline structures. These preliminary results are further strengthened by our findings: lamin B shares a number of distinct epitopes with intermediate filament proteins. These epitopes include the aIF epitope know to be present in a region of intermediate filament proteins forming a coiled-coil domain. Localization of the other epitopes identified by our monoclonal antibodies cannot be definitive at this point but may turn out to be also in α-helical regions involved in filament formation due to their presence on intermediate filament proteins. We are presently conducting in vitro reassociation experiments using purified rat liver lamins A, B, and C, and we have found that the aIF antibody will interfere with the reassociation of lamins into an insoluble structure, in agreement with the effect of microinjected aIF antibody on the disruption of intermediate filaments (Klymkowsky, 1981). Use of the monoclonal antibodies characterized in this study in reassociation experiments was hampered by the bulky nature of these IgM molecules: Fab fragments are being prepared for this purpose.

The primary structure of lamin B is not known. Our monoclonal antibodies have identified three distinct epitopes shared by lamin B with lamins A and C. A fourth epitope is recognized by the 41CC4 antibody (Burke et al., 1983) on all three lamins: this antibody did not compete with ours and did not appear to recognize intermediate filament proteins in immunofluorescence tests [see Figure 1 of Burke et al. (1983)], nor when it was used to probe blots of whole cell homogenates known to contain intermediate filament proteins (Lebel et al., 1987). Further knowledge of the primary structure of lamin B will be required to determine whether these shared epitopes play a similar role in all three molecules.

Finally, the functions and roles of the lamins are not known. These are indications that lamin B is involved in the attachment of the lamina to the inner nuclear membrane and in interaction with intermediate filament structures (Georgatos & Blobel, 1987). There are indications that lamins A and C interact with chromatin (Bouvier et al., 1985; Burke & Gerace, 1986) although the presence of all three lamins appears to be required in nuclei re-formation at the end of mitosis (Benavente & Krohne, 1986) and although a number of cell types possess a functional lamina composed of lamin B exclusively (Lebel et al., 1987).

In conclusion, we have prepared monoclonal antibodies against rat liver lamins that have allowed a further characterization of the primary and, possibly, secondary structures lamin B. These antibodies should be useful tools in setting up in vitro assays of the functional aspects of the lamins which are far from being clear at this point.

Added in Proof

Recent cDNA sequencing of the amphibian equivalent of mammalian lamin B (Krohne et al., 1987) has shown important similarities with lamins A and C and with intermediate filament proteins.

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Cytoplasmic Membrane Is the Target Organelle for Transition Metal Mediated Damage Induced by Paraquat in *Escherichia coli*†

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ABSTRACT: Bacterial survival indicates that copper or iron is an essential mediator in paraquat toxicity in *Escherichia coli* [Kohen, R., & Chevion, M. (1985) *Free Radical Res. Commun. 1*, 79–88; Korbashi, P., Kohen, R., Katzhendler, J., & Chevion, M. (1986) *J. Biol. Chem. 261*, 12472–12476]. In this study we have identified the cytoplasmic membrane as a target organelle in metal-mediated paraquat toxicity and have demonstrated the complete correlation of the membrane damage with the levels of adventitious copper (or iron). The extent of membrane damage was related by use of four parameters: (a) the level of cellular ATP, (b) the level of cellular potassium, (c) the cellular capacity to accumulate and retain radiolabeled leucine, and (d) the cellular integrity as reflected by transmission electron microscopy (TEM). Exposure of bacterial cells to a combination of paraquat and copper caused a marked decline in parameters a, b, and c. This decline was found to occur in parallel with, or even to precede, the sharp loss of survival of *E. coli* under the same conditions. Likewise, TEM micrographs clearly indicated alterations in cellular structure that possibly reflect sites of detachment of the cytoplasmic membrane from the bacterial capsule. In contradistinction, copper alone or paraquat alone could not bring about similar changes in cellular structure. These findings are in accord with the suggested site-specific metal-mediated Haber–Weiss mechanism for paraquat toxicity and support our notion that specific chelators of transition metals could reduce or prevent the biological deleterious effects of this herbicide.

The toxicity of the herbicide paraquat (PQ^{2+}),[1] also known as methyl viologen, has been extensively studied in a variety of biological models including *Escherichia coli* (Hassan & Fridovich, 1977, 1978, 1979a,b; Farrington et al., 1973; Kohen & Chevion, 1985a,b; Bagley et al., 1986). Fridovich and Hassan (1977, 1978, 1979a,b) showed that paraquat is concentrated by the bacterial cells and is reduced enzymatically to the monocation radical ($PQ^{\cdot+}$) and subsequently reacts with molecular oxygen to produce the superoxide radical within the cell. They also showed that its bactericidal effect requires molecular oxygen and a carbon source and that induction of endogenous superoxide dismutase and catalase provided considerable cellular protection.

In recent studies in both *E. coli* cells (Kohen & Chevion, 1985a,b; Korbashi et al., 1986) and mice (Kohen & Chevion, 1985c), we have demonstrated that the transition metals copper

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[1] Abbreviations: PQ^{2+}, paraquat dication, methyl viologen; $PQ^{\cdot+}$, paraquat radical monocation; detapac, diethylenetriaminepentaacetate; EDTA, ethylenediaminetetraacetate.

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