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Keratin-like proteins that coisolate with intermediate filaments of BHK-21 cells are nuclear lamins

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ABSTRACT **Four proteins of $M_r \approx$ 60,000, 65,000, 67,000, and 70,000 coisolate with the major intermediate filament (IF) structural proteins of BHK-21 cells. These proteins are keratin-like, they form distinctive paracrystals *in vitro*, and they are concentrated at the nuclear surface. Since these properties indicate similarities with the nuclear lamins, we have prepared conventional fractions of BHK-21 nuclei from which the same type of paracrystal is obtained. Furthermore, biochemical and immunological data demonstrate that the lamins are identical to the M_r 60,000–70,000 proteins found in IF preparations, and both sets of proteins are similar to keratin. These results suggest that an IF-like protein network is present in the nuclear lamina. We speculate that in some unknown way this network connects to the cytoplasmic IF network that courses from the juxtanuclear region to the cell surface. These proposed interconnecting networks may form part of the infrastructure of cytoskeletal–nuclear matrix-connecting links involved in signal transmission between the nuclear and cytoplasmic compartments of eukaryotic cells.**

Intermediate filaments (IF) are major cytoskeletal elements that are found in most types of mammalian cells (1–4). The molecular weights and immunological properties of IF differ according to cell type. However, their overall physical-chemical properties and morphology are conserved (1–4). Recently, several IF-associated proteins have also been described that appear to function in IF–IF crosslinking (5–9).

IF are usually apparent in large numbers in the perinuclear region, from which they radiate throughout the cytoplasm towards the plasma membrane (10–12). Ultrastructural studies suggest that IF are closely associated with or attached to the nuclear surface (10–15) and are also closely associated with the plasma membrane (16, 17). Based on these types of observations, it appears that IF may represent cytoskeletal-connecting links between the cell and nuclear surfaces (12).

Recently we described four M_r 60,000–70,000 keratin-like proteins in cultured fibroblasts that coisolated with IF (18). The keratin-like nature of these proteins was suggested by their physical, chemical, and immunological properties as well as the morphology of paracrystals that formed during their purification (18). Preliminary results indicated that these proteins were localized primarily at the nuclear surface (12, 18, 19).

In this study, we report that these latter proteins are very similar or identical to the nuclear lamins, a set of proteins thought to be involved in the organization of the nuclear envelope and in regulating interphase chromatin (20–24). In addition, nuclear lamins also appear to be members of the IF protein family, especially the keratins.

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MATERIALS AND METHODS

Cell Cultures. Baby hamster kidney cells (BHK-21) were grown in Dulbecco's modified Eagle's medium as described (25).

Isolation of Native IF. IF were prepared from cells grown to confluence in Corning roller bottles (26). This preparation, termed native IF (to distinguish it from *in vitro* reconstituted IF preparations) or cytoskeleton, is enriched in IF structural proteins along with some minor proteins, one of which is an IF-associated protein (8, 9).

Purification of the M_r 60,000–70,000 Proteins. Enrichment of the M_r 60,000–70,000 proteins from native IF was achieved by a previously published procedure (18). Pellets of native IF from five roller bottles were solubilized in 20 ml of disassembly buffer [8 M urea/5 mM sodium phosphate, pH 7.2/0.2 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$)/0.2% 2-mercaptoethanol] (room temperature, 30–60 min). Urea-insoluble material was removed by centrifugation at 30,000 × *g* (30 min, 15°C), followed by centrifugation at 250,000 × *g* (30 min, 15°C). The resulting supernatant was dialyzed against 6 mM sodium/potassium phosphate, pH 7.4/171 mM NaCl/3 mM KCl (PBSa) containing 0.2 mM $PhMeSO_2F$/0.2% 2-mercaptoethanol for 4 hr or overnight (room temperature) to polymerize IF. The IF were removed by centrifugation at 250,000 × *g* (30 min, 4°C) and the supernatant was dialyzed against 5 mM sodium phosphate, pH 6.6/0.2 mM $PhMeSO_2F$/0.2% 2-mercaptoethanol (4 hr, 4°C). Discrete paracrystals of the M_r 60,000–70,000 proteins formed, which were concentrated by centrifugation at 12,000 rpm in an Eppendorf centrifuge.

Nuclear Lamin Preparation. BHK-21 nuclear lamins were prepared from isolated nuclei by using a published method (24). Lamins were extracted with IF disassembly buffer, and paracrystals formed following the dialysis procedures described above.

Biochemical Techniques. Electrophoresis was carried out on 7.5% polyacrylamide gels with 4.5% polyacrylamide stacking gels (27). Amino acid analyses were carried out on proteins prepared from bands excised from gels following the removal of contaminating glycine (18). The α-helical content of individual proteins was determined by optical rotary dispersion and circular dichroism (ORD/CD) (18).

Immunological Methods. A rabbit antiserum was raised against the M_r 60,000 protein of paracrystals from native IF using polyacrylamide gel purification (see above).

A mouse monoclonal antibody (no. 19) directed against the M_r 60,000–70,000 proteins was prepared from a mouse hybridoma line obtained following immunization with a native IF preparation (8). This monoclonal antibody reacts with each of the M_r 60,000–70,000 proteins.

Two mouse monoclonal antibodies, J-9 and J-16, obtained following immunization with rat liver nuclear envelope frac-

Abbreviations: IF, intermediate filament(s); BHK, baby hamster kidney.

tions, have been described in Maul *et al.* (28). J-9 reacts with lamins A and C and J-16 reacts with A, B, and C.

To determine the specificity and cross-reactivity of these different antibodies, the electrophoretic transfer immunoblotting procedure was used (29). Indirect immunofluorescence with monoclonal and polyclonal antibodies is described elsewhere (8).

Electron Microscopy. Paracrystals were centrifuged (12,000 rpm, Eppendorf centrifuge, 15 min, room temperature) into pellets that were fixed in glutaraldehyde/osmium tetroxide, embedded in resin, thin-sectioned, and stained using established methods (8). Sections were viewed in a JEOL 100CX electron microscope.

RESULTS

Native IF were obtained from BHK-21 cells (26). These preparations are greatly enriched in IF structural proteins and also contain several minor components, primarily in the M_r 60,000–70,000 range and the M_r 200,000–300,000 range (Fig. 1, lane a).

Previously we described the properties of enriched preparations of the M_r 60,000–70,000 proteins, which consist of M_r 60,000, 65,000, 67,000, and 70,000 species (18). These proteins are keratin-like according to their amino acid composition, their α-helical content (30–35%), and, in some cases, their crossreactivity with mouse epidermal keratin antibodies (18). These proteins also form paracrystals with substructures consistent with existing domain maps of IF structural proteins (1–4); however, we have been unable to form IF from them *in vitro* (18).

The rabbit antiserum directed against the M_r 60,000 protein (see *Materials and Methods*) reacts with the M_r 60,000, 65,000, and 70,000 proteins as determined by immunoblotting (Fig. 2, lanes a and b). In indirect immunofluorescence assays, this antiserum produces a ring of fluorescence at the edge of the nucleus, with less intense staining seen throughout the nuclear matrix (Fig. 3*a*). A mouse monoclonal antibody that reacts with all four of the M_r 60,000–70,000 proteins (Fig. 2, lane c) produces a similar staining pattern (Fig. 3*b*).

Since this perinuclear staining pattern is similar to that produced with nuclear lamin antibodies (21, 22) and since the major polypeptides described in nuclear lamin preparations possess very similar molecular weights (e.g., 60,000–70,000; refs. 21–23), we decided to compare directly the M_r 60,000–70,000 proteins present in native IF preparations with nuclear lamins.

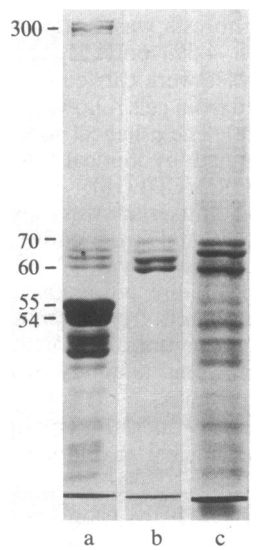

FIG. 1. NaDodSO$_4$/PAGE analyses of IF-enriched cytoskeletons obtained from BHK-21 cells (lane a), paracrystals derived from native BHK IF showing great enrichment for the M_r 60,000–70,000 proteins seen as minor constituents in lane a (lane b), and a BHK-21 nuclear lamin preparation showing enrichment for the M_r 60,000–70,000 proteins (lane c). All gels are stained with Coomassie blue R-250. Molecular weights are shown as $M_r \times 10^{-3}$.

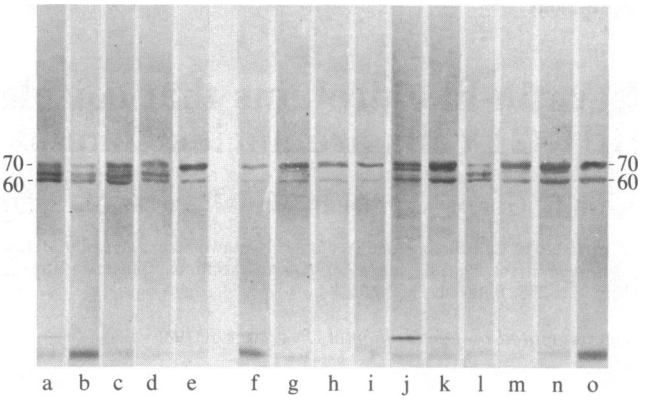

FIG. 2. Summary of the electrophoretic transfer immunoblotting results. Lanes a–e, results obtained by reacting various antibody preparations against paracrystals obtained from native IF: lane a, amido black stain of the transferred protein; lane b, reaction seen with rabbit anti-M_r 60,000 antibody; lane c, reaction with the monoclonal antibody directed against the M_r 60,000–70,000 IF-related proteins (no. 19); lanes d and e, reactions with the two monoclonal antibodies directed against rat liver nuclear lamins. Lane d is J-16 and lane e is J-9. Lanes f–j, results obtained by reacting the BHK-21 nuclear lamin preparation seen in Fig. 1, lane c: lane f, anti-M_r 60,000 antibody reaction; lane g, reaction with monoclonal antibody no. 19; lane h, reaction with J-16; lane i, reaction with J-9; lane j, amido black stain of the transferred protein. Lanes k–o, results obtained by reacting BHK-21 nuclear lamin-derived paracrystal proteins as seen in Fig. 5 with various antibodies: lane k, the transferred protein stained with amido black; lane l, reaction seen with J-9; lane m, J-16; lane n, no. 19; lane o, rabbit anti-M_r 60,000. All of the antibodies show significant crossreactivities with each of the fractions tested. Molecular weights are shown as $M_r \times 10^{-3}$.

Nuclear lamin fractions obtained from isolated nuclei have been compared with the M_r 60,000–70,000 proteins of native IF by using a two-step dialysis procedure (*Materials and Methods*). In the case of native IF, extraction in 8 M urea solubilizes virtually all of the IF structural proteins and the M_r 60,000–70,000 proteins. The resulting preparation is centrifuged at high speed to remove undissolved material. The supernatant is dialyzed against PBSa (*Materials and Methods*), which promotes the polymerization of IF from

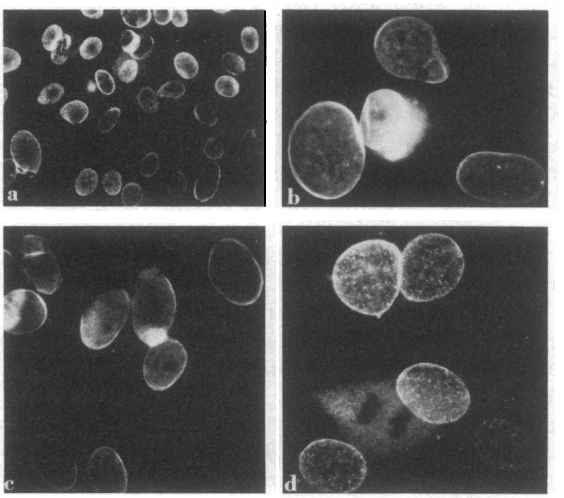

FIG. 3. Indirect immunofluorescence micrographs. (*a*) Rabbit anti-M_r 60,000 pattern. (*b*) Pattern generated by the monoclonal antibody preparation that reacts with all of the M_r 60,000–70,000 proteins found in native IF preparations (no. 19). (*c*) A monoclonal anti-rat nuclear lamin stain (antibody J-16). (*d*) Staining pattern seen with a rabbit polyclonal antibody directed against the BHK-21 lamin fraction. (*a*, ×335; *b*, ×1120; *c*, ×880; *d*, ×960.)

their structural subunits (M_r 54,000 and 55,000; ref. 18). The polymerized IF are removed from the dialysate by high-speed centrifugation. $NaDodSO_4$/PAGE analysis of the resulting pellet shows that the majority of M_r 60,000–70,000 proteins is absent (18). This second supernatant was dialyzed against a low ionic strength buffer followed by centrifugation to obtain a pellet of distinct paracrystals (Fig. 4 *a* and *b*). These paracrystals are morphologically similar to those obtained from native IF preparations (18). When these pellets are analyzed by $NaDodSO_4$/PAGE, only the M_r 60,000–70,000 proteins are obvious (Fig. 1, lane b).

Isolated nuclei subjected to the standard protocols for nuclear lamin enrichment (ref. 24; *Materials and Methods*) contain three major proteins, M_r 60,000, 67,000, and 70,000, as well as a minor M_r 65,000 component and other minor constituents (Fig. 1, lane c). Following extraction under the same conditions used for native IF and the dialysis and centrifugation steps described above, a pellet enriched in paracrystalline arrays is obtained (Fig. 4 *c* and *d*). The center-to-center spacing between either dark or light bands in both sets of paracrystals is ≈34–36 nm, and the thickness of the lighter staining bands is ≈20–21 nm, as determined from electron micrographs of thin-sectioned preparations. These latter bands contain electron-dense filaments of ≈2 nm in diameter with their long axes appearing to interconnect adjacent dark bands. These pellets show enrichment for the lamin proteins that comigrate with the four polypeptides seen in native IF-derived paracrystals (Fig. 5). Lamin antibodies produce nuclear staining patterns that are similar to those obtained with antibodies directed against the similar proteins obtained from native IF (compare Fig. 3 *a* and *b* with *c* and *d*).

Since these results suggest that the M_r 60,000–70,000 native IF-derived proteins and the nuclear lamins are very similar, electrophoretic transfer immunoblotting has been used to determine the crossreactivity of antibodies directed against both fractions. The results are summarized in Fig. 2. It appears that all of the antibodies crossreact with one or more proteins in both paracrystal preparations.

Further support for the similarities between the nuclear lamins and the M_r 60,000–70,000 proteins from native IF stems from the comparative amino acid analysis data presented in Table 1. All of the proteins are similar to each other and are also similar to a mouse skin keratin known to be a subunit of IF (18). In addition, the data show that there are distinctive differences in the compositions of these keratin-like proteins and the BHK M_r 55,000 IF structural protein (vimentin). ORD/CD analyses show that the nuclear lamins have high α-helical contents (41% ± 8% for the M_r 60,000 protein, 36% ± 5% for M_r 67,000 and 37% ± 4% for M_r 70,000). These percentages are consistent with those that we have reported for the M_r 60,000–70,000 native IF proteins and

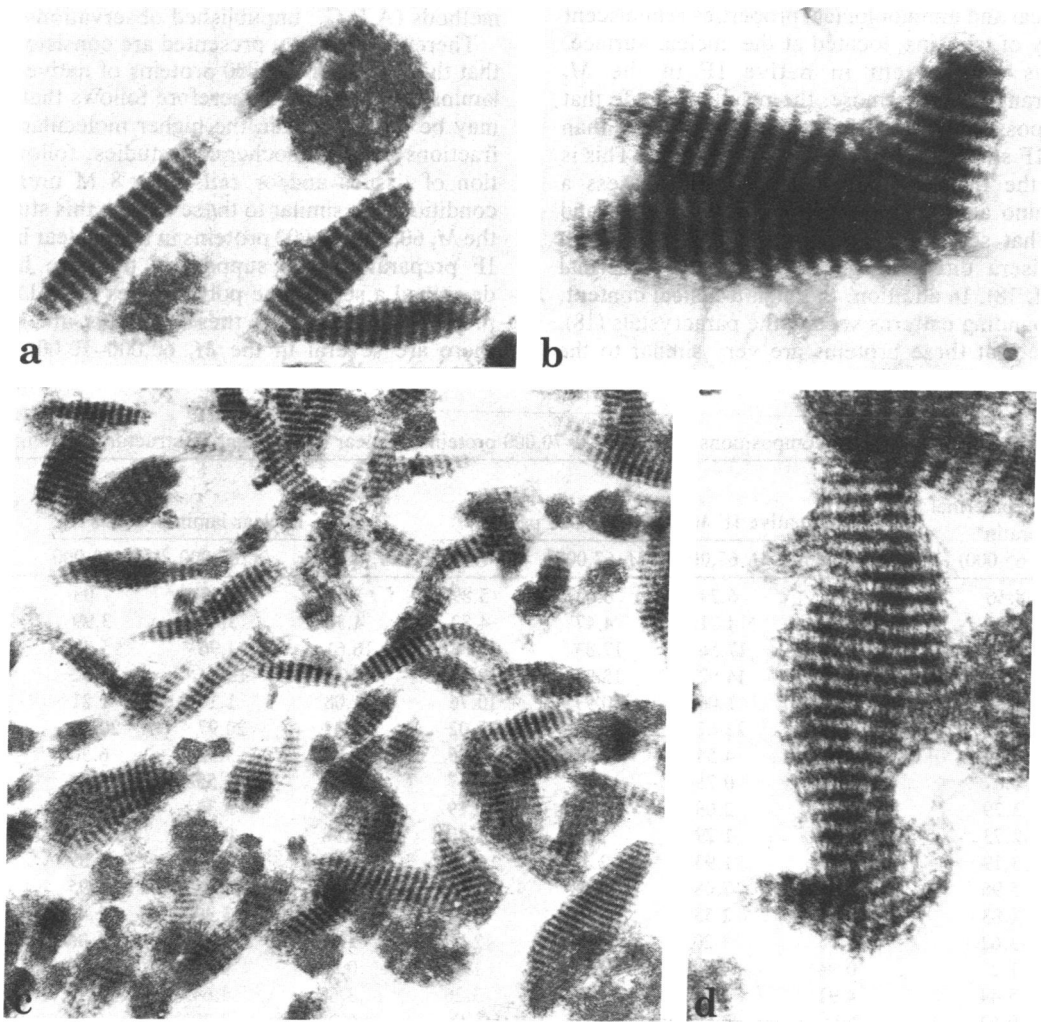

FIG. 4. Ultrastructural observations of paracrystals. (*a* and *b*) Thin-section and negative-stain views, respectively, of paracrystals obtained from native IF preparations. (*c* and *d*) Thin sections of pellets of the paracrystals obtained from a nuclear lamin preparation. These appear to be identical to those obtained from the native IF preparations. (*a*, ×50,400; *b*, ×80,000; *c*, ×32,000; *d*, ×80,000.)

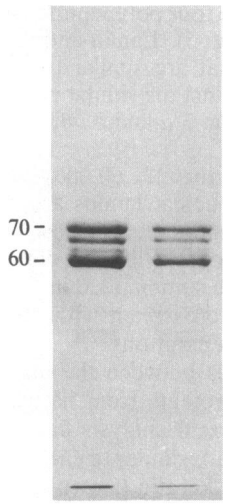

FIG. 5. $NaDodSO_4$/PAGE analyses of the proteins seen in a fraction of native IF (lane a) and nuclear lamin (lane b)-derived paracrystal preparations. Molecular weights are shown as $M_r \times 10^{-3}$.

they are also consistent with the values obtained for various keratins (18).

DISCUSSION

Our results support the idea that there are proteins with physical-chemical and immunological properties reminiscent of the IF family of proteins, located at the nuclear surface. These proteins are present in native IF in the M_r 60,000–70,000 range. Furthermore, the results indicate that these proteins possess greater similarity to the keratins than other types of IF structural proteins (e.g., vimentin). This is supported by the findings that these proteins possess a keratin-like amino acid composition (see refs. 1–4, 18 and Table 1) and that some of these proteins crossreact with polyclonal antisera directed against bona fide epidermal keratin (see ref. 18). In addition, the high α-helical content, as well as the banding patterns seen in the paracrystals (18), further indicate that these proteins are very similar to the keratins characterized to date (1–4, 18).

It should be emphasized, however, that the M_r 60,000–70,000 proteins have not yet been shown to form IF *in vitro*; rather, they tend to form other insoluble arrays, such as the paracrystals (ref. 18; and unpublished observations). Since we have not found such paracrystalline arrays *in situ* (R.D.G., unpublished observations), it appears that these proteins are probably in an organizational state that is not readily distinguished in conventional electron microscopic preparations.

The finding that the native IF-derived, keratin-like paracrystal-forming proteins are localized in the nuclear envelope region and the demonstration that similar protein paracrystals are formed from standard nuclear lamin preparations provide convincing proof that these two protein fractions are probably identical. Additional data supporting this identity are derived from the findings that the major polypeptides comprising the nuclear lamin fraction possess high α-helical contents and that their amino acid compositions are very similar to epidermal keratin and the M_r 60,000–70,000 proteins found in native IF (18). In addition, antibodies directed against the M_r 60,000–70,000 proteins of native IF or the nuclear lamins exhibit extensive crossreactivity.

It is also important to mention that the purification of the nuclear lamins by the formation of paracrystals represents a relatively simple method for the purification of these major nuclear matrix components. Indeed, rat liver nuclear lamins can be purified as similar paracrystals employing the same methods (A.E.G., unpublished observations).

Therefore, the data presented are consistent with the idea that the M_r 60,000–70,000 proteins of native IF and nuclear lamins are identical. It therefore follows that nuclear lamins may be present within the higher molecular weight keratin fractions used in biochemical studies, following solubilization of tissues and/or cells with 8 M urea (30, 31). The conditions are similar to those used in this study to solubilize the M_r 60,000–70,000 proteins in the nuclear lamin and native IF preparations. In support of this, we have previously described a set of nine polypeptides in HeLa cell native IF preparations. Most of these proteins are keratin-like and there are several in the M_r 60,000–70,000 range that are readily solubilized in 8 M urea (32). Recently we have found

Table 1. Comparisons of amino acid compositions of M_r 60,000–70,000 proteins, nuclear lamins, and IF structural proteins

Amino acid	Mouse epidermal keratin* (M_r 65,000)	BHK native IF M_r 60,000–70,000 proteins				BHK nuclear lamin proteins			BHK IF structural protein (vimentin)* (M_r 55,000)
		M_r 60,000	M_r 65,000	M_r 67,000	M_r 70,000	M_r 60,000	M_r 67,000	M_r 70,000	
Asp	8.36	6.45	6.29	6.03	5.89	5.33	6.48	7.06	9.1
Thr	3.21	3.99	4.21	4.47	4.82	4.30	3.48	3.99	4.6
Ser	12.29	17.17	17.56	17.83	18.04	16.61	14.96	15.48	9.5
Glu	12.34	15.22	14.92	13.25	10.69	15.72	15.41	13.02	15.2
Pro	1.52	1.34	1.06	0.97	10.76	1.08	1.10	1.21	0.7
Gly	22.43	21.07	23.62	24.92	27.02	21.32	20.97	20.89	9.8
Ala	6.44	4.96	4.54	4.78	4.95	4.94	5.03	6.30	6.4
Cys	0.67	0.71	0.75	0.74	0.67	0.46	0.55	0.84	0.8
Val	3.29	2.18	2.08	2.23	3.19	4.02	3.36	4.08	5.7
Met	2.73	1.22	1.29	1.17	1.49	0.98	0.99	1.49	2.2
Ile	3.19	2.89	1.93	2.37	2.50	3.50	3.50	2.89	3.5
Leu	5.96	8.06	7.08	6.98	7.04	7.82	7.94	6.95	11.6
Tyr	1.83	2.51	2.53	2.31	2.02	2.52	3.40	2.43	2.8
Phe	3.62	2.75	3.20	2.98	2.0	2.42	2.97	1.96	2.6
His	1.2	0.94	1.04	1.03	1.21	0.99	1.25	1.94	1.8
Lys	5.44	4.91	3.53	3.39	3.26	3.60	4.64	5.15	6.0
Try	0.63	ND	ND	ND	ND	0.28	0.20	0.24	0.38
Arg	4.75	4.16	4.13	4.36	4.60	4.39	3.73	4.04	8.2

All data are expressed as residues per 100 residues. ND, not determined.
*Data reproduced from Zackroff *et al.* (18).

by immunoblotting analyses that these latter HeLa proteins react with the nuclear lamin antibodies (A.E.G., unpublished observations). Thus, great caution must be taken in the interpretation of data regarding the presence of IF structural proteins that involve solubilization in urea-containing solutions—i.e., they may contain the nuclear-associated keratin-like proteins (lamins) and not only the cytoplasmic IF-forming proteins (keratins or "cytokeratins").

These findings also call into question the use of schemes developed to classify cell types by their IF composition (e.g., ref. 33, 34). According to such schemes, cells such as fibroblasts are considered to be devoid of keratin. Since the lamins appear to be conserved in different vertebrate and invertebrate cell types (28), then the consideration that most mesenchymal cells do not possess proteins similar to keratin is misleading (33, 34). This is especially important in those studies in which the various members of the "keratin" family are being used in tumor diagnosis (31, 34).

Probably the most exciting and provocative result of this study is that there is a set of IF-like proteins that form a stable polymer system devoid of obvious IF [≈10-nm (diameter) filaments] at the nuclear surface, which is the same region in which many cytoplasmic IF appear to terminate (12). Thus, it is conceivable that these two chemically related polymer systems are linked to each other. The mechanism of this linkage is, to date, not understood. Since the nuclear lamins are thought to reside primarily at the inner face of the nuclear envelope, it is difficult to imagine how such an interaction might occur. One possibility is that the two systems are connected indirectly through a series of transmembrane linkage molecules that have yet to be discovered or that there are more direct connections at the level of the nuclear pore complex (11).

On the other hand, it should be noted that the presence of nuclear lamins and IF in "cytoskeletal" preparations may represent the fortuitous coisolation of two very insoluble and functionally unrelated polymer systems. Therefore, the coisolation of these two sets of proteins may represent an artifactual relationship. However, we feel that this is an unlikely possibility, due to the biochemical data that demonstrate that the nuclear lamins are IF-like proteins and the morphological data regarding the close association of IF with the nuclear surface (10–12). In support of this latter contention, other laboratories have also proposed that IF interact with the nuclear surface–nuclear matrix (14, 15, 35).

Despite the above mentioned problems, we feel that these findings lend credence to the hypothesis that nuclear–cytoplasmic interactions may be in part mediated through the connections of cytoplasmic IF with the nuclear surface. It has been proposed that the nuclear lamina may be attached to chromatin and the inner membrane of the nuclear envelope (36) and that these associations are important in determining the molecular organization of interphase chromatin (36). In light of this latter possibility and our finding that the nuclear lamina may also be associated with cytoplasmic IF, which, in turn, may connect directly with the plasma membrane (12, 37), it is intriguing to speculate that these potential cytoskeletal–nuclear matrix interactions may be involved in regulating the bidirectional flow of information that ultimately may alter genomic and cytoplasmic functions (12, 37).

Note. Recently, McKeon *et al.* (38) demonstrated that there is extensive amino acid sequence homology between the A and C lamins and IF structural proteins in general.

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