Human Epithelial Cell Intermediate Filaments: Isolation, Purification, and Characterization

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ABSTRACT Intermediate filaments (IF) isolated from human epithelial cells (HeLa) can be disassembled in 8 M urea and reassembled in phosphate-buffered solutions containing >0.1 mg/ml IF protein. Eight proteins were associated with HeLa IF after several disassembly-reassembly cycles as determined by sodium dodecyl sulfate gel electrophoresis (SDS PAGE). A rabbit antiserum directed against HeLa IF contained antibodies to most of these proteins. The immunofluorescence pattern that was seen in HeLa cells with this antiserum is complex. It consisted of a juxtanuclear accumulation of IF protein and a weblike array of cytoplasmic fibers extending to the cell border. Following preadsorption with individual HeLa IF proteins, the immunofluorescence pattern in HeLa cells was altered to suggest the presence of at least two distinct IF networks. The amino acid composition and α-helix content (~38%) of HeLa IF proteins was similar to the values obtained for other IF proteins. One-dimensional peptide maps show extensive homology between the major HeLa IF protein of 55,000-mol-wt and a similar 55,000-mol-wt protein obtained from hamster fibroblasts (BHK-21). HeLa 55,000-mol-wt homopolymer IF assembled under conditions similar to those required for BHK-21 55,000-mol-wt homopolymers. Several other proteins present in HeLa IF preparations may be keratin-like structural proteins. The results obtained in these studies indicate that the major HeLa IF protein is the same major IF structural protein found in fibroblasts. Ultrastructural studies of HeLa cells revealed two distinct IF organizational stages including bundles and loose arrays. In addition, in vitro reconstituted HeLa IF also exhibited these two organizational states.

Intermediate filaments (IF)[1] constitute a family of cytoplasmic fibrous proteins that are involved in cytoskeletal functions including cell shape formation and maintenance (1–3). Biochemical and immunological data obtained from various cell and tissue types indicate that this family contains numerous subdivisions or classes of IF (4). These IF classes are categorized by differences in their protein subunit compositions and molecular weights. In spite of these differences, certain features of IF subunits are conserved, which place them in the k-m-e-f class of fibrous proteins. These include their ability to form IF in vitro, their amino acid composition, and their coiled-coil α-helical domains (5, 6).

The possibility that epithelial cells may contain more than one type of IF was first suggested by in vitro reconstruction studies employing epidermal keratin containing IF (7). More recently, using immunolocalization methods, it has been suggested that cultured human epithelial cells (HeLa) contain two major types of IF (8–10). One of these types appears to consist of IF bundles which resemble the tonofilaments seen in cells comprising epithelial tissues. Several properties of these IF bundles have been suggested by the findings that antibodies to bovine prekeratin decorate them following indirect immunoferritin and immunofluorescence labeling (8–10); and that the distribution of IF bundles is unaltered by colchicine (8, 9). There are also loose aggregates of IF that are believed to be similar to the major type of IF found in fibroblasts, since they appear to bind antibodies against IF protein (vimentin) obtained from fibroblasts; and they form juxtanuclear aggregates when colchicine is present (10).

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; hmw, highest molecular weight proteins; IF, intermediate filaments; lmw, lowest molecular weight proteins; PBSa, 6 mM Na^+-K^+ phosphate, 171 mM NaCl, 3 mM KCl, pH 7.4.

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Hela cytoskeletons prepared using high and low salt solutions with Triton X-100 contain IF (8). When analyzed by SDS Page, these preparations are seen to contain several proteins, one of which co-migrates with the major 3T3 cell IF protein (vimentin) and others that co-migrate with bovine prekeratin proteins (8). These studies also suggest the presence of two different IF classes in HeLa cells, although there is no direct evidence that the proteins in the Triton-extracted cytoskeletons are IF structural proteins. The best and most direct methods for determining which proteins are IF structural subunits include in vitro reconstitution of IF from candidate subunits, and the determination of subunit structural and biochemical properties (See e.g., 11, 12). There is the possibility that one or more of the polypeptides present in these "cytoskeletons" are actually IF-associated proteins such as the IF cross-linking protein, filaggrin (13, 14), or nonspecifically-bound contaminants. In addition, there are conflicting reports in the literature as to the presence of fibroblastic IF proteins in HeLa cells in the absence of colchicine (compare 8, 9, and 15), and thus there is the possibility that the deleterious side effects of colchicine, such as the induction of a multinucleated state (16), might induce the formation of a second form of IF.

The present study was initiated to obtain more direct proof that HeLa cells contain more than one IF type. To this end, a protocol has been developed for the purification of HeLa IF using cycles of disassembly/reassembly in vitro. This results in the purification of eight distinct HeLa IF associated proteins. Morphological, biochemical, and immunological studies using these in vitro reconstituted HeLa IF, as well as in vitro reconstitution studies using individually purified HeLa IF polypeptides, all support the contention that cultured HeLa cells contain several types of IF structural proteins, and that their intracellular distribution is different in different regions of the cytoplasm. Due to the complexity of the HeLa IF system and the rather remarkable number of possible structural subunits in this one cell type, we have decided for the sake of clarity, to refer to these proteins as HeLa IF proteins in general; and more specifically, HeLa 55,000-mol-wt IF protein, HeLa 43,000-mol-wt IF protein, etc.

MATERIALS AND METHODS

Cell Culture: Hela cells were grown at 37°C in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY), supplemented with 10% calf serum and 50 U/ml of penicillin and 50 μg/ml of streptomycin. Confluent cultures grown in plastic tissue culture dishes were treated with 0.05% trypsin, and EDTA (Gibco Laboratories) to remove cells prior to replating in other dishes or roller bottles. Rat kangaroo kidney (PtK_2) cells were grown in Eagle's minimum essential medium (Gibco Laboratories), 10% fetal calf serum, and 50 U/ml penicillin, 50 μg/ml streptomycin. BHK-21 cells were grown in Dulbecco's modified Eagle's medium containing 10% calf serum, 10% tryptose phosphate broth, and 50 U/ml penicillin, 50 μg/ml streptomycin. In some experiments, subconfluent dishes of cells were exposed to 5×10^{-6} M colchicine (Sigma Chemical Co., St. Louis, MO) in culture medium for 18–24 h to induce the formation of IF caps (FC) (16).

Isolation, Solubilization, and Reassembly of HeLa IF: Preparations of HeLa IF were obtained as described previously (3, 17) with minor modifications. Confluent cultures in roller bottles were rinsed twice with Ca^{2+}-Mg^{+2}-free phosphate-buffered saline (PBSa: 6 mM Na^+-K^+ phosphate, 171 mM NaCl, 3 mM KCl, pH 7.4). 10 ml of lysis buffer (0.6 M KCl, 10 mM $MgCl_2$, and 1% Triton X-100 [Rohm and Haas Co., Philadelphia, PA] in PBSa) containing 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.) and 1 mM *p*-tosyl-L-arginine methyl ester-HCL (TAME; Sigma Chemical Co.) were added to each bottle. The bottles were placed on a Bellco roller culture apparatus (Bellco Glass, Inc., Vineland, NJ) at 37°c until the cells lysed. The lysed cells were removed from the bottles with a rubber policeman and homogenized by three strokes in a Potter-Elvehjem homogenizer at 4°C. Deoxyribonuclease I (DNase I) (Sigma Chemical Co.) was added (0.5 mg/ml) and the homogenate was incubated for 10 min/4°C. The resulting IF networks were pelleted by centrifugation at 2,200 *g* for 3–5 min in a Beckman TJ-6R (4°C) (Beckman Instruments, Inc., Fullerton, CA). The pellet was washed three times in PBSa containing 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride.

Solubilization of HeLa IF was achieved by suspending the above pellets in urea buffer (8 M urea, 10 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, pH 7.6). This suspension was stirred for 2 h at room temperature. Cell debris was then removed by an initial centrifugation at 30,000 *g* for 30 min/4°C, followed by a second centrifugation at 250,000 *g* for 2 h/4°C, to remove smaller particles in a Beckman model L5-65 ultracentrifuge (Beckman Instruments, Inc.) using a type 65 rotor. The resulting supernatant solution contained IF subunits (18).

Optimal conditions for IF assembly in vitro were determined (see Results). The yields of IF and their associated proteins were established by pelleting the IF from suspension by centrifugation at 125,000 *g* for 1 h at 4°C, and by measuring the protein concentration of the solution and the resulting supernatant solution before and after centrifugation (19).

SDS PAGE and One-dimensional Peptide Mapping by Limited Proteolysis: Routine electrophoretic analysis of HeLa IF proteins was performed on 7.5% (or 10%) polyacrylamide slab gels with 4.5% stacking gels (20). One-dimensional peptide mapping by limited proteolysis was carried out as described by Cleveland et al. (21). Slab gels were silver stained by the method of Oakley et al. (22) to enhance the resolution of the peptides. Destaining, when necessary, was performed according to the method of Switzer et al. (23).

Isolation and Separation of the HeLa IF Proteins: Hela IF were isolated and purified by one cycle of disassembly/reassembly. HeLa IF polypeptides were separated using either a gel slice method or DE-52 cellulose chromatography (Whatman Chemical Separation, Inc., Clifton, NJ) followed by preparative PAGE (24). The gel slice system consisted of visualizing bands of protein by immersion in 0.5 M KCl for ~2 min following SDS PAGE (20). The desired bands were excised from each lane and minced with a razor blade. The pieces were placed in Laemmli sample buffer (without Pyronin Y) on 2 × 0.5 cm 4.5% Laemmli tube gels and electrophoresed at 2–5 mA/tube into dialysis tubing. Following removal of SDS, the proteins were solubilized in urea buffer and subsequently dialyzed against reassembly buffer under the conditions described in Results.

For chromatographic separation, a column (28 × 1.5 cm) of DE52-cellulose was equilibrated at 23°C with a buffer containing 8 M urea, 20 mM Tris-HCl (pH 7.6), 25 mM B-mercaptoethanol, 1 mM EDTA, and 2% isopropanol. Proteins were eluted in steps with increasing concentrations of KCl (0, 25, 50, 75, and 100 mM). Precautions were taken to remove cyanate ions from the urea buffer (18). Fractions under each peak containing the same polypeptides were pooled and dialyzed against 10 mM Tris-HCl, pH 7.6, at 23°C. The samples were transferred to centrifuge tubes and 0.2 vol of 1 M sodium acetate buffer pH 5.0 were added to each sample. The resulting precipitates were pelleted, solubilized in a small volume of urea buffer, and dialyzed against 100 vol of the cathode buffer used for preparative gel electrophoresis.

Preparative gel electrophoresis was performed using a Uniphor 7900 apparatus (LKB) and a discontinuous gel buffer system (25) in which 1.0% SDS or 0.5% sodium decyl sulphate (NaDS) (wt/vol) was included in the cathode buffer. A 7.5% T (total acrylamide concentration), 3% C (concentration of cross-linker) gel (15 × 2.4 cm) was used (26). The sample (in cathode buffer) was heated at 90–95°C for 2 min in the presence of β-mercaptoethanol prior to loading the gel. Electrophoresis was at 20 mA at 20.0 ± 0.5°C for 20–24 h. Material that was electrophoresed from the end of the gel was eluted with anode buffer containing 0.1% SDS (0.5% sodium decyl sulphate) at 12 ml/h and was collected into fractions of 1.5 ml. Initial localization of the fractionated polypeptides was determined by measuring the rate of migration of bands made visible as refractive-index discontinuities; this permitted accurate prediction of the position of elution of each band. Analytical tube gel electrophoresis (9% T/3% C) was carried out on fractions at and around the predicted regions (25). Fractions containing the same proteins from several preparative gel procedures were pooled prior to further use. SDS was removed (see below) and the proteins were solubilized in urea buffer followed by dialysis against reassembly buffer under conditions described in Results.

Removal of protein bound SDS was accomplished using the method of Henderson et al. (26). The protein-SDS complex was precipitated from solution at 0°C with 20 vol of acetone/triethylamine/glacial acetic acid (88:6:6, vol/vol/vol). The protein precipitate was re-extracted twice with the same solvent made 5% (vol/vol) with water. Finally, the protein was redissolved (0.3–1 mg/ml) in urea buffer and dialyzed against 1,000 vol of reassembly buffer (see Results).

Amino Acid Analysis: Amino acid analysis of one-cycle reassembled HeLa IF was performed on a Durrum D-500 autoanalyzer. Reassembled IF

were desalted by dialysis, lyophilized, and then hydrolyzed in vacuo in 6 M HCl at 100°C for 24 h. Individual purified HeLa IF proteins were desalted by dialysis following removal of SDS and then lyophilized. These samples were hydrolyzed in vacuo in 5.7 M HCl at 100°C for 24 h and then analyzed on a Beckman 121 amino acid analyzer (Beckman Instruments, Inc.)

Determination of α-Helix Content and X-ray Diffraction: The α-helix contents of reassembled HeLa IF and individually purified proteins were estimated by circular dichroism measurements (5).

A pellet of reassembled HeLa IF was drawn into fibers on a cross-wire support and allowed to dry at 5°C. The fibers were mounted in a Norelco microcamera with a specimen to film distance of 30 mm and exposed to Cu-K alpha radiation (λ-1.54 Å) for ~48 h in an atmosphere of dry helium. Finely powdered NaCl was dusted onto selected fibers before exposure and the 2.82 Å spacing was used for calibration purposes.

Electron Microscopy: IF preparations were negatively stained with 1–3% uranyl acetate on formvar-carbon coated copper grids. Pellets of isolated IF or cells grown on 60 mm Falcon cell culture dishes (Falcon Labware, Oxnard, CA) were fixed in 1% glutaraldehyde in PBS (PBSa + 1 mM $CaCl_2$ + 0.5 mM $MgCl_2$) for 15 min, washed 1 h in PBS, postfixed in 1% osmium tetroxide in PBS for 30 min, rinsed in distilled water, and dehydrated and embedded as previously described (e.g., 3, 16). Thin sections were made on an LKB Ultrotome III using a diamond knife and were mounted on uncoated copper grids. These were stained with uranyl acetate and lead citrate (3, 16). Electron micrographs were taken on either a Philips 201C or 401 electron microscope.

Immunological Procedures: A column (90 × 2.6 cm) of Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, NJ) was used to separate the HeLa IF polypeptides from any high molecular weight proteins remaining after one cycle of reassembly in vitro. The disassembly and elution buffers contained 8 M urea, 50 mM Tris-HCl, 0.2 M KCl, 1 mM EDTA, and 1 mM dithiothreitol, at pH 7.6. The column was run at maximum flow rate at room temperature (23°). The fractions containing IF protein were pooled, dialyzed exhaustively against distilled water, and then lyophilized.

Prior to immunization, one New Zealand rabbit was bled and the preimmune serum prepared. 2 mg of HeLa IF protein in 0.5 ml of water was emulsified with an equal volume of Freund's adjuvant and injected subcutaneously into the neck of the rabbit two days later. The rabbit was boosted once intramuscularly 21 d after the initial injection with 3 mg of protein in 50% Freund's incomplete adjuvant. One week after the last injection, blood was collected from the ear vein, left to clot at 37°C for 2 h and overnight at 4°C. The serum was clarified by two centrifugations at 2,000 *g* for 30 min at 4°C. 1-ml aliquots were frozen and stored at −20°C. A DEAE-affi-gel blue column (Bio-Rad Laboratories, Richmond, CA) was used to separate the IgG from the serum.

Determination of those proteins containing antigenic determinants with which the HeLa IF antiserum, BHK-21 IF antiserum (3), and antisera to individually purified bovine stratum corneum (prekeratin) proteins reacted, were carried out using HeLa IF, BHK-21 IF, bovine prekeratin, and human callus proteins. The technique employed was the ^{125}I-protein A binding procedure of Adair et al. (27).

For several experiments, HeLa IF antiserum was preabsorbed with HeLa IF proteins prior to use in the ^{125}I-protein A procedure and immunofluorescence: immediately following SDS PAGE, HeLa IF protein bands were made visible by immersion of the gel in 0.5 M KCl for ~2 min. The major polypeptides were carefully excised with a razor blade. These gel slices were homogenized with a small volume of antiserum in a ground glass homogenizer. The homogenate was stirred overnight at 4°C and then clarified twice at 2,200 *g* for 10 min in a Beckman TJ-6R centrifuge (Beckman Instruments, Inc.). The preabsorbed serum was used immediately or frozen for later use.

For immunofluorescence studies cells grown to subconfluence on glass coverslips were rinsed in PBS and fixed in 3.7% formaldehyde/PBSa for 3–5 min at room temperature followed by distilled water rinses and 1 min in acetone at −20°C. 50 μl of IgG or diluted antiserum were applied to each coverslip. Coverslips were incubated in a humidified chamber for 30 min at 37°C. Excess serum was removed by three washes in glass distilled water. 50 μl of goat-anti-rabbit IgG conjugated with fluorescein (FI GAR) (0.5 mg/ml) (Miles Laboratories, Inc. Elkhart, IN) were added to each coverslip. Coverslips were again incubated in a moist chamber for 30 min at 37°C. the FI GAR was removed by rinsing in water. The coverslips were again drained, mounted on slides with Gelvatol (Monsanto Co., St. Louis, MO), and were examined with a Zeiss Photomicroscope III equipped with a IIIRS epifluorescence system and an XBO 75W xenon lamp. Photomicrographs were taken on Plus-X film (Kodak) and developed in Diafine (Acufine).

RESULTS

Isolation of HeLa IF

Native HeLa IF were obtained by the method of Zackroff and Goldman (17) as determined by electron microscopy and SDS PAGE. Pellets of these IF preparations contained loose IF, IF bundles, centrioles, and vacuoles (Fig. 1). SDS PAGE revealed that these preparations contained major proteins that co-migrated with the 54,000- and 55,000-mol-wt proteins obtained from native BHK-21 IF (Fig. 2, *b* and *c*). Most of the HeLa IF proteins lie in the same molecular weight range (~40,000–70,000) as those seen in bovine prekeratin and human plantar callus IF preparations (Fig. 2, *d* and *e*). The major HeLa IF proteins in the crude isolate consist of at least nine distinct bands or regions, 43,000-, lmw (the two proteins between 43,000 and 54,000), 54,000, 55,000, 60,000, 65,000, 70,000, and hmw (the highest molecular weight proteins) (see Fig. 4).

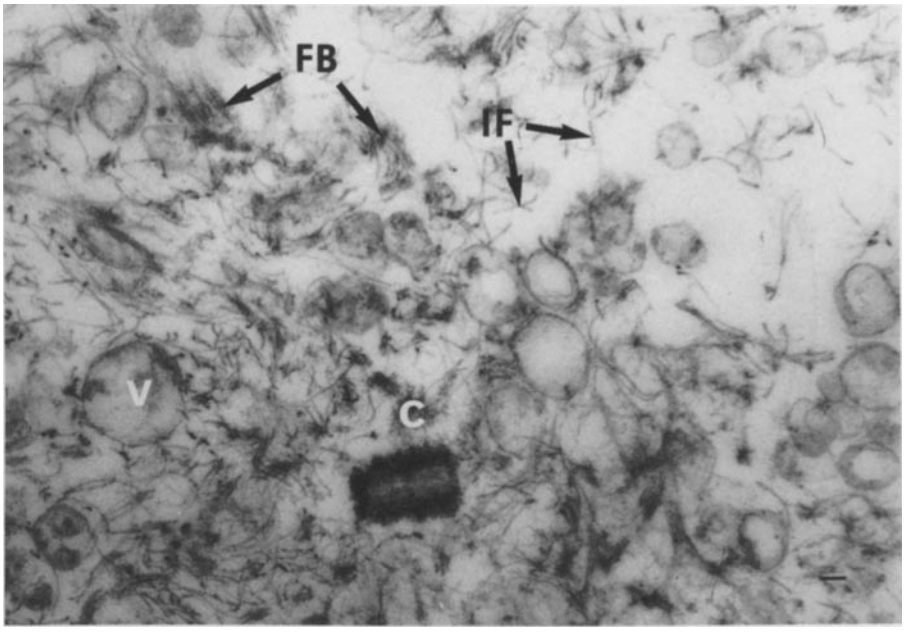


FIGURE 1 Electron micrograph of a thin section through the pellet of a crude HeLa IF preparation. Note the presence of loose IF (*IF*), IF bundles (*FB*), a centriole (C), and vacuoles (*V*). Bar, 0.1 μm. × 37,250.

Disassembly and Reassembly of HeLa IF In Vitro

Disassembly of HeLa IF was achieved in 8 M urea (see Materials and Methods, 7, 18, 24, 28). Attempts at disassembly either in low ionic strength buffers (as in the case of BHK-21 IF) (17) or high ionic strength buffers (as in the case of squid brain neurofilaments) (29), in the absence of urea, were unsuccessful. Optical density at 320 nm was used to estimate the proportion of particulate matter remaining in solution following solubilization of the IF in urea and clarification by ultracentrifugation. The average OD 320-nm value of solubilized protein (following ultracentrifugation, see Materials and Methods) at a concentration of 0.4 mg/ml, was <0.010. After 5 min in reassembly buffer, this value increased up to 0.400 using the same protein concentration.

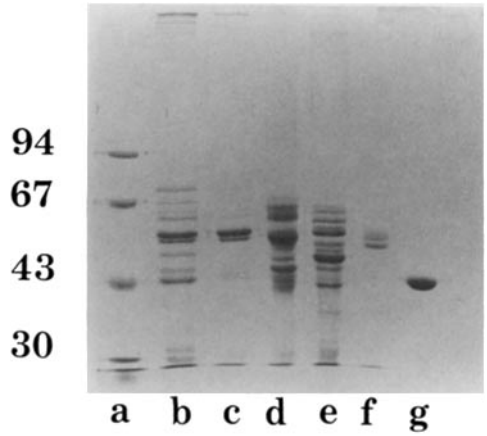


FIGURE 2 SDS polyacrylamide slab gel (7.5%) comparing IF proteins from various sources. (*a*) Standards: 94,000 (*94*), rabbit muscle phosphorylase b; 67,000 (*67*), bovine serum albumin; 43,000 (*43*), chick egg white ovalbumin; 30,000 (*30*), bovine erythrocyte carbonic anhydrase. (*b*) HeLa IF proteins. (*c*) BHK-21 IF proteins. (*d*) Human plantar callus. (*e*) Bovine stratum corneum prekeratin. (*f*) Bovine brain tubulin. (*g*) Rabbit skeletal muscle actin.

Optimal conditions for reassembly of HeLa IF from solubilized, unfractionated proteins were determined by negative staining (Fig. 3) and ultracentrifugation. IF assembly depended on pH, temperature, protein concentration, and ionic strength. No other specific co-factors such as ATP, Ca^{++}, etc., were required. Optimal conditions for reassembly, were obtained when urea was removed by dialysis against 1,000 vol of 5–10 mM $NaPO_4$ (or Tris-HCl) buffer (pH 6.6), 0.1 M KCl, 1 mM dithiothreitol (or 25 mM β-mercaptoethanol), 0.1 mM phenylmethylsulfonyl fluoride at 23°C for 16 h. No IF formed at $5 \leq pH \geq 8$ or in salt concentrations >0.25 M KCl. IF reassembled at 23–37°C were much longer than those assembled at 0°–10°C for the same time intervals. A critical protein concentration of ~0.1 mg/ml was necessary for assembly. When concentrations above ~1.0–1.5 mg/ml were used, some IF protein would precipitate in an amorphous form. Yields of reassembled IF containing as much as 85% of the proteins present in freshly isolated native IF were achieved

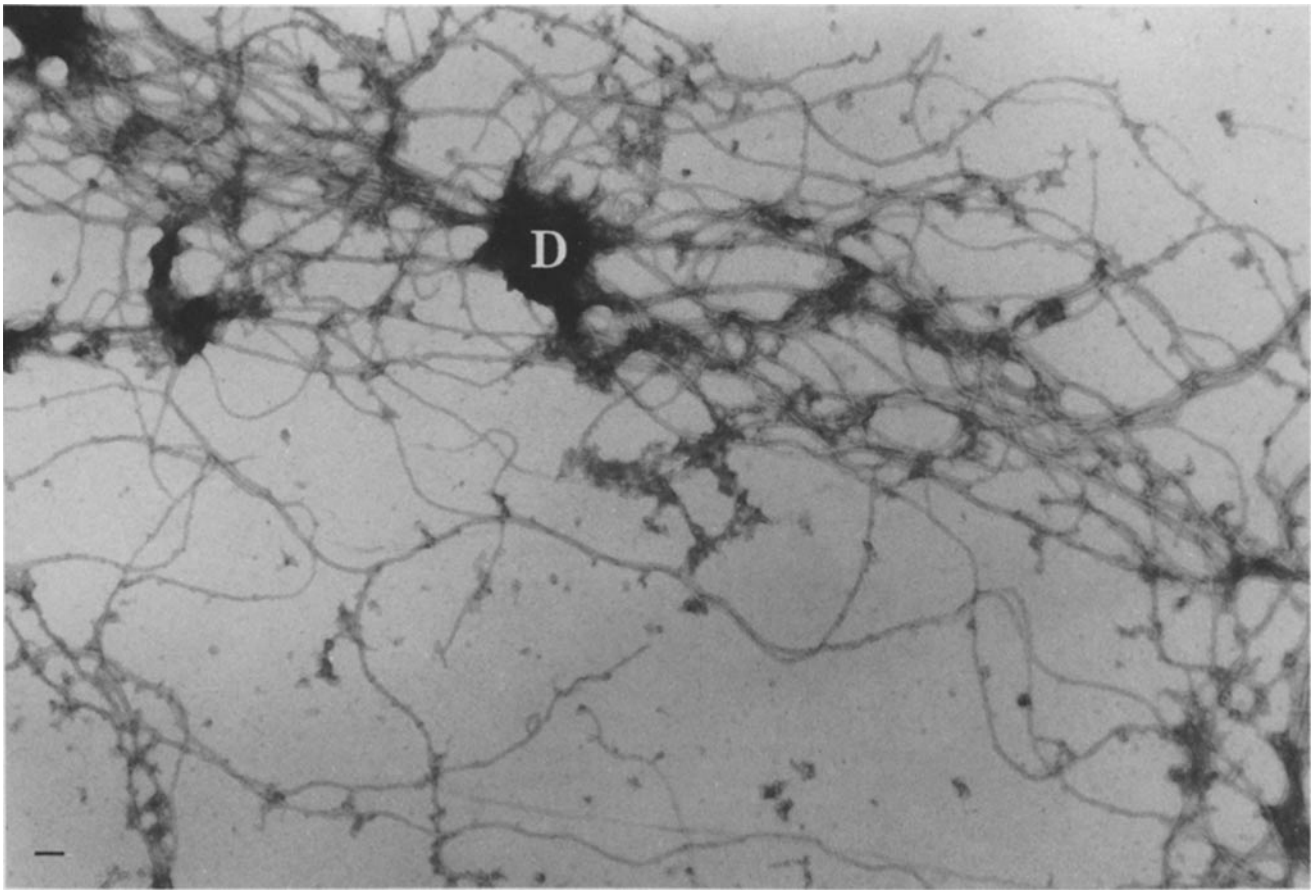


FIGURE 3 Electron micrograph of a negative stain preparation of twice-cycled HeLa IF. Optimal conditions for reassembly were employed. The size and protein composition of these IF are similar to those of IF isolated intact from HeLa cells and one-cycle reassembled IF. The dark region (*D*) is believed to contain closely packed HeLa into which the negative stain cannot penetrate. Bar, 0.1 μm.

for one cycle of reassembly, however the efficiency for two cycles of reassembly was somewhat lower (~50–65%). The hmw proteins would not be detected in stained Laemmli gels after two cycles of disassembly/reassembly. Densitometric scans of the same gels, however, indicated that the relative stoichiometric ratio of all of the other HeLa IF proteins was retained (Fig. 4).

Peptide Mapping

One-dimensional peptide mapping by limited proteolysis (21) was used to determine the extent of homology amongst the 54,000- and 55,000-mol-wt IF proteins isolated from BHK-21 and HeLa cells. The peptides produced by the 55,000-mol-wt proteins were virtually identical (Fig. 5, *c–d*). However, there was little homology between the BHK-21 and HeLa 54,000-mol-wt proteins (Fig. 5, *a–b*). The HeLa 43,000-mol-wt protein map was compared with that of rabbit skeletal muscle actin (42,000), since actin is often present in cytoskeletal preparations. The results indicated that the majority of protein from the 43,000-mol-wt band mapped differently from actin (Fig. 5, *f–g*).

Assembly of IF from Purified Subunits

Another approach to deciphering whether the HeLa proteins are structural subunits or associated proteins has been to determine which of the eight major proteins can form IF in vitro either individually (e.g., BHK-21 55,000-mol-wt homopolymers [30]), or in combinations of two or more (e.g., keratin IF co-polymers [7]). Two methods were employed for the purification of the HeLa IF proteins. The gel slice method was used to successfully purify the HeLa 55,000 protein, however, subsequent in vitro assembly resulted in unusually short homopolymers. As a result, preparative gel electrophoresis was employed to purify individual proteins. Attempts were then made to reassemble these into IF using conditions that have been established for bovine keratin IF and fibroblast IF assembly in vitro.

Prior to preparative gel electrophoresis, partial separation of the HeLa IF proteins was achieved by chromatography on DE52-cellulose. The KCl concentrations of the elution buffer containing enriched fractions of the 60,000- and 70,000-mol-wt protein; 65,000- (a doublet), 55,000-, lmw, and 43,000-mol-wt protein; and 54,000- and 43,000-mol-wt proteins were 25, 50, and 75 mM, respectively (see Fig. 6, *g–i*). These fractions were subsequently loaded onto preparative gels for further purification (Fig. 6, lanes *a–f*). The only proteins not separated were those that migrated between 54,000 and 43,000 (lmw).

The purified proteins and the fraction containing lmw were dialyzed overnight under conditions known to favor either keratin (5 mM Tris-HCl, pH 7.6 [7]) or HeLa IF assembly (5 mM Tris-HCl, pH 7.6 containing 0.1 M KCl). Only the HeLa 55,000 formed homopolymers of ~10-nm diam under the conditions for HeLa IF assembly (Fig. 7*a*). HeLa 55,000

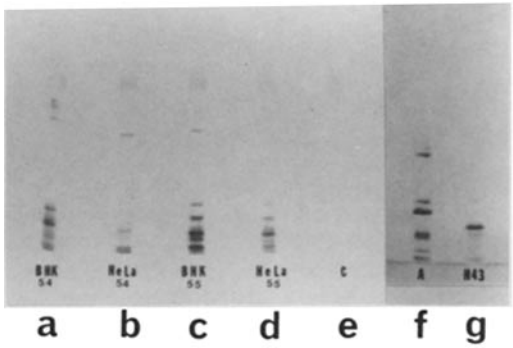


FIGURE 5 One-dimensional peptide mapping by limited proteolysis in a 15% SDS polyacrylamide slab gel. Comparison of HeLa 54- and 55-Kdalton IF polypeptides with BHK-21 54- and 55-Kdalton IF polypeptides (lanes *a–d*). Maps of HeLa and BHK-21 55-Kdalton polypeptides are very similar. (*a*) BHK-21 54-Kdalton polypeptide; (*b*) HeLa 54-Kdalton polypeptide; (*c*) BHK-21 55-Kdalton polypeptide; (*d*) HeLa 55-Kdalton polypeptide; (*e*) 0.25 μg of protease *S. aureus* V8. Comparison of rabbit skeletal muscle actin (lane *f*) with HeLa 43-Kdalton polypeptide (lane *g*).

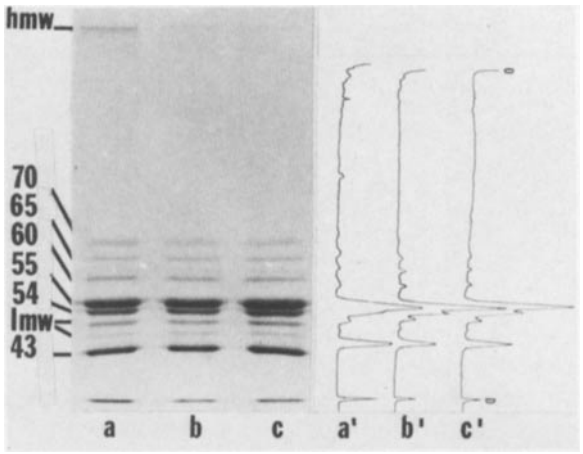


FIGURE 4 Electrophoretic and densitometric analyses of uncycled, one-cycle, and two-cycle reassembled HeLa IF proteins. SDS polyacrylamide gels (*a–c*) and gel scans (*a'–c'*) correspond to uncycled, one-cycle, and two-cycle reassembled HeLa IF, respectively. Note that, with the exception of the HMW proteins which are lost gradually during reassembly, the relative stoichiometric ratio of all of the other HeLa IF proteins is retained through two cycles of assembly.

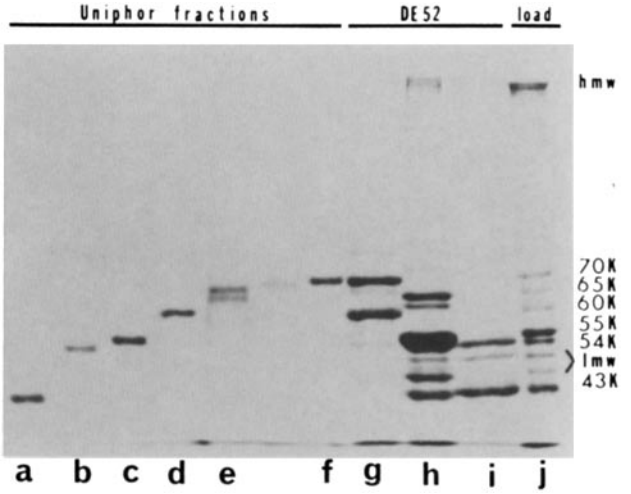


FIGURE 6 SDS polyacrylamide gel (7.5%) containing the electrophoretically purified HeLa IF polypeptides (lanes *a–f* containing the 43,000 (*43K*), 54,000 (*54K*), 55,000 (*55K*), 60,000 (*60K*), 65,000 (*65K*) doublet, and 70,000 (*70K*) polypeptides, respectively), partially purified HeLa IF polypeptide samples from DE-52 cellulose chromatography that were loaded onto the Uniphor (lanes *g–i*), and the original unfractionated sample of HeLa IF polypeptides that was applied to DE52-cellulose (lane *j*).

dialyzed under conditions for keratin IF assembly did not form IF.

It was not surprising that the majority of IF proteins did not form homopolymers, since it has been shown that at least two proteins are required for keratin IF assembly in vitro (7, 31). Keratins from different sources (i.e., mouse epidermal K_1 or K_2 and bovine prekeratin band 5) will also co-polymerize into IF under keratin IF assembly conditions (32). To determine whether HeLa IF proteins other than 55,000-mol wt were keratin-like in vitro (e.g., obligate co-polymers), various mixtures of two proteins were combined in equimolar amounts and dialyzed under keratin IF assembly conditions. Although most combinations did not form IF, short IF (<2 μm long) that were ~10-nm diam did form when mouse K_1 was added to HeLa lmw (Fig. 7*b*) and when lmw was added to HeLa 54,000 (Fig. 7*c*). These IF were comparable in length to the short IF formed by some mixtures of bovine epidermal keratin proteins (7), and to those seen shortly after the addition of salt to induce BHK-21 IF assembly (17).

Physical and Chemical Characterization of HeLa IF Proteins

The amino acid analysis of one-cycle reassembled HeLa IF as well as the composition of IF proteins from several other sources are shown in Table I. The amino acid values for HeLa IF are similar to those published for BHK-21 fibroblast IF, prekeratin, and a variety of other IF from diverse cell types (3, 4, 30), suggesting that these sources contain related proteins.

HeLa IF reassembled from the unfractionated mixture of proteins contain ~38% α-helix as determined by circular dichroism measurements. This value is very close to the range cited for other classes of IF (40–60%) including prekeratin and BHK-21 IF (4).

Wide angle x-ray diffraction studies were performed to obtain more detailed information about the structure of HeLa IF. Analysis of the x-ray diffraction patterns shows that these IF have prominent meridional arcs at 5.15 Å and equatorial spots at 9.7 Å (data not shown). Similar reflections have been observed for IF assembled from bovine epidermal keratin (28), BHK-21 fibroblasts (5), α-keratin (33), and *Myxicola* axoplasm (34).

Similarities in the amino acid values of the seven purified HeLa IF proteins are also shown in Table I. Almost half of each of the polypeptides analyzed consists of just four amino acids: glycine, glutamic acid, leucine, and serine. Furthermore most of these amino acid compositions are very similar to those of purified keratins obtained from other species (24, 35–38). However, there are still noticeable differences in amino acid values amongst the HeLa IF proteins. For example, HeLa 55,000, the only protein capable of forming homopolymers, has the lowest serine and glycine levels and the highest glutamic acid, aspartic acid, threonine, methionine, histidine, and arginine levels. The values for HeLa 55,000 are comparable to those for BHK-21 55,000 and the major IF

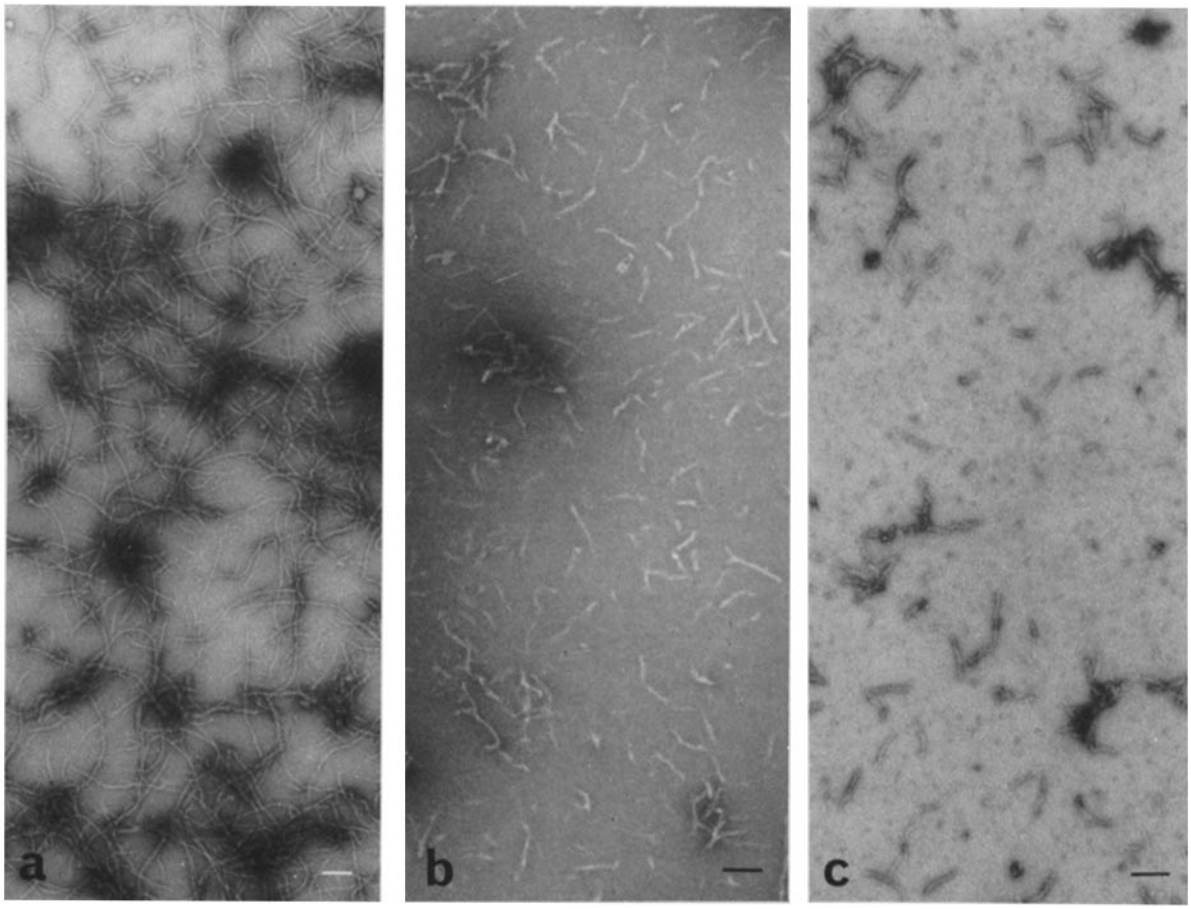


FIGURE 7 Electron micrographs of negatively stained HeLa 55-Kdalton homopolymer IF (*a*); HeLa I mw + mouse K1 heteropolymer IF (*b*); HeLa 54-Kdalton + I mw heteropolymer IF (*c*). Bars, 0.1 μm. × 40,000 (*a*); × 48,000 (*b*); × 46,000 (*c*).

TABLE I

Amino Acid Composition of Unfractionated and Purified IF Proteins from HeLa and Other Sources

	Unfractionated IF					Purified IF polypeptides								
	HeLa	BHK-21*	Human stratum corneum‡	Squid neural§	Bovine prekeratin‖	HeLa 43,000	HeLa LMW	HeLa 54,000	HeLa 60,000	HeLa 65,000	HeLa 70,000	HeLa 55,000	BHK-21 55,000	CHO
Aspartic Acid	9.4	10.3	8.5	10.5	9.2	7.66	8.14	8.59	6.83	7.18	6.73	9.24	9.1	9.0
Threonine	5.2	4.8	3.7	6.5	3.9	3.76	4.01	3.89	3.85	4.31	3.85	4.89	4.6	4.5
Serine	8.4	6.5	12.4	7.8	9.4	13.32	14.17	11.88	16.57	16.47	18.19	10.39	9.5	9.3
Glutamic acid	16.7	18.7	13.3	20.8	14.7	13.69	14.31	13.66	13.88	14.41	14.50	15.26	15.2	14.7
Proline	2.9	2.9	1.3	1.6	1.6	1.80	1.11	0.90	1.49	1.75	1.56	0.98	0.7	0.8
Glycine	7.3	5.7	20.9	5.6	15.5	12.68	15.21	18.51	19.95	21.08	17.39	11.91	9.8	9.6
Alanine	8.9	8.4	5.5	7.9	6.5	5.99	5.84	4.71	5.75	5.43	6.17	6.08	6.4	6.3
Half-Cysteine	ND	0.8	1.5	1.4	ND	0.93	0.75	1.19	0.69	0.93	0.89	0.93	0.8	0.9
Valine	5.2	5.4	3.7	2.6	5.2	3.48	3.17	4.06	2.41	2.41	3.25	3.95	5.7	5.9
Methionine	1.8	2.0	1.5	3.2	1.8	1.15	1.87	1.37	1.28	1.37	1.41	2.17	2.2	2.1
Isoleucine	3.5	3.8	4.0	4.4	4.0	5.04	3.25	4.35	3.34	2.78	3.59	3.27	3.5	3.7
Leucine	10.5	11.2	8.4	8.5	8.8	11.52	10.73	8.71	8.67	7.75	8.40	11.27	11.6	11.7
Tyrosine	2.5	2.3	3.6	3.3	3.0	2.08	2.13	3.38	2.41	2.33	1.34	2.74	2.8	2.7
Phenylalanine	2.3	2.3	3.2	2.0	3.6	2.81	1.79	3.45	2.45	1.48	2.17	2.40	2.6	2.8
Histidine	1.6	1.5	1.5	1.4	1.1	0.79	0.79	0.95	0.95	0.98	0.89	1.21	1.8	1.5
Lysine	5.5	5.5	4.5	5.7	5.0	7.25	5.84	4.92	5.08	4.71	4.53	5.66	6.0	6.1
Arginine	8.0	8.1	5.5	7.5	6.1	6.01	6.91	5.51	4.14	4.20	4.79	7.67	8.2	8.3
Tryptophan	ND	ND	<0.1	0.58	ND	ND	0.58	ND	0.51	0.51	0.61	ND	0.38	0.41

* Data expressed in residues/100 residues.
† See Reference 3.
‡ See Reference 3.
§ R.V. Zackroff, Department of Anatomy and Cell Biology, Northwestern University Medical School, Chicago, IL, personal communication.
‖ See Reference 18.
¶ See Reference 39.

subunit from Chinese hamstery ovary (CHO) cells (see Table I). The highest molecular weight proteins have the highest contents of serine and glycine and the lowest leucine and arginine levels.

The α-helix contents of the 43,000-, lmw, 54,000-, 55,000-, 60,000-, 65,000-, and 70,000-mol-wt proteins have been estimated by circular dichroism measurements to be 46.2, 46.5, 45.0, 45.1, 37.6, 33.8, and 30.5%, respectively. The proteins showing the lowest α-helix contents (60,000, 65,000, and 70,000) show no tendency to form IF under the conditions chosen for assembly. The α-helix content of HeLa 55,000 (45.1%) is very close to the values reported for CHO and BHK-21 55,000, which are both 43% (6, 39).

HeLa IF Morphology In Situ and In Vitro

Electron microscopic methods were used to compare the morphology of IF in situ with those assembled in vitro. Thin sections of HeLa cells indicated the presence of at least two morphologically distinct IF organizational states—one similar to the IF bundles (tonofilaments) seen in various epithelial cells in situ (40), and the other, loose aggregates (Fig. 8). The IF bundles were seen mainly near the adhesive surface of cultured HeLa cells and occasionally in the vicinity of the nucleus. This distinction was much more pronounced in colchicine-treated cells in which microtubules were depolymerized. After this treatment, large aggregates (or caps) of relatively loosely packed IF were found in the juxtanuclear region (Fig. 8). When caps were present, few loose IF were found in the peripheral cytoplasm; however, IF bundles were still present in regions located near the lower surface of the cell. These results are agree with published observations (8, 10).

Isolated HeLa IF were also examined by electron microscopy. Fig. 3 was obtained from a negative stain preparation of two-cycle reassembled HeLa IF. Dark regions (*D*) which appeared to contain closely packed IF were still present. In some cases, IF within these dark regions were particularly conspicuous (Fig. 9). Thin sections of two-cycle reassembled IF (Fig. 10) revealed that these regions closely resembled the tonofilament bundles seen in a variety of epithelial cells in situ (40). Loose IF are also present. Therefore, even after two cycles of reassembly the HeLa IF system still appears to contain both types of IF organizational states, which are similar to those seen in situ.

Immunological Properties of HeLa IF Proteins

Another approach to determining the overall organization of HeLa IF proteins has been through the use of indirect immunofluorescence. The aim of these studies was to determine whether antiserum directed against HeLa IF reassembled from the unfractionated mixture of proteins, was directed against several IF systems including those usually considered to be of fibroblast and epithelial origins.

When HeLa cells were stained with antiserum directed against the 55,000 IF structural protein of BHK-21 fibroblasts, the immunofluorescence pattern suggested an enrichment in the juxtanuclear region (Fig. 11 *a*). This effect was enhanced when HeLa cells were exposed to colchicine prior to fixation. This enhancement was visualized as bright juxtanuclear caps (Fig. 11 *b*). Accumulations of IF in the juxtanuclear region

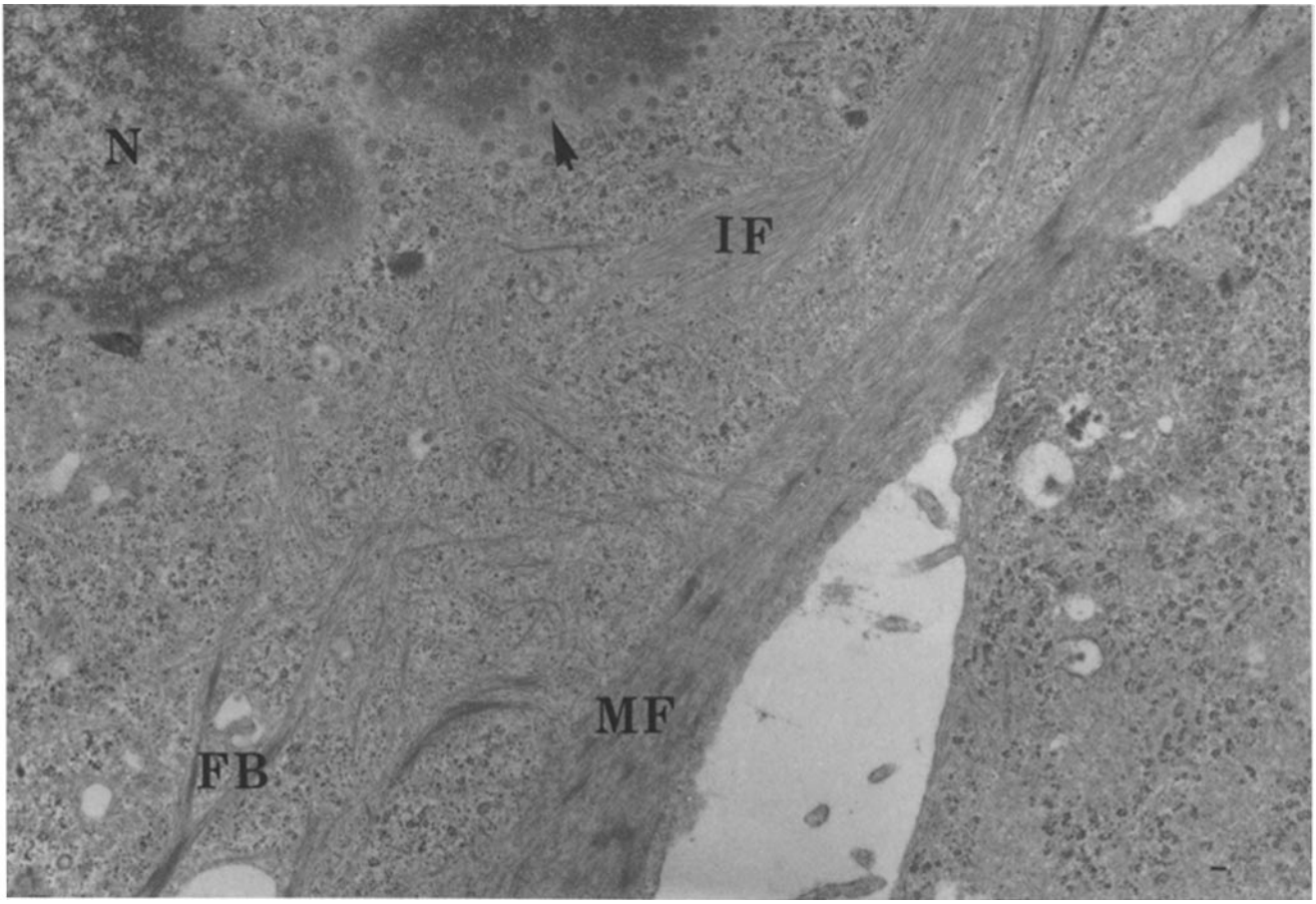


FIGURE 8 Electron micrograph of a thin section through a HeLa cell after 24 h in colchicine (5×10^{-6} M). Note aggregates of loose IF (*IF*), IF bundles (*FB*), microfilament bundles (*MF*), nucleus (*N*), and nuclear pores (arrowhead). Bar, 0.1 μm. × 19,200.

where these caps were found have been observed by electron microscopy in many cell types including HeLa cells (e.g., Fig. 8) and BHK-21 fibroblasts (3, 41).

In contrast, when HeLa cells were stained with antiserum directed against bovine prekeratin, a cytoplasmic filamentous network was visible which extended from the nuclear region to the cell periphery (Fig. 11*c*). This pattern, which remained unaltered in the presence of colchicine, most likely represents the tonofilament bundles seen at the electron microscopic level. These results confirm the observation of Osborn and co-workers (9).

When HeLa cells were stained with HeLa IF antiserum (Fig. 11*d*), the immunofluorescence pattern appeared similar to both the BHK-21 IF (Fig. 11*a*) and prekeratin (Fig. 11*c*) patterns; giving the impression that both patterns were superimposed. In the presence of colchicine (Figs. 11, *e* and *f*) perinuclear IF caps formed, and the cytoplasmic fibrous network remained in its normal distribution.

To test the cross-reactivity of the HeLa IF antiserum with IF systems in other cells, both the epithelial cell line, PtK_2, and the fibroblastic cell line, BHK-21 were examined. Cytoplasmic tonofilament-like arrays were stained in PtK_2 (rat kangaroo epithelial) cells (Fig. 12*a*) and in the presence of colchicine large perinuclear caps were also visible with this antiserum. BHK-21 fibroblasts displayed an IF pattern (Fig. 12*b*) similar to that obtained with antiserum to BHK-21 55,000-mol-wt IF protein (Fig. 12*c*). In the presence of colchicine, the pattern produced with HeLa IF antiserum resembled the juxtanuclear caps seen in BHK-21 cells stained with BHK-21 55,000-mol-wt IF antiserum (Fig. 12, *d* and *e*). When BHK-21 cells were stained with prekeratin antiserum, no obvious cytoplasmic fluorescence pattern could be detected.

When HeLa IF antiserum was adsorbed with SDS polyacrylamide gel slices containing HeLa IF proteins, the immunofluorescence patterns seen in HeLa cells were altered. Cells stained with antiserum adsorbed with the HeLa 54,000- and 55,000-mol-wt proteins revealed primarily the weblike cytoplasmic fluorescence (Fig. 12*f*) which was similar to that observed when HeLa cells were stained with antiserum directed against bovine prekeratin (See Fig. 11*c*). This pattern appeared unaltered in the presence of colchicine. The bright juxtanuclear fluorescence that was present in cells stained with total HeLa IF antiserum was not present in these preparations. When HeLa IF antiserum was adsorbed with the 43,000-mol-wt protein, the resulting immunofluorescence pattern (Fig. 12*g*) was similar to that seen in HeLa cells stained with antiserum against BHK-21 55,000-mol-wt protein (compare Figs. 11*a* and 12*g*); e.g., juxtanuclear fluorescence was most obvious and did not extend to the outer edges of the cell as seen in the prekeratin pattern (see Fig. 11*c*). In the presence of colchicine this juxtanuclear pattern was greatly enhanced (Fig. 12*h*) using the HeLa antiserum adsorbed with the 43,000-mol-wt protein.

The ^{125}I-protein A binding procedure of Adair et al. (27) was used to determine the complexity of the HeLa IF antiserum and its cross-reactivity with IF proteins from other

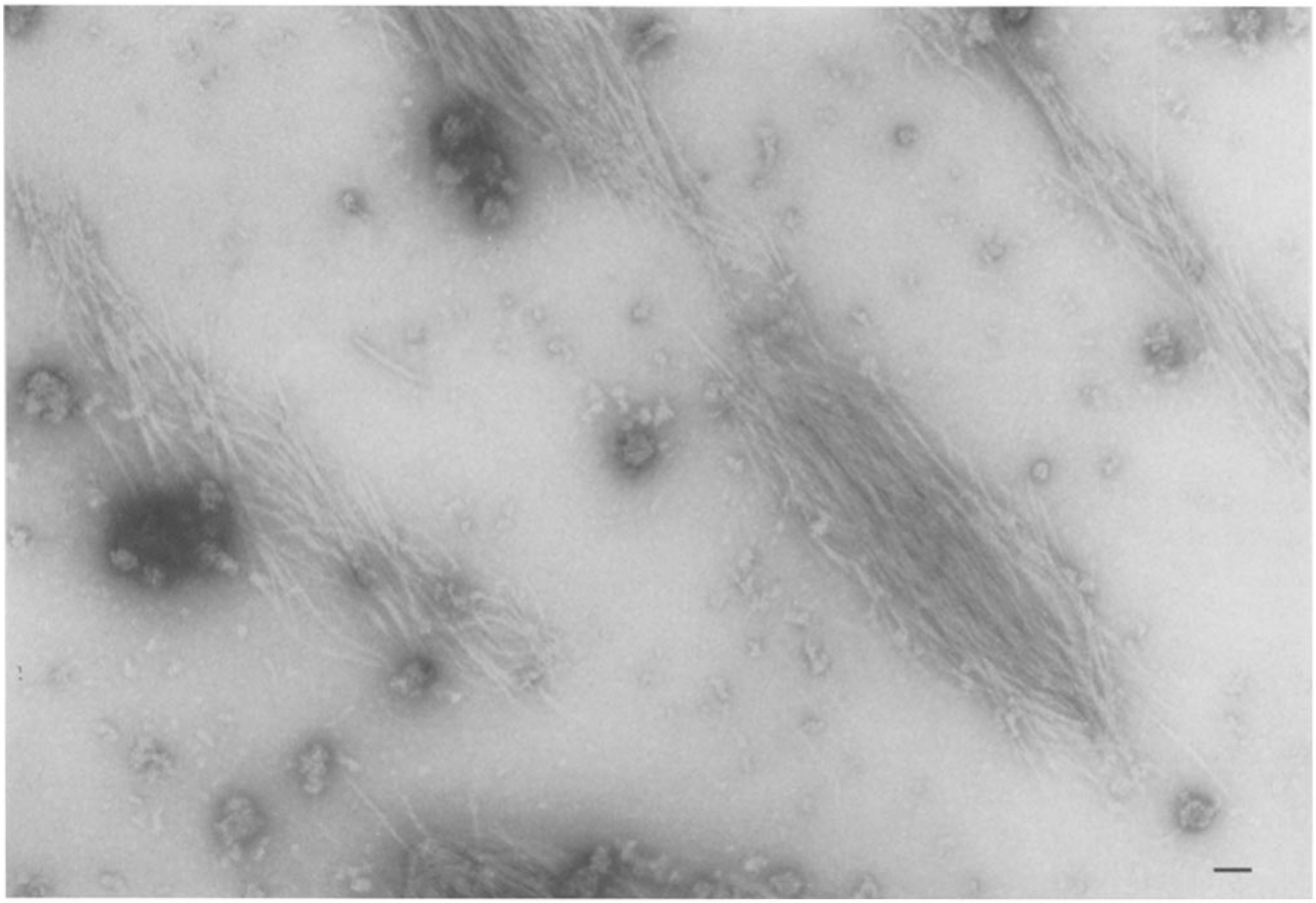


FIGURE 9 Electron micrograph of two-cycle reassembled HeLa IF that were negatively stained with uranyl acetate. Closely packed IF within the filament bundles are clearly visible. Bar, 0.1 μm. × 52,400.

sources—in particular those of fibroblast and epithelial origins. The results of this procedure demonstrated that this antiserum contained antibodies directed against all of the major HeLa IF associated proteins except for the hmw proteins (Fig. 13*c*). Preimmune serum did not react with any of the HeLa proteins (Fig. 13, lane *g*; Fig. 14, lane *e*). Specific bands were absent in the autoradiographs of gels that had been treated with antiserum adsorbed with the appropriate purified antigens (See Fig. 13, *d* and *e*).

The HeLa antiserum also reacted with BHK-21 fibroblast IF proteins (Fig. 13, lane *f*), and with some of the lower molecular weight bovine prekeratin proteins from a stratum corneum preparation (Fig. 14, lane *m*). In addition, the BHK-21 55,000-mol-wt IF antiserum cross-reacted with the HeLa 55,000-mol-wt protein (Fig. 14, lane *g*), while antisera against some bovine prekeratin proteins reacted with lower molecular weight HeLa IF proteins (Fig. 14, lane *h–l*). Bovine prekeratin antiserum (Fig. 14, lane *n*) showed cross-reactivity with human plantar callus (Fig. 14, lane *o*) and HeLa IF antigens (not shown). In some autoradiographs, nonspecific binding of the ^{125}I-protein A occurred at the ends of the gel strips and at the dye front. This phenomenon has been reported by several other laboratories (e.g., 42, 43).

DISCUSSION

In this study HeLa cells have been used as a model to explore further the possibility that cultured epithelial cells may contain more than one type of IF. The isolation of intact IF from HeLa cells can be accomplished rapidly and easily using the procedure of Zackroff and Goldman (17). The presence of centrioles in the crude pellet of IF is not surprising since IF-centriole associations have been observed in several cell types in situ (44, 45, 48) and since centrioles are known to co-isolate with BHK-21 IF caps (3). In all probability the major proteins seen by SDS PAGE are IF associated components, and not centriole-related polypeptides. This is based on the finding that the relative amounts of the major proteins associated with freshly isolated IF remain constant even after several disassembly-reassembly cycles in vitro, at which time centrioles are no longer observed.

Disassembly and Assembly of HeLa IF In Vitro

The studies involving the disassembly and reassembly of HeLa IF were aimed at determining the nature of the major IF structural proteins. An initial clue regarding the nature of the HeLa IF came from their solubility properties in vitro that, for the most part, were very similar to those seen in the case of epidermal keratin (e.g., 8 M urea; 18, 46, 47). Furthermore, all of the proteins present in the crude IF extracts, except for the hmw proteins, were retained stoichiometrically after two cycles of assembly in vitro using 8 M urea for solubilization. The loss of the hmw proteins during such cycling indicates that they are not essential for polymerization. This observation also indicates that these proteins are not necessary for the cross-linking of IF into the bundles that form during assembly in vitro. It is still possible, however,

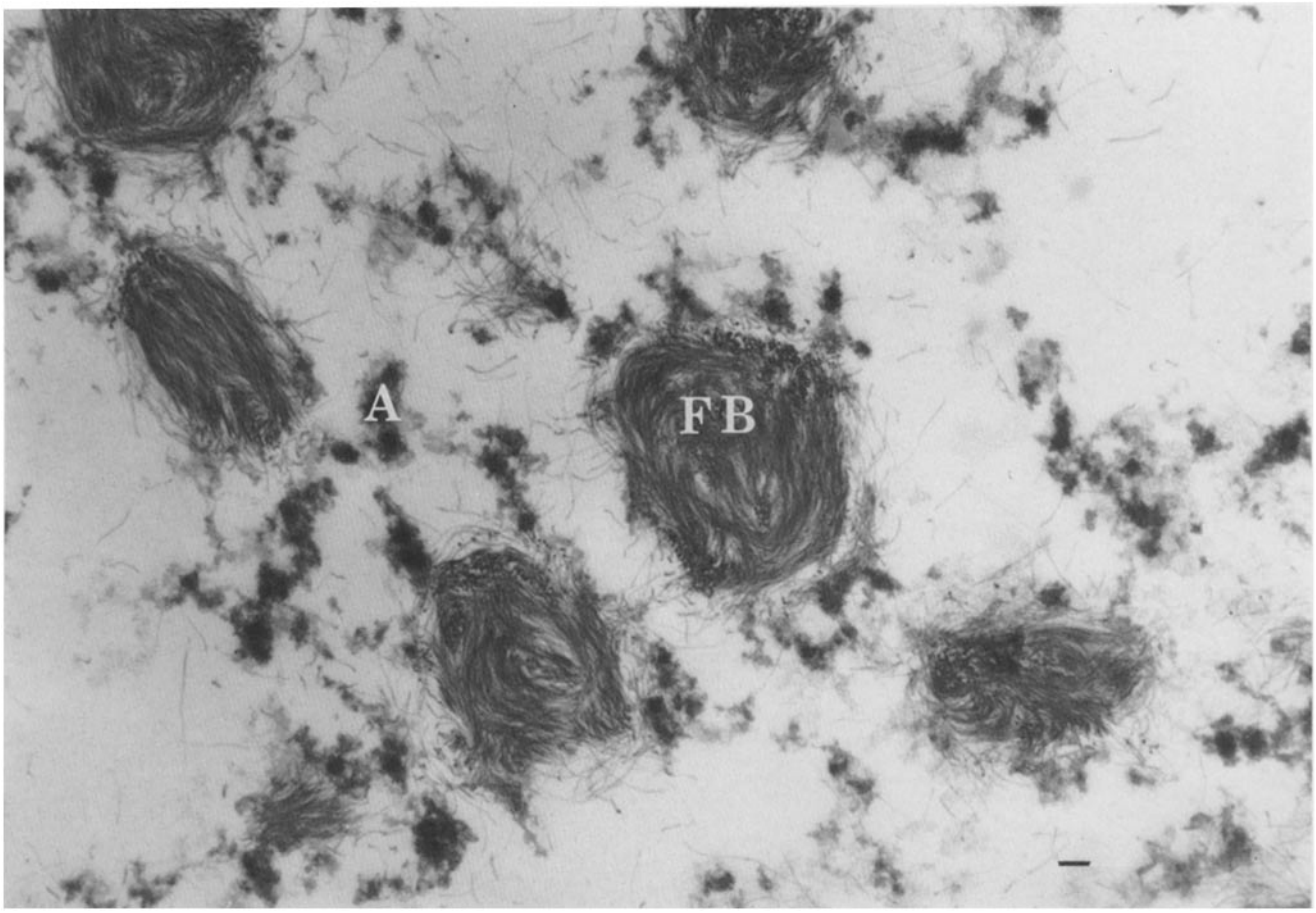


FIGURE 10 Electron micrograph of a thin section of two-cycle reassembled HeLa IF. Bundles of IF (*FB*) containing closely packed IF were present as well as occasional loose IF and some amphorous material (*A*). Bar, 0.1 μm. × 37,250.

that the hmw proteins may be involved in the association of IF with other cytoskeletal structures such as microtubules or other organelles (e.g., 2, 16).

The conditions for HeLa IF disassembly and reassembly differ from those used for keratin (7) and BHK-21 fibroblast IF (17, 30). In general, HeLa IF are as insoluble as epidermal keratin IF. However, they require 0.1 M KCl for optimum reassembly, which in terms of ionic strength required for IF assembly, represent conditions that are intermediate between those required for unfractionated epidermal keratin IF ($I<0.01$ [mol/liter], pH 7–8) and BHK-21 IF ($0.10<I<0.20$) assembly (17, 30). Although optimal reassembly conditions require the presence of 0.1 M KCl, lower yields of IF are obtained under conditions that favor keratin IF assembly. These observations suggest that the complex of reassembled HeLa IF contain keratin-like IF. Furthermore, it is known that keratin can only form IF if more than one subunit is present (7). This is in contrast to the finding that the two major BHK-21 IF subunits (54,000- and 55,000-mol wt) assemble into homopolymers (30). If some HeLa IF are composed of epidermal keratin-like proteins and others are composed of fibroblast type IF proteins, then, when purified, the fibroblast-like proteins should form homopolymers in buffers similar to those used in the BHK-21 in vitro assembly system (0.1–0.2 M KCl) (30). Furthermore, keratin-like proteins should form co-polymers in solutions of lower ionic strength (e.g., 10 mM Tris-HCl, pH 7–8). Therefore, it is not surprising to find that the HeLa 55,000-mol wt polypeptide can form homopolymers in vitro since it appears identical to the major 55,000-mol wt subunit obtained from BHK-21 and CHO cells which also form homopolymers in vitro (30, 39).

The ability of the HeLa 54,000-mol wt and lmw proteins to form copolymer IF under conditions usually permissive for keratin IF assembly, indicates that the lmw proteins are similar to keratin. In support of this finding, it has been demonstrated that individually purified epidermal keratins from diverse sources can form co-polymers (32). Therefore, the ability of HeLa lmw to form co-polymers with the mouse K1 protein is another indication of their keratin-like nature. The inability of these co-polymers to form lateral aggregates (bundles) suggests that other associated proteins are necessary for this phenomenon.

It is clear from the in vitro assembly results that at least two types of IF with different reassembly properties can form from the mixture of HeLa IF polypeptides. These two IF types are the homopolymers that form in solutions of $0.10<I<0.20$ and the co-polymers that form in solutions of $I<0.01$. However, it is not clear why the HeLa 43,000-mol-wt protein does not form homopolymer or co-polymer IF, since it is retained stoichiometrically through several cycles of disassembly/reassembly in unfractionated HeLa IF preparations. Steinert et al. (7) have made similar observations and have demonstrated that some combinations of keratin polypeptides do not form co-polymers possibly due to structural changes in the subunits that occur during isolation and purification. However, it does appear that the 43,000-mol-wt protein is an IF type of protein

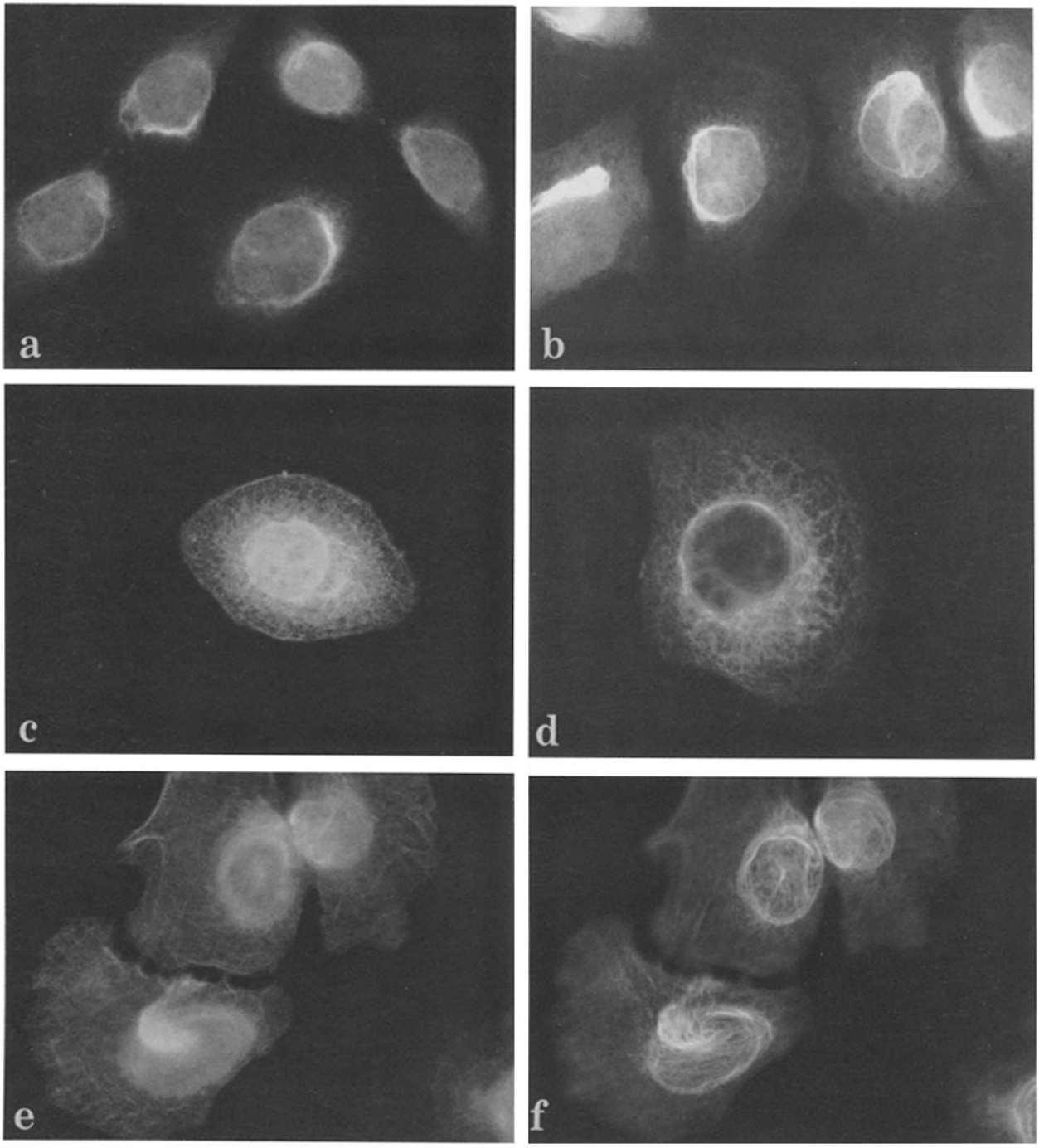
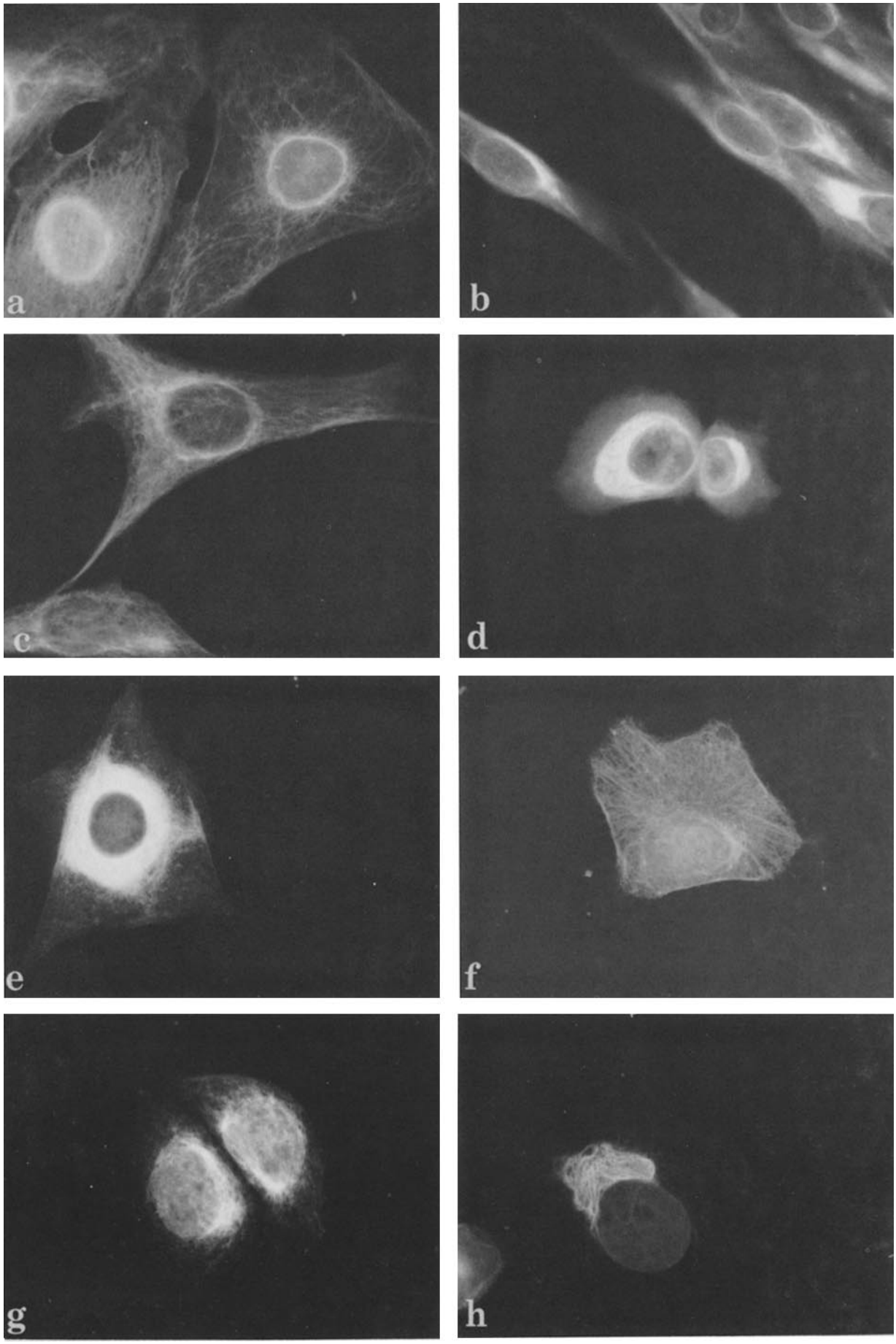


FIGURE 11 (a) Fluorescence micrograph of HeLa cells stained with antiserum to the BHK-21 55-Kdalton structural IF polypeptide. Note juxtanuclear fluorescence which does not extend to the cell periphery. (*b*) Fluorescence micrograph of HeLa cells stained with antiserum to the BHK-21 55-Kdalton structural IF polypeptide following exposure to colchicine (18 h). Note enhanced juxtanuclear fluorescence. Compare with *a*. (*c*) Fluorescence micrograph of a HeLa cell stained with bovine prekeratin antiserum. This pattern, which is distinct from the juxtanuclear fluorescence obtained using antiserum to the BHK-21 55-Kdalton structural IF polypeptide, is unaltered by exposure of these cells to colchicine. (*d*) Fluorescence micrograph of a HeLa cell stained with HeLa IF antiserum. This immunofluorescence pattern looks like the BHK 55-Kdalton and prekeratin patterns superimposed. Note bright juxtanuclear fluorescence and the fine fibrous network that extend to the cell periphery. (e) Fluorescence micrograph of HeLa cells stained with HeLa IF antiserum following exposure to colchicine (18 h) and fixation. The fine fibrous network that is located toward the attached surface of the cell below the nucleus is in focus. Juxtanuclear fluorescence is out of focus. (*f*) Fluorescence micrograph of the same HeLa cells seen in e, but the focal plane is in the nuclear region. The IF caps are now in focus and the fine fibrous network is out of focus. *e* and *f* demonstrate that HeLa IF antiserum cross-reacts with IF organized near the nucleus as well as with IF located toward the adhesive surface of cells, which extend from the nucleus to the cell periphery. (*a–f*) epifluorescence. × 750.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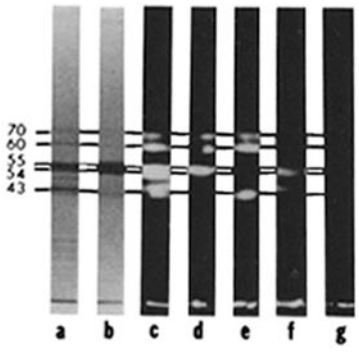

FIGURE 13 Immunochemical analysis of HeLa IF antiserum by the ^{125}I-protein A procedure. Lanes *a–b*, Coomassie Blue stained gels; lanes *c–g*, autoradiographs of antigen-antibody complexes labeled with ^{125}I-protein A. Lanes *c–g* were separated prior to incubation with antiserum and the protein A preparation to prevent spillage from one gel lane to the next. No spillage of antigen was observed between lanes of the Coomassie Blue stained gel. (*a*) HeLa IF polypeptides stained with Coomassie Blue; (*b*) BHK-21 IF polypeptides stained with Coomassie Blue; (*c*) HeLa IF polypeptides + HeLa IF antiserum; (*d*) HeLa IF polypeptides + HeLa IF antiserum preadsorbed with HeLa 43-kdalton IF polypeptide; (*e*) HeLa IF polypeptides + HeLa IF antiserum preadsorbed with HeLa 54- and 55-kdalton IF polypeptides; (*f*) BHK-21 IF polypeptides + HeLa IF antiserum; (*g*) HeLa IF polypeptides + HeLa IF preimmune serum.

due to its reaction with antisera directed against several purified keratins, its amino acid composition which is similar to the keratins, and the fact that it appears to contribute to the IF pattern seen in HeLa cells by immunofluorescence following preadsorption of HeLa IF antiserum with 43,000-mol-wt protein.

Chemical and Structural Features of HeLa IF

One-dimensional peptide mapping by limited proteolysis shows that there is extensive homology between the HeLa and BHK-21 55,000-mol-wt proteins. Therefore this protein appears to be a major component of IF isolated from epithelioid and fibroblastic cell lines. Immunofluorescence studies have also suggested the presence of a common IF structural protein in epithelial cells and fibroblasts (e.g., 8–10), but none of these observations have attempted to determine the biochemical properties of these proteins and none have addressed the issue of how much of the fraction contains such common subunits. On the other hand, the HeLa 54,000-mol-wt and BHK 54,000-mol-wt proteins exhibit major differences in their 1-d peptide maps, which indicates that they are different proteins. Since it has been demonstrated that the BHK-21 54,000-mol-wt protein is very similar to the major IF protein (termed skeletin [49], desmin [50], and 100-Å-filament protein [51]) of muscle (30, 52), it is very unlikely, based on our results, that the 54,000-mol-wt HeLa protein is a similar form of IF structural protein.

All of the major HeLa IF proteins exhibit several of the features that the extensive family of IF proteins have in common. For example, there are extensive similarities in the amino acid compositions of the HeLa IF proteins and those values obtained for both unfractionated preparations and individual purified proteins from many sources ranging from squid neural-IF to human astrocyte IF (4, 18, 38, 49, 53–56). Specifically, the HeLa 55,000-mol-wt protein contains the greatest amounts of glutamic acid, arginine, and leucine, and the lowest amounts of glycine and serine. These values are similar to those obtained for the CHO and BHK-21 55,000-mol-wt proteins. The amino acid compositions of all other purified HeLa IF proteins are similar to both unfractionated and purified IF proteins obtained from human stratum corneum and other sources (e.g., 38). In addition, these compositions are very similar to those of unfractionated or partially fractionated keratins from other species (i.e., 24, 35–37).

Another general feature of IF and their structural proteins is their relatively high α-helix content. The values obtained for IF (reassembled from unfractionated proteins) from HeLa cells (38 ± 10%), and BHK-21 cells (44 ± 10%) are comparable. The range of values observed for separately purified HeLa proteins is 30.5–46.2%. These values fall within the range of values cited for individually purified bovine epidermal keratins (25–56%) (24). Furthermore, the α-helix content of HeLa 55,000 (45.1%) is very close to the values obtained for the similar protein isolated from BHK-21 and CHO cells (both 43%) (5, 39). In addition, x-ray diffraction studies of HeLa IF place them in the α-type category which is the same as all IF studied by this technique (4, 5, 33, 57, 58).

Morphology of HeLa IF

Electron microscopic observations of normal and colchicine-treated HeLa cells reveal the presence of two morphologically distinct IF organizational states; bundles and loose arrays. Similar IF bundles have been observed in a wide variety of epithelial cells. The loose arrays of IF are frequently associated with microtubules in HeLa cells and in other cell types. In the presence of colchicine and other antimicrotubule

FIGURE 12 (*a*) Fluorescence micrograph of PtK_2 cells stained with HeLa IF antiserum. Bright juxtanuclear fluorescence is accompanied by a fibrous network that extends through the cytoplasm; (*b*) Fluorescence micrograph of BHK-21 fibroblasts stained with HeLa IF antiserum. Note bright juxtanuclear region and cytoplasmic staining; (*c*) Fluorescence micrograph of BHK-21 fibroblasts stained with antiserum to BHK-21 55-kdalton IF protein. Note bright juxtanuclear fluorescent fibers that extend out into the cytoplasm; (*d*) Fluorescence micrograph of BHK-21 cells stained with HeLa IF antiserum following exposure to colchicine (18 h) and fixation. Cells exposed to colchicine have lost their characteristic fibroblast shape. Juxtanuclear caps fluoresce brightly; (*e*) Fluorescence micrograph of a BHK-21 cell stained with BHK-21 55-kdalton IF antiserum following exposure to colchicine (18 h). This immunofluorescence pattern is comparable with that observed in *d*; (*f*) Fluorescence micrograph of a HeLa cell stained with HeLa IF antiserum that had been preadsorbed with the HeLa 54- and 55-kdalton IF polypeptides. Notice that a fine fibrous network is present which extends from the nucleus to the cell periphery and that much of the bright juxtanuclear fluorescence is gone. See also Fig. 11*c*; (*g*) Fluorescence micrograph of HeLa cells stained with HeLa IF antiserum that had been preadsorbed with the HeLa 43-kdalton IF polypeptide. Note that much of the fine fibrous network, seen throughout the cytoplasm using unadsorbed HeLa IF antiserum is now gone. The juxtanuclear fluorescence pattern is retained; (*h*) Fluorescence micrograph of HeLa cells stained with HeLa IF antiserum that had been preadsorbed with the HeLa 43-kdalton IF polypeptide. These cells were exposed to colchicine (18 h) prior to fixation. Bright juxtanuclear caps are easily distinguished after staining with this antiserum, but cytoplasmic fluorescence is greatly diminished. × 750 (*a* and *c–h*); × 425.

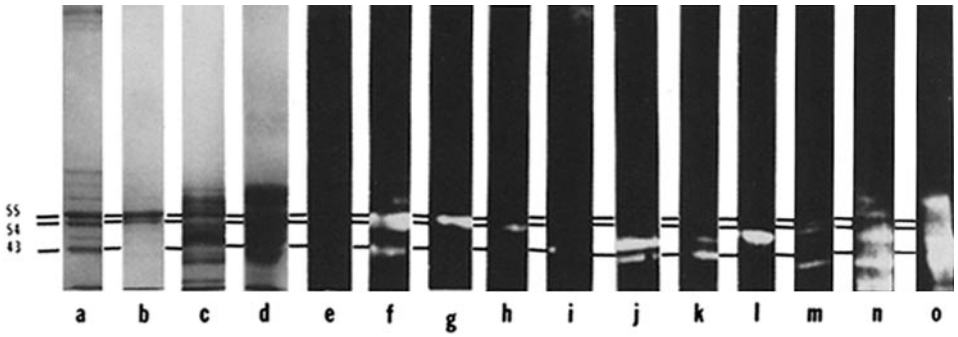

FIGURE 14 Immunochemical analyses of HeLa IF, BHK-21 55,000, and prekeratin antisera by the ^{125}I-protein A procedure. Lanes *a–d*, Coomassie Blue stained gels; lanes *e–o*, autoradiographs of antigen-antibody complexes labeled with ^{125}I-protein A. Lanes *e–o* were separated prior to incubation with antiserum and label to prevent spillage from one lane to the next. No spillage of antigen was observed between lanes of the Coomassie Blue stained gel. (*a*) HeLa IF polypeptides stained with Coomassie Blue; (*b*) BHK-21 IF polypeptides stained with Coomassie Blue; (*c*) Prekeratin polypeptides stained with Coomassie Blue; (*d*) Human plantar callus polypeptides stained with Coomassie Blue; (*e*) HeLa IF polypeptides + HeLa IF preimmune serum; (*f*) HeLa IF polypeptides + HeLa IF antiserum; (*g*) HeLa IF polypeptides + BHK-21 55,000 IF antiserum; (*h*) HeLa IF polypeptides + antiserum to bovine prekeratin 54-kdalton polypeptide; (*i*) HeLa IF polypeptides + antiserum to bovine prekeratin 48-kdalton polypeptide; (*j*) HeLa IF polypeptides + antiserum to bovine prekeratin 52-kdalton polypeptide; (*k*) HeLa IF polypeptides + antiserum to bovine prekeratin 58-kdalton polypeptide; (*l*) HeLa IF polypeptides + antiserum to bovine prekeratin 65-kdalton polypeptide; (*m*) Bovine prekeratin polypeptides + HeLa IF antiserum; (*n*) Bovine prekeratin polypeptides + total bovine prekeratin antiserum; (*o*) Human plantar callus polypeptides + total bovine prekeratin antiserum.

compounds these loose arrays form juxtanuclear caps (4, 16). These observations are comparable with the immunofluorescence and immunoelectron microscopic studies of Weber and co-workers (8, 10), and therefore support the idea that HeLa cells contain at least two major organizational types of IF. These two organizational states of IF are also seen in vitro following two cycles of disassembly/assembly of HeLa IF preparations. This latter observation suggests that bundling or cross-linking proteins or other factors are associated with HeLa IF. IF cross-linking proteins, termed filaggrins (14), have been isolated from keratinocytes, and we are now searching for similar cross-linker(s) in the HeLa IF preparations.

Immunological Properties of HeLa IF

The immunochemical studies employing ^{125}I-labeled protein A demonstrate that the complex rabbit antiserum directed against the unfractionated mixture of HeLa IF proteins contains antibodies against each of the major HeLa IF components (except for the hmw). In addition, the findings that IF structural proteins from BHK-21 fibroblasts and bovine epidermal prekeratin preparations react with the HeLa IF antiserum, also suggest that proteins thought to be characteristic of both fibroblast and epithelial IF are present in HeLa cells. Further support for this contention is found in the cross-reactivity between HeLa IF proteins and BHK-21 IF antibodies (which react with HeLa 55,000), as well as bovine prekeratin antibodies.

The immunofluorescence observations reported in this study and by other laboratories (see e.g., 8, 9) also suggest that HeLa cells contain two organizationally distinct IF systems. One of these is colchicine-sensitive and reacts with antiserum directed against the 55,000-mol-wt protein of BHK-21 IF. The other IF system reacts with antiserum to bovine epidermal keratin. These two IF systems are observed simultaneously when HeLa cells are stained with HeLa IF antiserum, but due to the complexity of the antiserum it is not clear which IF proteins are responsible for the observed immunofluorescence patterns. Preadsorption of the HeLa IF antiserum has helped to begin to clarify this issue. Using antiserum preabsorbed with both the HeLa 54,000-mol-wt and 55,000-mol-wt proteins, it is evident that visualization of the colchicine-sensitive pattern is due to a reaction between IF containing these subunits and the antibodies directed against at least one of these proteins. Similarly, visualization of the keratin-like pattern appears to be due in large part to a reaction with the 43,000-mol-wt polypeptide.

Possible Functions of IF in HeLa Cells

The redistribution of one class of HeLa IF from a cytoplasmic distribution to a juxtanuclear accumulation in the absence of microtubules (i.e., during colchicine treatment) indicates that these IF are dependent on microtubules for their distribution through the cytoplasm (e.g., 2). If further suggests that a functional association may exist between these two cytoskeletal elements within HeLa cells, as has been suggested in BHK-21 cells with regard to saltatory organelle movements and cell shape formation (e.g., 2). It has been suggested that some of the high molecular weight proteins, such as those present in the freshly isolated HeLa preparations, may be involved in the formation of IF-microtubule complexes. Thus hmw IF associated proteins (IFAPs) may be involved in cross-bridging IF and microtubules, IF and organelles, or IF with other IF (3). In contrast, the bundles of keratin containing IF, which are localized primarily towards the lower surface of cultured HeLa cells, and whose distribution appears unaltered in the presence of colchicine, are most likely involved in shape maintenance.

One of the most intriguing features of IF in general is their diversity with regard to subunit number, molecular weight, and antigenicity. This holds true within singled cultured cell lines in which several types of IF exist, such as HeLa cells, or in situ in which cells comprising different tissues contain different IF proteins (4). Thus, IF are not conserved throughout nature in the same fashion as the other two major cytoskeletal components, microtubules and actin-containing microfilaments. Although we do not yet understand the func-

tional basis of this diversity, it is quite possible that this diversity is in itself a major factor in distinguishing different cell types. Thus IF may function in a type of cytoplasmic differentiation that may underlie differences in cell shape, distribution of intracellular organelles and molecules, and variation in subcellular molecular architecture.

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