

# Dynamics of Keratin Assembly: Exogenous Type I Keratin Rapidly Associates with Type II Keratin In Vivo

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**Abstract.** Keratin intermediate filaments (IF) are obligate heteropolymers containing equal amounts of type I and type II keratin. We have previously shown that microinjected biotinylated type I keratin is rapidly incorporated into endogenous bundles of keratin IF (tonofilaments) of PtK<sub>2</sub> cells. In this study we show that the earliest steps in the assembly of keratin subunits into tonofilaments involve the extremely rapid formation of discrete aggregates of microinjected keratin. These are seen as fluorescent spots containing both type I and type II keratins within 1 min post-injection as determined by double label immunofluorescence. These observations suggest that endogenous type II keratin subunits can be rapidly mobilized from their endogenous state to form complexes with the injected type I protein. Furthermore, confocal micros-

copy and immunogold electron microscopy suggest that the type I-type II keratin spots form in close association with the endogenous keratin IF network. When the biotinylated protein is injected at concentrations of 0.3–0.5 mg/ml, the organization of the endogenous network of tonofilaments remains undisturbed during incorporation into tonofilaments. However, microinjection of 1.5–2.0 mg/ml of biotinylated type I results in significant alterations in the organization and assembly state of the endogenous keratin IF network soon after microinjection. The results of this study are consistent with the existence of a state of equilibrium between keratin subunits and polymerized keratin IF in epithelial cells, and provide further proof that IF are dynamic elements of the cytoskeleton of mammalian cells.

EPITHELIAL cells contain large bundles of 10-nm-diam intermediate filaments (IF)<sup>1</sup>, termed tonofilaments, comprised of heteropolymers of the types I and II IF proteins, the keratins. At the center of the cell tonofilaments are associated with the nuclear envelope and nuclear pore complexes (32). These appear to be continuous with tonofilaments radiating throughout the cytoplasm, forming an anastomosing network which at the cell surface interacts with microfilaments (34) and the plaque structures of hemidesmosomes and desmosomes (43). The equimolar ratios of type I and II keratins expressed in vivo are a direct consequence of the requirement for heterodimers in the assembly of keratin IF (15, 37, 74). The two protein chains of the heterodimer appear to be aligned parallel and in register (63). While the large family of keratins are often expressed as cell specific pairs in vivo (78, 80), keratin IF are formed from nearly any combination of the two types in vitro (35).

In vitro assembly studies with mutant keratins (16, 36–38), transient transfection studies (1, 2, 82), and transgenic mouse experiments (18, 83) have helped to determine which domains within the keratin molecule are essential for assembly of IF. These studies have shown that the amino- and carboxy-terminal ends of the  $\alpha$ -helical central rod domain

are important for IF formation in vitro and in vivo (1, 2, 38, 53, 54, 82). However, deletion of the non- $\alpha$ -helical end domains still results in IF assembly (1, 2, 36, 54).

Experiments designed to determine the domains within the keratin molecule required for de novo keratin IF network assembly have utilized the co-transfection of type I and II keratin genes into cells devoid of keratin. These experiments have produced conflicting results. Transient co-transfection with both mutant genes lacking the non- $\alpha$ -helical COOH-terminal domain indicate that this domain is not necessary for IF assembly (4). However, experiments involving retroviral mediated expression of a pair of mutant keratins indicate that one of the keratins must contain both the non- $\alpha$ -helical head and tail domains for efficient de novo filament formation to occur (54).

These studies on assembly are also relevant to the etiology of epidermolysis bullosa simplex (17, 24, 51) and epidermolytic hyperkeratosis (9, 10, 14, 30, 42, 67), blistering skin diseases in which there is disruption of tonofilament integrity and cytolysis (7, 41). Significantly, point mutations within keratin chains have been found in these disease states (17, 51, 67) leading to the suggestion that keratin IF may play a role in the mechanical integrity of keratinocytes within the physiological milieu of skin tissue (7, 9, 10, 17, 18, 24, 51, 67).

The prevailing view of IF as relatively inert cytoskeletal

1. *Abbreviation used in this paper:* IF, intermediate filament.

elements is now being replaced in favor of the idea that IF are dynamic cytoskeletal structures (26, 32, 70, 77). The former notion was in part based on the low solubility coefficient of IF in most neutral physiological-strength buffers *in vitro* (68), the concept of disulfide bond stabilization of keratin IF in the epidermal differentiation process (76), the slow rates of IF protein turnover (19, 56), and the existence of barely detectable "soluble pools" of IF subunits in cell homogenates (6, 31, 40, 71). Recently, however, evidence has become available which supports a more dynamic model of IF. In mitosis, for example, various cell types disassemble their IF and subsequently reassemble them following cytokinesis (28, 44, 50, 66). It has been demonstrated that the disassembly of both vimentin IF (11, 12) and the nuclear lamina (20, 64, 87), are accompanied by hyperphosphorylation catalyzed by p34<sup>cdc2</sup>. In further support of the role of phosphorylation in the regulation of IF polymerization states, *in vitro* studies have shown that vimentin and/or desmin IF are phosphorylated by several kinases which induce disassembly (39, 46) and that protein kinase C appears to be closely associated with vimentin (59, 72) and keratin (62). In addition, the rapid turnover of phosphate demonstrated for vimentin (25) is mediated in part by protein phosphatases whose function is essential for the structural integrity of vimentin IF (25).

Biochemical approaches have also provided information about the IF assembly process. Pulse chase experiments indicate that newly synthesized vimentin is rapidly incorporated into IF networks using either co-translational (40) or posttranslational mechanisms (6, 40). Interestingly, different mesenchymal cell types may use these two mechanisms to vastly varying extents (6, 40). While the "soluble pool" of IF in cells may be extremely small, experiments conducted at steady state using fluorescence recovery after photobleaching techniques demonstrate that a pool of vimentin IF is indeed kinetically active, allowing for the rapid exchange of vimentin subunits along IF fibers (86). Furthermore, fluorescence energy transfer experiments demonstrate that neurofilament IF (3) and glial fibrillary acidic protein (60) subunits exhibit a dynamic exchange equilibrium with IF *in vitro* and also indicate that a specific dissociation step may be rate limiting (3). Similar information concerning the dynamic aspects of keratin IF in epithelial cells is not available.

Information concerning the sites of IF assembly has been derived from several lines of experimentation. In the case of keratin IF, a juxtannuclear incorporation of newly synthesized subunits has been described following recovery from the effects of transiently transfected mutant genes (1, 2). Other transfection studies using vimentin genes suggest that incorporation of newly synthesized subunits occurs throughout the cell (61, 65). Similarly, microinjection of keratin mRNA demonstrates that *de novo* network assembly can also occur throughout the cytoplasm (29). Thus, it seems likely that different IF systems may use different processes for the incorporation of their subunits into pre-existing and newly developing IF networks.

Microinjection techniques have also been employed in determining the steps in the posttranslational assembly of keratin IF (57). Immediately after injection into either primary mouse epidermal cells or PtK<sub>2</sub> cells, aggregates of biotinylated type I keratin are distributed throughout the cytoplasm. Within minutes, these aggregates appear to form

short biotinylated tonofibrils that are coincident with endogenous tonofilaments. After 2 h a continuous biotinylated tonofilament network is seen which co-localizes with the endogenous tonofilament network. These experiments demonstrate that keratin subunit assembly into endogenous tonofilaments can take place at discrete sites throughout the cytoplasm. The different assembly sites seen when keratin is studied following either transfection with keratin genes or microinjection of purified keratin may be related to differences in co-translational or posttranslational mechanisms used by the cell (57, 70).

Microinjection methods have also been used in studies of posttranslational assembly for other types of IF during interphase. Upon microinjection into cultured fibroblasts (84) or PtK<sub>2</sub> cells (57), vimentin is assembled into the endogenous IF network in a juxtannuclear organization center. Furthermore, microinjection of phosphorylated GFAP suggests that the phosphorylation state of the injected protein can affect the temporal and spatial patterns of incorporation (88). Fluorescent-labeled desmin also incorporates into an IF pattern (58) and microinjection of the type V IF protein, lamin A, results in its rapid incorporation into the nuclear lamina during interphase (33). These observations indicate that IF polymer networks are dynamic with respect to their capacity to incorporate additional structural subunits.

These types of studies have provided new information regarding the sites of assembly of IF and some details of the mechanisms underlying their incorporation into polymerized networks. However, these studies have not revealed much information about the earliest steps in the assembly of IF subunits into their respective polymer systems. Heteropolymer keratin IF provide an advantageous system in which to study the early steps in the assembly process because of the availability of soluble and injectable biotinylated type I keratin (57) and specific immunological probes for type II keratin subunits (23). The results reported here demonstrate that incorporation of biotinylated type I keratin involves rapid association with an endogenous type II keratin, before formation of tonofilaments. Furthermore microinjection of high concentrations of type I keratin results in the alteration of the endogenous keratin network. These results support a dynamic equilibrium model for keratin IF based on a rapidly exchangeable pool of keratin within the cell.

## Materials and Methods

### Preparation of Type I Keratin

Biotinylated type I keratin was prepared from bovine tongue epithelium as described elsewhere (57). Protein concentrations for injection were determined after dialysis into injection buffer (10 mM Tris, pH 7.4) (57) using the method of Bradford (8) with BSA as a standard. Biotinylated bovine type II keratin is not soluble under these conditions and we have not found alternate suitable conditions for its injection. The percentage of type I keratin biotinylated in the injection sample was determined using avidin affinity column chromatography. 90–95% of the injected protein was biotinylated, as determined by densitometric scanning of Coomassie blue-stained gels obtained from bound vs. unbound fractions of avidin affinity columns (Pierce Chemical Co., Rockford, IL). Unbound protein was shown to be non-biotinylated by immunoblotting analysis with goat anti-biotin antibody (Sigma Immunochemicals, St. Louis, MO) (data not shown).

Glutaraldehyde cross-linking studies were carried out to determine the oligomeric state of the biotinylated type I keratin. Fresh aliquots of glutaraldehyde (EM grade; Electron Microscopy Sciences, Fort Washington, PA) were diluted to 1.2% in 10 mM Tris, pH 7.4, immediately before use. This

glutaraldehyde stock solution was added to 100- $\mu$ l aliquots of type I keratin (100  $\mu$ g/ml) in the same buffer to yield final glutaraldehyde concentrations in the range of 0.1–0.6%. Samples were mixed by vortexing. For each concentration of glutaraldehyde, cross-linking was carried out for 2, 3, 5, 7, 9, 11, and 13 min. At appropriate times, the cross-linking reaction was quenched using an equal volume of SDS-sample buffer containing 0.3% BME. As a positive control, vimentin IF subunits were also cross-linked. In this case, a band corresponding in size to tetramers was identified on Coomassie-stained SDS-gels (Eriksson, J. E., and R. D. Goldman, personal communication). Samples were analyzed by SDS-PAGE and the resulting gels were stained with Coomassie brilliant blue R-250 (Sigma Immunochemicals). A single band at 48 kD was observed at all glutaraldehyde concentrations and for all times of incubation. No cross linking into higher molecular weight forms was seen, indicating that the injected type I keratin is monomeric. Non-biotinylated type I bovine tongue keratin was extracted and prepared on a DE-52 ion-exchange column exactly as described for biotinylated keratin (57).

## Cell Culture

PtK<sub>2</sub> cells were grown in MEM (Gibco Laboratories, Grand Island, NY) with 10% FCS, penicillin, and streptomycin as previously described (57). For microinjection, these cells were grown on glass locator coverslips (Bellco Glass, Vineland, NJ).

## PtK<sub>2</sub> IF Preparations

IF were prepared from confluent dishes of PtK<sub>2</sub> cells as described elsewhere (90).

## Immunoblotting

Immunoblotting was carried out by the method of Towbin et al. (81). The mAbs, AE1 and AE3 (23), directed against type I and II keratins, respectively, were provided by Dr. T.-T. Sun (New York University College of Medicine, New York) and were used at a dilution of 1:50 as previously described (57). Troma 1, a rat mAb directed against mouse keratin 8, was provided by Dr. Robert G. Oshima (La Jolla Cancer Research Foundation, La Jolla, CA) and was used undiluted. Goat anti-biotin antibody (Sigma Immunochemicals) showed no cross-reacting proteins in immunoblot analyses of whole-cell extracts and IF preparations obtained from PtK<sub>2</sub> cells.

Peroxidase-conjugated secondary antibodies included rabbit anti-goat (Amersham, Arlington Heights, IL), goat anti-rat (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and goat anti-mouse (Southern Biotechnologies Associates, Inc., Birmingham, AL), all used at a dilution of 1:1,000.

## Microinjections

Microinjections were carried out as previously described (57). Microinjections of type I keratin at concentrations >2.0 mg/ml could not be carried out. This was due to clogging of the tip of the needle. In control assays, biotinylated BSA, containing 8–10 biotin moieties per molecule (Pierce Chemical Co., Rockford, IL), was resuspended in 10 mM Tris, pH 7.4, and clarified at 12,000 *g* for 2 min before microinjection.

## Indirect Immunofluorescence

PtK<sub>2</sub> cells were processed for indirect immunofluorescence using cold methanol fixation as described elsewhere (57, 90). Alternatively, cells were fixed for 5 min in 1% formaldehyde/0.5 mM MgCl<sub>2</sub>/1 mM CaCl<sub>2</sub>/PBS, extracted in three washes of 0.1% NP-40/PBS over 5 min at room temperature, and rinsed in PBS for 2 min before the addition of antibodies (33). This latter fixation procedure produced staining results identical to those obtained with methanol fixation, except that background mitochondrial staining due to endogenous biotin was eliminated. Biotinylated keratin was detected using goat anti-biotin antibody (Sigma Immunochemicals) and donkey anti-goat secondary antibody conjugated with FITC (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) as described elsewhere (57, 85). Endogenous keratin networks were revealed using either Troma 1 or a rabbit polyclonal directed against mouse epidermal type I keratin as previously described (45). Another rabbit polyclonal (#140) raised against type II mouse epidermal keratin was used in some experiments to detect the keratin aggregates which formed soon after microinjection. To monitor the fate of microinjected keratin at short time points (30 s to 3 min), immunofluores-

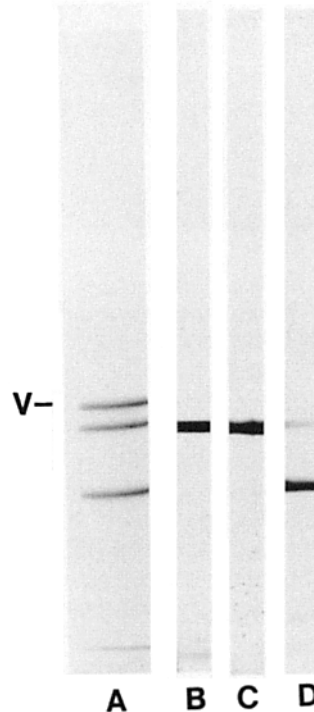
cence was carried out on cells grown and injected on 2 mm  $\times$  4 mm coverslips cut from larger locator coverslips with a diamond edge. These latter preparations were fixed, washed, and stained in 96-micro-well plates containing 200  $\mu$ l of the appropriate solutions. Cells were examined by epifluorescence with a Zeiss Axiophot microscope or a Zeiss Laser Scan Microscope (LSM) (Carl Zeiss, Oberkochen, Germany) equipped with a 488  $\lambda$  argon laser and a 543  $\lambda$  helium-neon laser. Confocal images were photographed with a Matrix multicolor high-resolution monitor (Matrix Instruments, Inc., Orangeberg, NY) equipped with a 35-mm camera.

## Overlay Analysis of Tonofilaments and Spots Containing Biotinylated Type I Protein

PtK<sub>2</sub> cells were injected with biotinylated type I keratin. After 20 mins, cells were fixed and stained for double label indirect immunofluorescence with anti-biotin and anti-keratin antibodies. Confocal microscopy was used to obtain both rhodamine and fluorescein images. The brightest biotinylated spots were identified by their visibility through a single sheet of vellum paper and traced onto clear plastic using an extra-fine Sharpie marker. This spot pattern was overlaid onto the endogenous keratin pattern and aligned using the assistance of the "protect-image" mode of the LSM software. Spots were scored as either associated with tonofilaments or not associated. Furthermore, of those spots associated with tonofilaments, we also scored those in close proximity with apparent branch points in the tonofilament network.

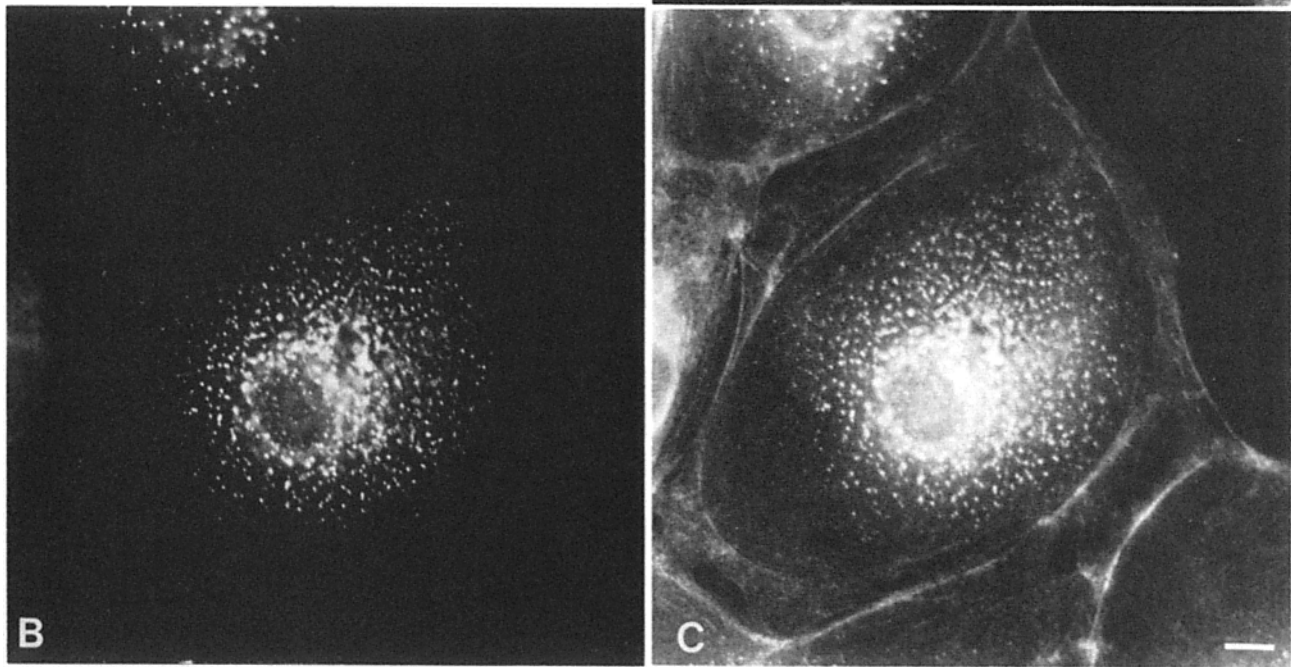
## Immunogold Electron Microscopy

A procedure modified from Yang et al. (90) was used. Injected cells were fixed on locator coverslips in dry methanol ( $-20^{\circ}$ C) for 3 min, and rinsed in PBS for 1 min. Goat anti-biotin (Sigma Immunochemicals) and normal donkey serum (Janssen Inc., Olen, Belgium) in cacodylate buffer (0.1 M sodium cacodylate trihydrate, pH 7.2) (Ted Pella Inc., Redding, CA) were applied to the cells for 30 min at 37 $^{\circ}$ C at a dilution of 1:20. Coverslips were rinsed three times over 9 min in cacodylate buffer. Rabbit anti-goat secondary antibody conjugated with 5 nm gold particles (Amersham Corp., Arlington Heights, IL) was used at a 1:5 dilution in cacodylate buffer for 30 min at 37 $^{\circ}$ C. Cells were rinsed as above and fixed for 1 h in 1% glutaraldehyde/cacodylate buffer. After three rinses (20-min each) in cacodylate buffer, cells were postfixed for 2–6 h in 1% OsO<sub>4</sub>, 0.8% potassium ferricyanide (K<sub>3</sub>Fe[CN]<sub>6</sub>) (Sigma Immunochemicals) in cacodylate buffer at 5 $^{\circ}$ C in the dark. Preparations were then counterstained with 1% uranyl acetate, dehydrated in sequential ethanol baths, and embedded in eponaldehyde as detailed in an earlier publication (85). Embedded, injected cells were removed from the glass coverslip, and located via the impression in



**Figure 1.** AE3 antibody recognizes a type II keratin in PtK<sub>2</sub> cells, but does not react with injected type I keratin. AE3 has been shown to react primarily with type II keratin, and AE1 with type I keratin (23). PtK<sub>2</sub> IF (lane A), SDS-PAGE, Coomassie blue) were transferred to nitrocellulose and probed with anti-keratin 8 (lane B) AE3 (lane C), and AE1 (lane D). v, vimentin. Previous work by Miller et al. (Fig. 2 in reference 57) has demonstrated by immunoblotting criteria that bovine tongue type I keratins do not react with AE3 antibody.

**Figure 2.** Type II spots are revealed with AE3 antibody and colocalize with biotinylated type I spots in cells injected with type I keratin. PtK<sub>2</sub> cells were injected with biotinylated type I keratin. After 2 min (A) or 20 min (B and C), cells were fixed and stained for indirect immunofluorescence using AE3 (A and C) or anti-biotin (B). Staining of PtK<sub>2</sub> cells with AE3 antibody shows weak staining of endogenous keratin IF networks, but not the bright spots seen after microinjection. Bar, 10 μm.



the Epon left by the locator coverslip (85), sectioned en face, stained, and viewed on a 1200EX electron microscope (Jeol Ltd. Tokyo, Japan) as described elsewhere (85).

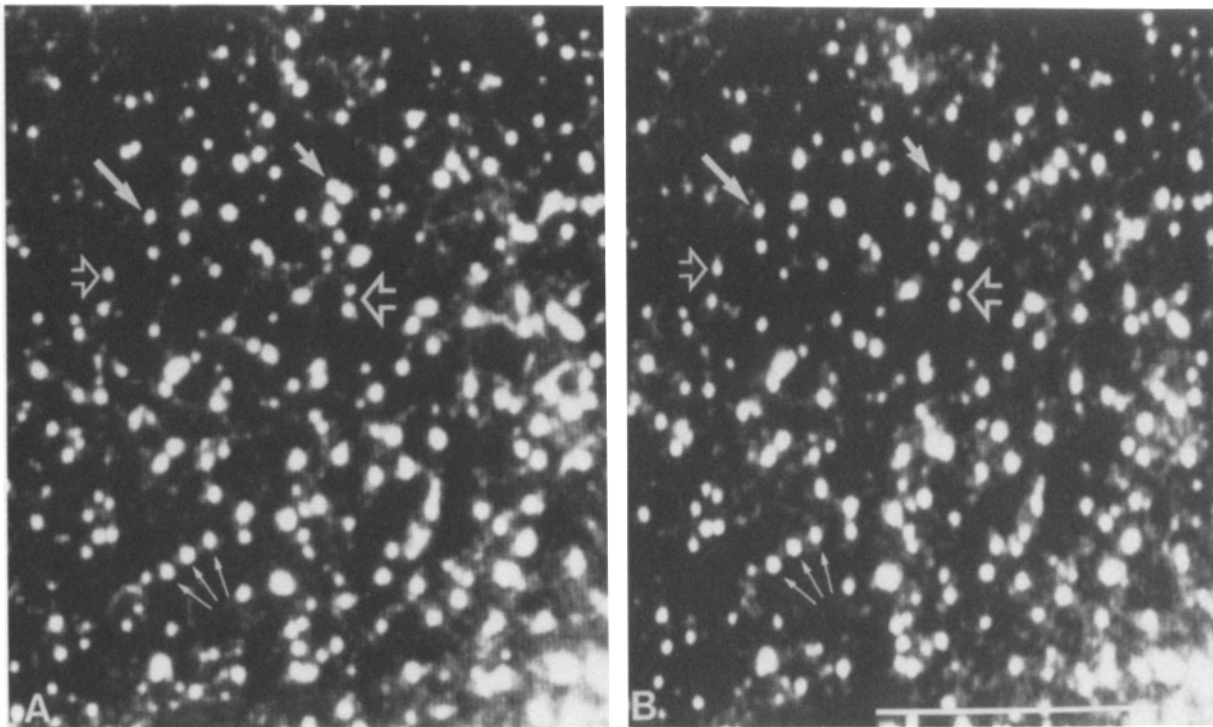
## Results

Our previous work at the light microscope level of resolution showed that microinjected biotinylated type I keratin formed discrete spots immediately after microinjection (57). Elongated “squiggles” of biotinylated keratin were then observed in close association with endogenous tonofilaments as the spots disappeared. After 2–4 h, a biotinylated keratin IF network which colocalized with the endogenous keratin network was seen (57). However, these studies did not examine

the incorporation process with respect to interactions with endogenous type II keratin, with which the injected type I keratin should pair to form IF (15, 37, 63, 74).

### *Type II Keratin Rapidly Associates with Microinjected Type I Keratin*

A characterization of the antibodies used to analyze the earliest steps in the incorporation process was carried out. AE1 and AE3 antibodies have been used previously to distinguish type I and II keratins (23). AE1 recognizes type I keratin and AE3 recognizes type II. In agreement with the work of others (22), AE3 antibody did not recognize the injected biotinylated type I keratin by immunoblotting criteria (see

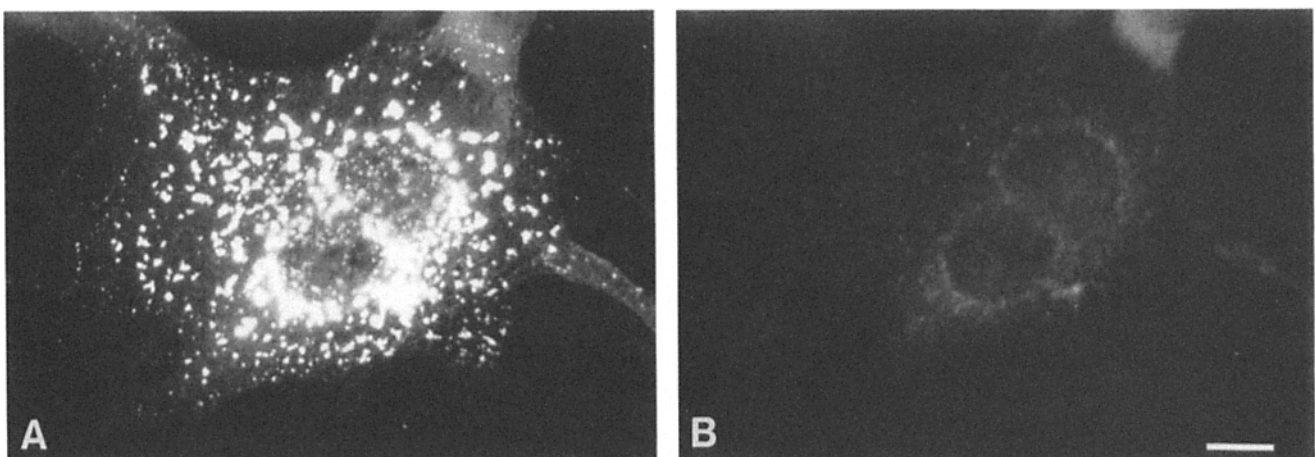


**Figure 3.** Confocal microscopy showing greater detail of the co-localization of type II spots and biotinylated type I spots. PtK<sub>2</sub> cells were injected with biotinylated type I keratin and fixed after 20 min. Cells were stained with anti-biotin (A), and AE3 (B). Arrows point to examples of individual spots which co-align. Bar, 10  $\mu$ m.

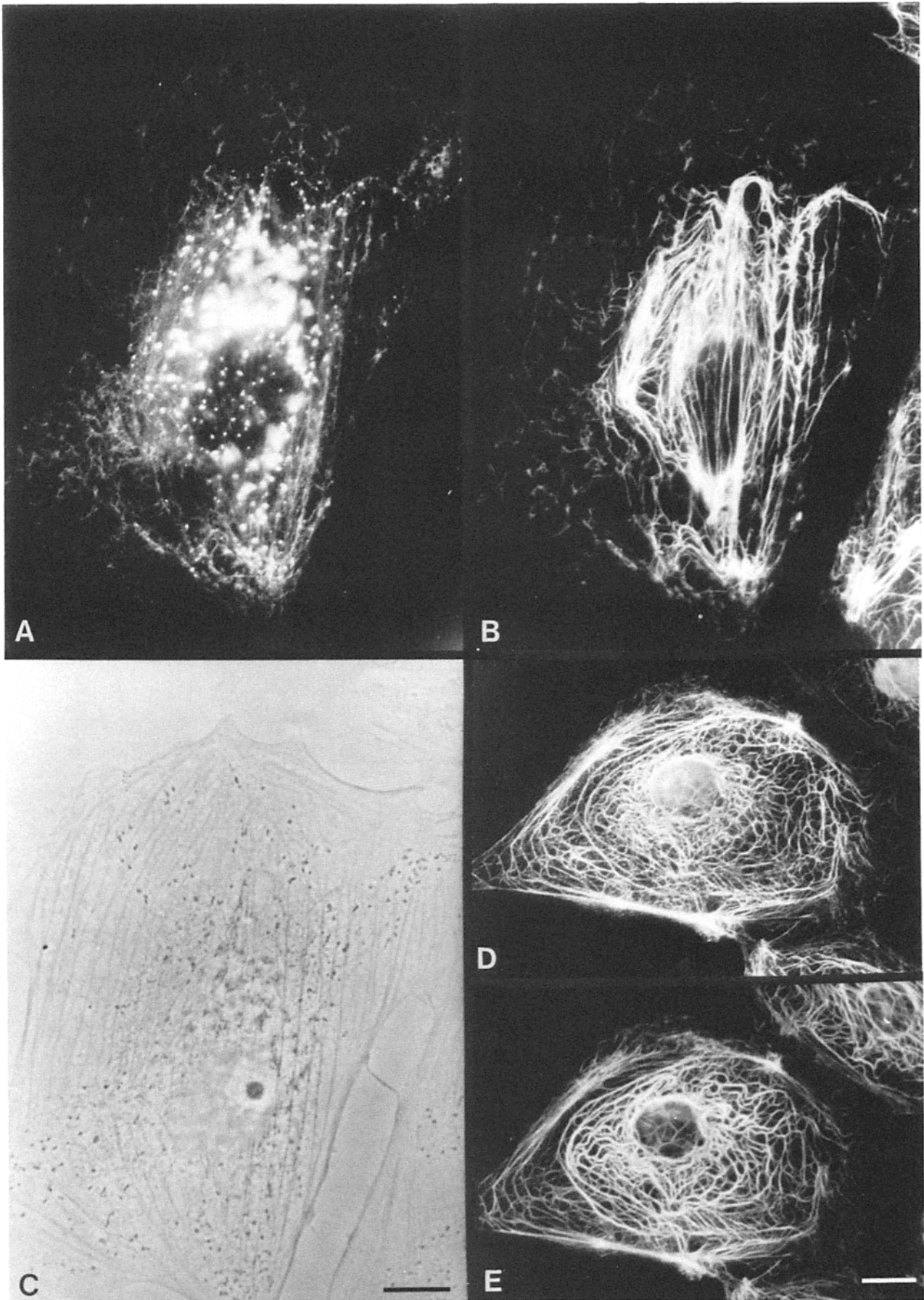
Fig. 2 in reference 57). Immunoblotting analysis was also carried out to confirm the reactivity of AE3 antibody with the endogenous type II keratin of PtK<sub>2</sub> cells. PtK<sub>2</sub> cells have been shown to contain primarily keratins K8 and K18 (27). AE3 recognized a single band corresponding to K8 (a type II) in immunoblotting analyses of both PtK<sub>2</sub> IF preparations (Fig. 1, lane c) and whole cell extracts (data not shown).

To determine the speed of association of endogenous type II keratin with the injected type I keratin, cells were fixed at short time points postinjection and stained with AE3 anti-

body. Within less than 1 min, a spot pattern was seen by indirect immunofluorescence (Fig. 2 A). To determine the relationship between these spots and the aggregates of biotinylated type I keratin, double label immunofluorescence was carried out. The AE3 pattern was virtually identical to that of the biotinylated keratin spot pattern (Fig. 2, B and C). Similar results were also obtained using a rabbit polyclonal antibody #140, see Materials and Methods) which recognized type II PtK<sub>2</sub> keratin, and did not recognize the injected biotinylated keratin as determined by immunoblotting



**Figure 4.** AE3 antibody does not react with injected biotinylated type I keratin in vivo. To show that AE3 antibody does not react with injected type I keratin in vivo, biotinylated type I keratin was microinjected into BHK-21 fibroblasts, which do not contain keratin, and stained after 20 min with anti-biotin (A) and AE3 (B) antibodies. Photography of anti-biotin (fluorescein) used a 30-s exposure, while AE3 (rhodamine) required a 5-min exposure to capture any image on film. After extended time periods after microinjection (24 h), the biotin-spots are not longer detectable with anti-biotin. Bar, 20  $\mu$ m.



analysis (data not shown). Confocal microscopy was used to show the co-occurrence of anti-biotin and AE3 staining in microinjected cells (Fig. 3, *A* and *B*). It should be noted that AE3 stained the endogenous keratin IF networks of PtK<sub>2</sub> cells very weakly (Fig. 2, *A* and *C*). This was most likely due to an epitope masking phenomenon, which has been documented previously with AE3 (47) and other keratin antibodies (28, 89). In contrast, the appropriate epitope appeared to be readily available in the spots seen early after microinjection. Therefore, most of the AE3 epitopes are probably unavailable within the structure of the fully polymerized keratin IF.

To demonstrate that the coincidence of anti-biotin and AE3 immunofluorescence patterns was not due to crossreactivity between AE3 and the injected protein in the *in vivo* situation, the following control was carried out. Biotinylated type I keratin (1.0–2.0 mg/ml) was microinjected into BHK-1 fibroblasts, which do not contain keratin IF by immunological criteria (Miller, R. K., and R. D. Goldman, unpublished observations). After 20–30 min, cells were processed in the same fashion for indirect immunofluorescence using AE3 and anti-biotin antibodies. While spots were clearly visible with anti-biotin (Fig. 4 *A*), AE3 exhibited no immunoreactivity (Fig. 4 *B*). As an additional control, non-biotinylated type I keratin was injected into PtK<sub>2</sub> cells and stained with AE3 antibody. Spot patterns, identical in appearance, were observed at 20 min after injection (data not shown).

#### ***High Concentrations of Exogenous Type I Keratin Induce Structural Alterations in Endogenous Keratin IF Networks***

The microinjection results described above suggested that the exogenous bovine type I keratin rapidly associated with endogenous type II keratin. Since a likely source of the type II protein is the polymerized IF comprising the tonofilaments, we decided to determine whether or not higher concentrations of an injected type I keratin would have an effect on the structural integrity or extent of polymerization of the endogenous keratin IF network. A concentrated (1.5–2.0 mg/ml) solution of biotinylated type I keratin was prepared and injected into PtK<sub>2</sub> cells. The endogenous keratin IF network was observed using the K8 antibody, Troma 1. Within 1 to 3 h after injection, alterations in the endogenous IF networks were observed (Fig. 5, *A* and *B*). These alterations were more pronounced in the peripheral regions of the cell, where very few or no continuous tonofilaments could be found. Normally this region contains numerous long tonofilaments (see Fig. 5, *D* and *E*). The central portion of the keratin IF network appeared less affected (Fig. 5, *A* and *B*). These alterations of the keratin IF network did not result in obvious changes in cell shape as determined by phase contrast microscopy (Fig. 5 *C*). At the standard concentrations used for microinjection (0.3–0.5 mg/ml), no detectable alter-

ations in the endogenous keratin IF networks were seen (Fig. 5, *D* and *E*). Interestingly, incorporation into the endogenous tonofilaments appeared to occur at a faster rate when lower concentrations of keratin were injected. Changes in keratin organization induced by injection of type I keratin at the highest concentrations were not fatal. Injected cells fully recovered and assembled a normal appearing keratin IF network over 24–36 h and frequently divided to produce daughter cells, each containing extensive networks detected with anti-biotin which were coincident with the endogenous networks as detected by double label immunofluorescence (Fig. 6, *A* and *B*).

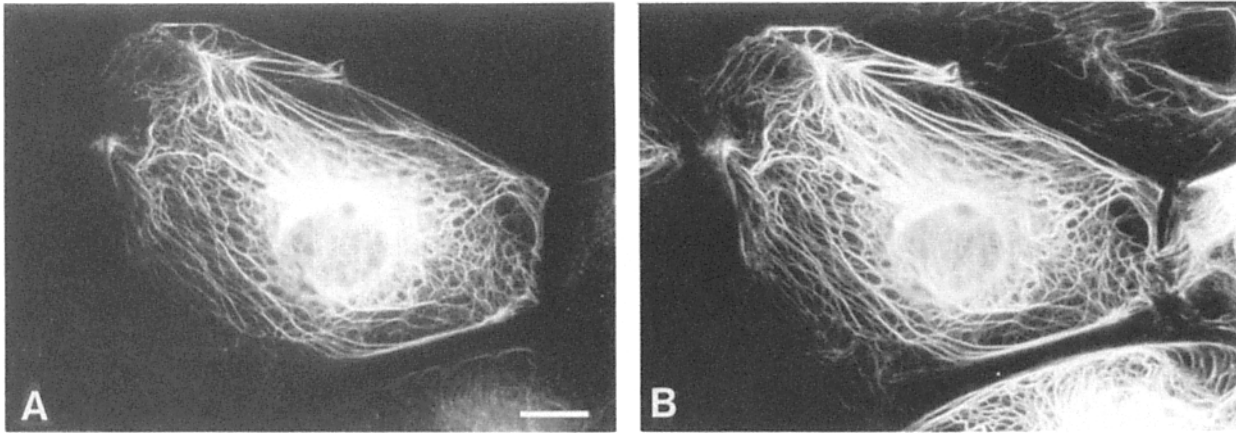
To eliminate the possibility that the biotinylation of type I keratin might be involved in the alteration of the endogenous network, 1.5 mg/ml of non-biotinylated type I keratin was injected into PtK<sub>2</sub> cells. 1–3 h later, cells were fixed and stained for immunofluorescence using anti-keratin 8 to visualize the endogenous keratin IF network. The endogenous network was disrupted in a fashion identical to that seen in cells injected with biotinylated keratin (see Fig. 5 *B*, data not shown). No alteration of the endogenous keratin network could be detected 1–4 h after the microinjection of non-biotinylated keratin at lower concentrations (0.3–0.5 mg/ml) (data not shown).

We also tested whether or not the more generalized effects of high concentrations of microinjected protein might be responsible for altering the endogenous IF network. To this end, we injected solutions containing high concentrations of biotinylated BSA (8 mg/ml). Cells were fixed and stained at time intervals after injection with anti-biotin and anti-keratin antibodies. Biotinylated BSA was found distributed diffusely throughout the cell with no obvious association with the endogenous keratin network, which appeared normal and undisturbed (data not shown).

#### ***The Type I–Type II Complexes Formed Immediately after Microinjection Are Associated with Endogenous Tonofilaments***

A morphometric analysis was carried out to determine the relationship of the rapidly formed type I–type II aggregates (fluorescent spots) and the endogenous keratin IF network using double label indirect immunofluorescence with anti-biotin and the anti-keratin antibody. In cytoplasmic regions containing well defined keratin IF networks, biotinylated spots were traced onto clear plastic and overlaid onto the corresponding keratin IF network. 2,861 spots were scored as either associated or not associated with tonofilaments. A large fraction ( $88 \pm 4\%$ ) of the biotinylated spots appeared in close association with the endogenous keratin IF network. Of those associated with tonofilaments, almost half were located near apparent branch points. A confocal-generated overlay of biotinylated spot association with tonofilaments is shown (Fig. 7).

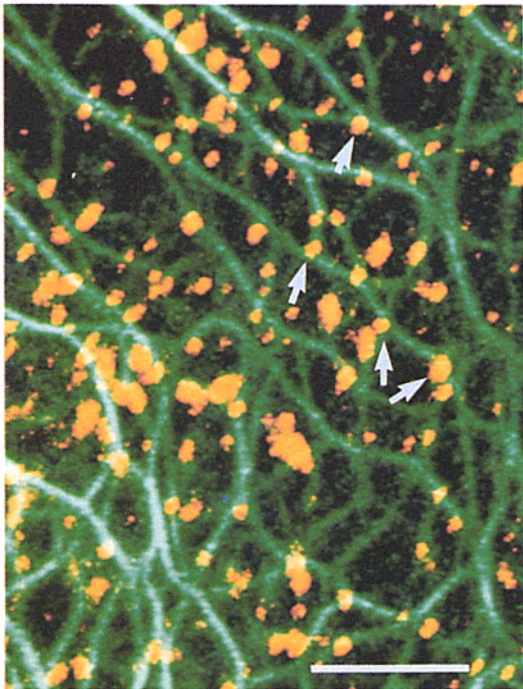
*Figure 5.* High concentrations of injected biotinylated type I keratin can alter the endogenous keratin IF network, while low concentrations do not. PtK<sub>2</sub> cells were injected with high concentrations (1.5–2 mg/ml, *A*, *B*, and *C*) and the normal, low concentration (0.3–0.5 mg/ml, *D* and *E*) of biotinylated type I keratin. Cells were fixed 2 h postinjection and stained with anti-biotin (*A* and *D*) and anti-keratin 8 (*B* and *E*). A phase contrast micrograph of *A*, *B* is shown in *C*. Bar, 20  $\mu$ m.



**Figure 6.** Altered keratin IF networks recover. PtK<sub>2</sub> were microinjected with high concentrations of biotinylated type I keratin and fixed after 24–36 h (A and B). Cells were stained with anti-biotin (A) and anti-keratin (B) antibodies. Bar, 10  $\mu$ m.

### Immunogold Localization

To determine the ultrastructural features of the fluorescent spots, their association with the IF bundles, and the general features of the incorporation process, an electron microscopic analysis was carried out. Small subconfluent groups of PtK<sub>2</sub> cells on locator coverslips were microinjected. At different time points, coverslips were processed for immunogold EM using an anti-biotin antibody and 5 nm gold conjugated secondary antibody. At 20 min, anti-biotin staining revealed gold-labeled keratin aggregates that appeared



**Figure 7.** A confocal generated overlay showing the association of biotinylated type I spots with the endogenous network. Cells were microinjected with biotinylated type I keratin. After 20 min cells were fixed and stained with anti-biotin (yellow/orange) and anti-keratin (green). Images were overlaid using the capabilities of the Zeiss confocal microscope and are displayed in pseudocolor. Bar, 5  $\mu$ m.

electron dense and slightly granular. These were frequently found in close association with the apparent branch points of the IF bundles which comprise tonofilaments (Fig. 8 A). No obvious IF or protofilamentous structures were seen in any of the aggregates examined up to 25 min after microinjection (Fig. 8 D). The peripheral gold labeling of the keratin aggregates was most likely the result of the pre-embedding labeling technique which probably inhibits antibody penetration into these densely packed proteinaceous structures. While at 20 min very little gold localization was detected along IF bundles, by 30–40 min after injection short segments of tonofilaments were found labeled with gold in addition to dense aggregates (Fig. 8 B). By 4–5 h, gold particles were found exclusively along tonofilaments and no dense aggregates could be detected (Fig. 8 C). A view at higher magnification is seen in Fig. 8 E. No gold particles were found associated with microfilaments, microtubules, or most other elements of the cytoplasm. However, some gold particles were found located within mitochondria (data not shown), confirming that the weak background staining previously observed with anti-biotin by indirect immunofluorescence (57) was due to endogenous biotin normally associated with mitochondrial functions. At all time points, control preparations of uninjected cells showed no labeling of IF bundles.

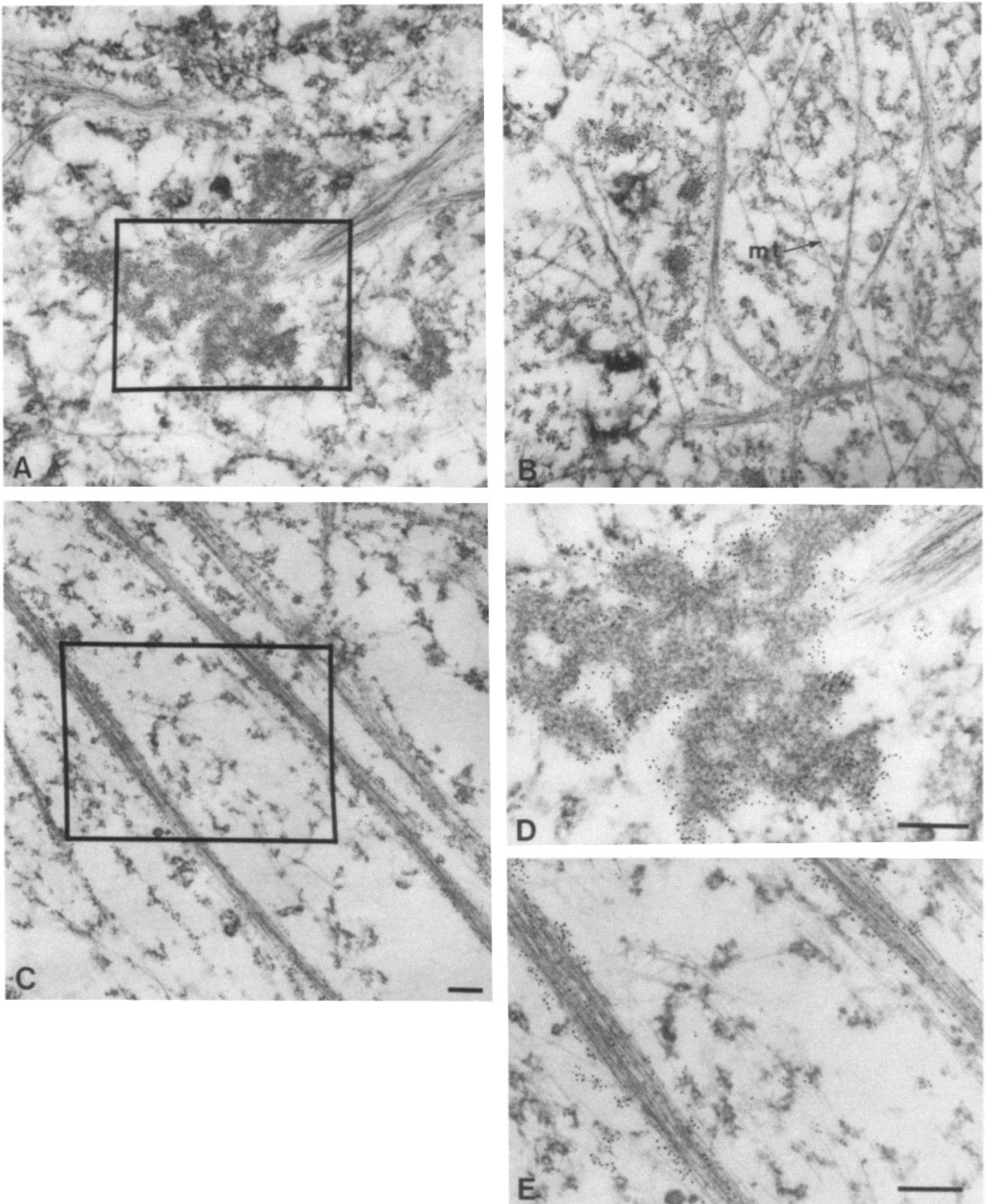
### Discussion

Immediately after microinjection, biotinylated type I bovine keratin forms aggregates in close association with the keratin containing tonofilaments. It then appears to become integrated into the endogenous keratin IF network at numerous sites (57). The biotinylated type I aggregates are seen to contain endogenous type II keratin within less than a minute after injection. Furthermore, at high concentrations, the injected type I keratin has a disruptive effect on the endogenous keratin IF network. Our results are consistent with a model of keratin IF as dynamic cytoskeletal elements capable of continual subunit exchange processes.

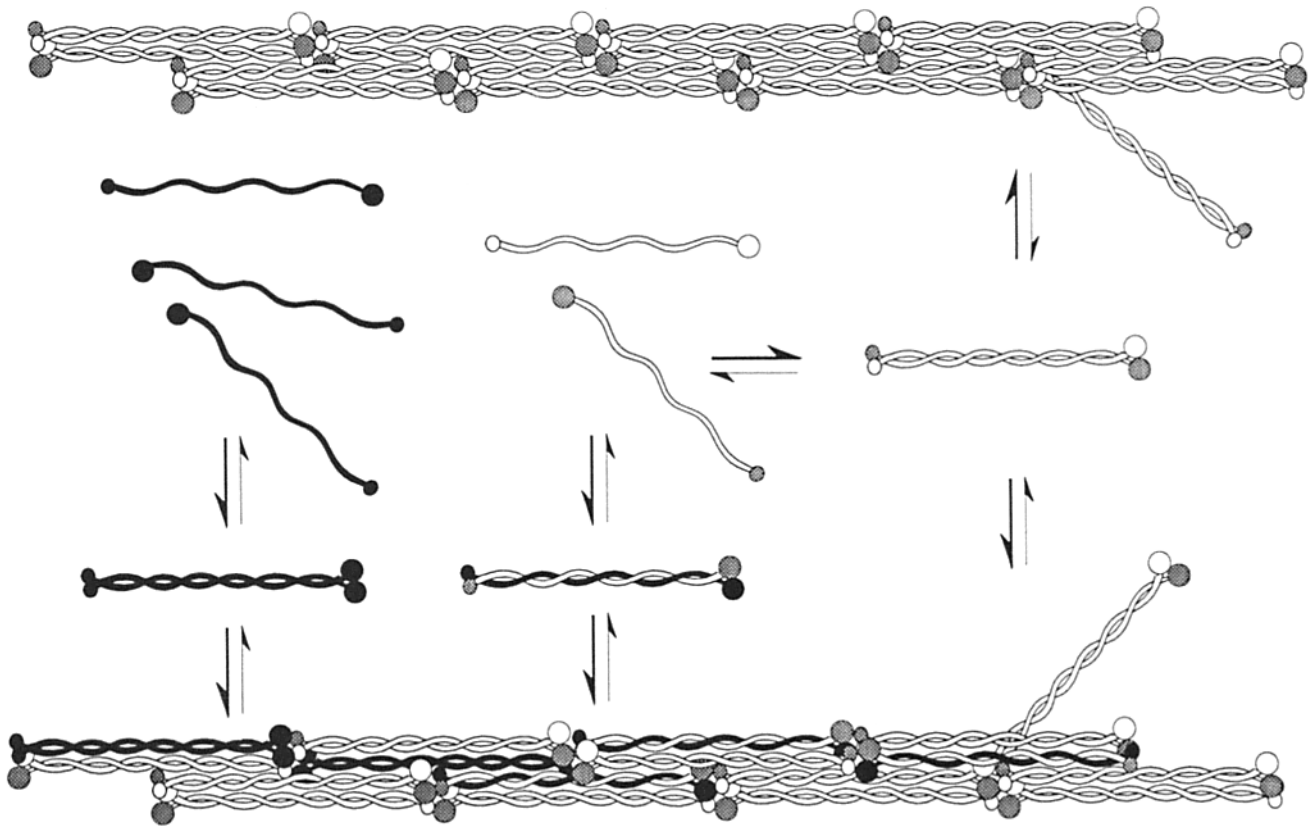
#### Type I–Type II Complexes Form Rapidly

Numerous studies indicate that keratin IF assembly involves the formation of type I and type II keratin heterodimers (15,





**Figure 8.** Immunogold localization showing biotinylated keratin incorporation into tonofilaments. PtK<sub>2</sub> cells were injected with biotinylated type I keratin and fixed after 20 min (*A* and *D*), 30–40 min (*B*), and 5 h (*C* and *E*). Samples were probed with goat anti-biotin and gold-conjugated rabbit anti-goat secondary antibody and embedded for EM. Higher magnification views of the areas marked by the rectangles in *A* and *C* are seen in *D* and *E*, respectively. *mt*, microtubule. Bars, 200 nm.



**Figure 9.** A possible model explaining the behavior of keratin IF in cells injected with biotinylated type I keratin. Keratin IF are comprised of heterodimers containing type I (shown with white globular end domains) and type II (shown with gray globular domains) keratin (37, 74). In this model, dimers (or higher-order oligomers) can exit keratin IF polymers (seen at top and bottom), and either re-enter the polymer directly or dissociate transiently into monomers. After injection, biotinylated type I keratin (shown in black) can pair with monomeric endogenous type II keratin. This heterologous dimer can then enter a pre-existing 10 nm keratin IF. Since the data derived from FRAP experiments on vimentin-IF *in vivo* strongly suggest that subunit exchange can occur all along the length of vimentin IF (86), this model has been drawn to follow that paradigm. While the steady-state equilibrium is likely to lie in the direction of the more polymerized forms (as indicated by the arrows), the addition of a large concentration of exogenous type I keratin chains will likely shift the steady state equilibrium. This would result in additional subunits being withdrawn from the polymer, and an increased rate of dimer to monomer dissociation. In turn, this could cause alterations to the endogenous network as seen at the high concentrations of microinjected keratin.

Several additional parameters must also be considered within the context of this model. For example, the fate of the displaced endogenous type I keratin is not known. However, homodimers have been shown to assemble into urea-cycled keratin IF *in vitro* (74). Similar incorporation *in vivo* could contribute to the experimental observations and is shown by the incorporation of a biotinylated homodimer (depicted in black). Another consideration is based on the finding that unpaired keratins can be degraded (21, 48, 49, 52). However, the extent of degradation of the excess type I keratin would be difficult to determine in microinjected cells.

37, 54, 74, 79). The finding that the type II keratin specific antibody, AE3, stains the same spots formed by the microinjected biotinylated type I protein lends further support to the idea of heterodimer formation *in vivo*. Although higher order associations cannot be ruled out using our methods, the association of injected biotinylated type I with endogenous type II probably involves the dimerization step described in numerous studies *in vitro* (15, 37, 74). Our results also suggest that type I-type II associations take place before incorporation into pre-existing tonofilaments.

***The "Capture" of Endogenous Type II Keratin within the Spots Suggests That Keratin Subunits Can Be Rapidly Mobilized***

FRAP studies of vimentin IF have supported the existence of a soluble pool which provides the underlying

mechanism for a steady state exchange between subunit and polymer, permitting a bleached zone to recover its fluorescence along the length of a vimentin fiber (86). In support of this, cell fractionation studies have shown that there is a small soluble pool of vimentin IF *in vivo* (6, 71) and that newly synthesized vimentin (6) and neurofilament proteins (5) are rapidly transferred through this soluble pool to a pelletable cytoskeletal fraction. However, the existence of a similar pool of keratin subunits has not been characterized. Therefore the association of type II keratin within the aggregates of the biotinylated keratin suggests that the injected protein "captures" endogenous subunits before its incorporation into the IF comprising tonofilaments. This association occurs rapidly, within less than 1 min after injection, and requires that endogenous type II keratin is available for such complex formation, moving from its native state within the cell to the biotinylated type I aggregates. Therefore, the type

II keratin subunits are likely to exist in at least two forms: that found in the fully polymerized keratin IF comprising tonofilaments and another in a pool, albeit most likely very small, not associated with tonofilaments. Considering the speed with which type II associates with the spots, it is possible that this association takes place during the process of spot formation. It is unlikely that the endogenous type II keratin is cotranslationally associated with the spots. In support of this, type I keratin can form extensive networks after injection into PtK<sub>2</sub> cells in the absence of protein synthesis (57). Therefore, the incorporation of exogenous subunits into the endogenous polymerized keratin IF network is a dynamic process (57) reflecting a surprisingly high rate of subunit mobility *in vivo*.

### **Models of Dynamic Equilibrium**

As indicated above, the rapid formation of aggregates containing both type I and II keratins suggests the availability *in vivo* of unpolymerized type II keratin. This pool could consist of type II protein available in the cytosolic compartment and/or it could be derived from polymerized IF as depicted in Fig. 9. This model is based on the existence of an equilibrium state *in vivo*. As a result injected type I keratin could pair with endogenous type II keratin, forming heterodimers. Once formed, dimers can subsequently re-enter the keratin polymer. As the concentration of biotinylated type I is increased, additional keratin subunits are drawn out of the polymer, resulting ultimately in a more significant shift in the equilibrium such that more type II subunits enter the "pool." Therefore, above a certain concentration of excess type I, the model would predict structural changes and disassembly of the endogenous keratin tonofilament network. In support of this latter contention, changes in the assembly state of the endogenous keratin IF network were seen when the highest concentrations of type I protein were injected and monitored by the immunofluorescence methods used in this study.

It must be kept in mind that homodimers have been shown to become incorporated into keratin IF to a limited extent *in vitro* (37, 74, 75). This possibility is also shown in the model depicted in Fig. 9. The alteration of the endogenous network may also be due in part to an incorporation into the network of an excess of such homodimers (37, 74). These could be formed either by biotinylated type I homodimers, biotinylated type I–endogenous type I keratin (heterologous homodimers), or endogenous type I homodimers which might be more likely to form if their type II partner has been sequestered by an excess of biotinylated type I keratin. The observation that the microinjection of lower concentrations of type I keratin seemed to incorporate faster than higher concentrations could also be explained by this model. Our results are consistent with the *in vitro* models of keratin assembly compiled by Coulombe and Fuchs (15).

Even at the highest concentrations of injected protein, the disrupted keratin network ultimately recovers its normal organization demonstrating that this type of alteration is not lethal to the cell. It is possible that the cell has restored the unbalanced equilibrium caused by microinjection, perhaps through new protein synthesis. It further suggests that the cell is capable of a continual remodeling of its keratin IF network in response to intracellular shifts in the concentrations of a specific type of keratin.

It is possible that alterations seen in the endogenous network using high concentrations may also be the result of titrating out not only the type II keratin partner from existing "pools," but may also result from altering the endogenous stoichiometric relationships among other factors important for keratin IF assembly. These might include keratin IF associated proteins, kinases, phosphatases, or other unknown elements involved in IF assembly (26, 69). Whatever the explanation, the alterations seen following the addition of only one keratin underscore the importance of maintaining correct stoichiometric relationships amongst all of the molecular constituents regulating the organization of IF networks. This concept can also be extended to the interpretation of stable or transient transfection results in which the levels of expression of the desired gene product have not been or cannot be adequately controlled or measured. In support of this, similar results have also been obtained in transient transfection experiments: the same mutant keratin construct transfected into PtK<sub>2</sub> cells caused more severe disruption of the keratin IF network than when expressed in SCC-13 cells which contain more total keratin (53).

Previous work has demonstrated that the products of single transfected keratin genes are rapidly degraded when expressed in fibroblasts, whereas the pair-wise expression of a type I and a type II keratin results in resistance to proteolysis. These observations suggested that specific proteolysis may represent a cellular mechanism for the maintenance of equimolar ratios of type I/type II keratin within the cell (21, 48, 49, 54). Consistent with these findings, we have found that type I keratin is no longer detectable by immunofluorescence 24 hours after microinjection into BHK-21 fibroblasts. The finding that type II colocalizes with the type I in the spot stage in epithelial cells immediately following injection may therefore represent an interesting correlate of the observations made in genetically engineered cells.

By EM, the spots formed immediately following microinjection into PtK<sub>2</sub> cells exhibit an electron-dense, granular appearance. They do not appear to contain protofilamentous structures seen in the type III IF protein aggregates which form following the breakdown of IF during the prometaphase–metaphase transition in BHK-21 fibroblasts (66) and the similar keratin-rich aggregates reported in some types of epithelial cells in mitosis (28). Frequently, the spots appear to associate with apparent branch points of the network by both light and electron microscopic criteria. Although the significance of this latter observation is not yet apparent, it is tempting to speculate that preferential incorporation may be occurring in these regions. However, the molecular mechanisms underlying the formation of these foci remains unknown.

The keratins exhibit an extensive array of posttranslational modifications, including phosphorylation (73), disulfide bonding (68, 76),  $\epsilon$ -( $\gamma$ -glutamyl)-lysine cross-links (76), and glycosylation (13). While the posttranslational modifications of acetylation and detyrosination of tubulin are believed to confer stability to microtubules (55), little is known concerning the role which such modifications play in regulating the dynamic properties of IF. Microinjection techniques now provide an avenue to test directly the role that these modifications play in the dynamics and assembly processes of keratin IF networks *in vivo*.

In summary, we present evidence consistent with the exis-

tence of a state of equilibrium between keratin subunits and polymerized keratin IF in epithelial cells. It appears that this equilibrium state provides a "soluble pool" of keratin which contributes to the initial steps in the incorporation of microinjected keratin subunits into polymerized IF. The speed at which the initial steps in this assembly process takes place is remarkably rapid and provides further proof that IF are dynamic elements of the cytoskeleton of mammalian cells.

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