

The Expression of Mutant Epidermal Keratin cDNAs Transfected in Simple Epithelial and Squamous Cell Carcinoma Lines

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Abstract. We have deleted cDNA sequences encoding portions of the carboxy-terminal end of a human type I epidermal keratin K14, and examined the molecular consequences of forcing the expression of these mutants in simple epithelial and squamous cell carcinoma lines. To follow the expression of our mutant products in transfected cells, we have tagged the 3' end of the K14 coding sequence with a sequence encoding an antigenic domain of the neuropeptide substance P. Using DNA transfection and immunohistochemistry (with an antibody against substance P), we have identified a collection of mutants that have a wide range of morphological effects on the endogenous keratin filament

networks of transfected cells. Mutants that are missing most of the nonhelical carboxy-terminal domain of K14 incorporate into the endogenous keratin filaments without any visible perturbations on the network. In contrast, mutants that are missing as few as 10 of the 310 amino acids of the central α -helical domain of the polypeptide cause gross alterations in the keratin network. In some cases, the entire cytoskeletal network of keratins was disrupted, leaving no evidence of 8-nm filaments. These results reveal the existence of a dynamic exchange between newly synthesized subunits and preexisting keratin filaments.

INTERMEDIATE filament (IF)¹ proteins are expressed in virtually all mammalian cells. Based on their tissue-specific expression, they can be subdivided into five distinct groups: vimentin (expressed in cells of mesenchymal origin), glial fibrillary acidic protein (glial cells), desmin (muscle cells), neurofilament proteins (neural cells), and keratins (epithelial cells) (for review, see Geisler and Weber, 1982; Steinert et al., 1985). The lamins form a sixth class of IFs, but unlike the others they are universally expressed (for review, see Franke, 1987). Both in vivo and in vitro, all IF proteins have the capacity to assemble into 8–10 nm filaments in the apparent absence of any auxiliary proteins or factors. Whereas the five classes of tissue-specific IFs form extensive fibrous networks in the cytoplasm, the lamins form a lattice of filaments on the inner side of the nuclear membrane.

Of all the IF proteins, the keratins are by far the most complex and diverse group. There are >20 keratins (40–70 kD) that are differentially expressed in different epithelia and at various stages of differentiation and development (Fuchs and Green, 1980; Moll et al., 1982; Wu et al., 1982; Tseng et al., 1982). As such, they seem to be tailored to suit the specialized structural needs of each higher eukaryotic epithelial cell. Keratins can be subdivided into two distinct classes: Type I keratins are acidic ($pK_i = 4.5$ – 5.5) and small (mol wt 40–56.5K), whereas type II keratins are basic ($pK_i = 6.5$ – 7.5) and generally larger (mol wt 53–67K) (Fuchs et al.,

1981; Schiller et al., 1982; Kim et al., 1983; Magin et al., 1983; Sun et al., 1984). Type I and type II keratins are frequently expressed as specific pairs, and at any one time, an epithelial cell will typically express one to three pairs of keratins (Eichner et al., 1984).

Like all IF proteins, type I and type II keratins have a central 310 amino acid residue domain consisting of four segments which are predicted to be largely α -helical (Hanukoglu and Fuchs, 1982; 1983; Crewther et al., 1983; Jorcano et al., 1984a, b; Steinert et al., 1983; 1984). The classification of keratins according to type is based on these sequences. Within the type I or type II keratin family, the helical regions of different keratins share 50–99% sequence identity; keratins of opposite type share only 25–35% homology in these domains. Across type lines, much of the homology resides at the end of the fourth helical segment, where a highly conserved sequence T Y R R L L E G E is found in nearly all intermediate filament proteins.

The basic subunit structure of all IFs seems to be a dimer which is formed through a coiled-coil interaction between the helical domains of two polypeptide chains. The core of the coiled-coil is stabilized by hydrophobic interactions, hence giving rise to a periodicity of hydrophobic residues in the sequence within this region (McLachlan, 1978). In addition, a conservation and periodicity in positively and negatively charged residues can be seen, indicating that electrostatic interactions may also play an important role in stabilizing the coiled-coil structure (Parry et al., 1977; McLachlan and Stewart, 1982).

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

Early *in vitro* assembly studies indicated that while some IF subunits (e.g., vimentin) can form homopolymers (Steinert et al., 1981; Geisler and Weber, 1981; Geisler et al., 1982), no keratin by itself is competent to form filaments (Steinert et al., 1976; Lee and Baden, 1976). However, when almost any two random type I and type II epithelial keratins are isolated and recombined *in vitro*, 8-nm filaments can form readily even across species lines (Hatzfeld and Franke, 1985). Since type I keratins are generally acidic, while type II keratins are more basic, it seems reasonable that the coiled-coil dimer of the keratin filament is composed of a type I and a type II keratin, held together via hydrophobic and ionic interactions. Some biochemical studies have supported this notion (Woods and Inglis, 1984; Parry et al., 1985). However, because stable tetramers form readily in solution (Quinlan et al., 1984; Soellner et al., 1985), the composition of the dimer has been difficult to determine. Thus, although the ratio of type I and type II keratins in the tetramer is 1:1, it has not yet been resolved unequivocally whether two coiled-coil homodimers or two coiled-coil heterodimers come together to form a stable tetrameric subunit.

The two polypeptide chains of the dimer are aligned in register, and in a parallel fashion, with the two amino termini at one end and the two carboxy termini at the other. The two dimers in the tetramer seem to be arranged in an antiparallel manner, but whether they are in register or staggered is still a controversial issue (Parry et al., 1985; Geisler et al., 1985). Once formed, tetramers assemble into a hierarchy of higher ordered structures: an undetermined number of protofilaments (2–3 nm) assemble into protofibrils (4.5 nm), and four protofibrils intertwine to form the resulting 8-nm filament (Aebi et al., 1983; Steven et al., 1983; Ip et al., 1985; Eichner et al., 1986). It is not known whether newly synthesized tetramers can freely exchange with tetramers in preexisting keratin filaments, or whether the filaments, once formed, are static.

While it seems clear that the helical domains of keratins are involved in the formation of the coiled-coil, the role of the nonhelical amino- and carboxy-terminal sequences in filament assembly has not been elucidated. These nonhelical termini are hypervariable in size and can be subdivided into at least three sequence groups: (a) the type I and type II keratins of epidermal cells have end domains that are unusually abundant in glycine and serine rich sequences (Hanukoglu and Fuchs, 1982; 1983; Steinert et al., 1983; 1984; Jorcano et al., 1984a; b); (b) wool and possibly hair keratins have terminal segments that contain a number of cysteine and proline residues, with very few glycines (Crewther et al., 1983; Dowling et al., 1986); and (c) simple epithelial keratins have termini that are not rich in any of these amino acids (Glass et al., 1985; Singer et al., 1986; Magin et al., 1986; Romano et al., 1986; Bader et al., 1986). In addition, the carboxy termini of simple epithelial keratins seem to be unusually short. If these end domains are involved in filament formation, then the differences in their sequences may influence the properties of the resulting filaments, as well as interfilament interactions and associations with other proteins and organelles.

To determine more precisely the role that the nonhelical and helical sequences play in filament assembly, and to examine the dynamics of keratin filaments *in vivo*, we have constructed a series of deletion mutations at the carboxy-terminal end of a cDNA encoding the complete human epi-

dermal type I keratin K14. By inserting the mutant cDNAs 3' to an SV-40 promoter and enhancer, we could drive their expression in transfected simple epithelial cells and in an epidermal cell line derived from a squamous cell carcinoma of the skin. Using immunoblot analyses, we have monitored the expression of the transfected products in these cells. Using immunofluorescence microscopy, we have assessed the effects of deleting carboxy-terminal sequences of K14 on its ability to incorporate into the endogenous keratin filament networks of the transfected cell types.

Materials and Methods

Cell Culture

PtK2 cells were maintained in a 3:1 mixture of DME and Ham's F12 medium supplemented with 10% FBS. SCC 13 cells were obtained from Dr. James Rheinwald (Dana Farber Cancer Center, Harvard Medical School, Boston, MA), and were cultivated as described previously (Wu and Rheinwald, 1981).

Construction of Plasmid pJK14-P

(a) *Construction of a Fragment Containing the 3' Portion of the K14 Coding Sequence Linked to Substance P.* Clone pKB-2 (Hanukoglu and Fuchs, 1982) was digested with *Ava* II restriction endonuclease, and the ends were filled in using DNA polymerase I (Klenow). A 570-bp fragment containing the 3' coding sequence of K14 cDNA was isolated and purified. This fragment was missing sequence coding for the last five carboxy amino acids of the K14 protein (see Fig. 1). Using T4 DNA ligase, the fragment was linked 5' to a 1.6-kb *Hinc* II fragment of plasmid pAHP.2 (Munro and Pelham, 1984), which contained the sequence encoding the neuropeptide substance P, followed by a TGA translation stop codon and the 3' noncoding sequence of a *Drosophila hsp70* gene (Fig. 1 B). The joining recreated the *Hinc* II site, which is marked (*H*) in Fig. 1 A. This ligated fragment was then cleaved at a unique *Sac* I site within the K14 sequence (see Fig. 1 A), and the 3' subfragment (containing the 3' portion of the K14 cDNA, substance P, and the 3' noncoding segment of the *Drosophila hsp70* gene) was purified for further use below. This fragment had a 5' *Sac* I end and a flush 3' end.

(b) *Construction of a Fragment Containing the 5' Portion of the K14 Coding Sequence.* A 500-bp *Ava* I/*Kpn* I fragment containing the transcription initiation site of the K14 gene (GK-1) and part of exon I (Marchuk et al., 1984) was ligated to a 990-bp *Kpn* I/*Stu* I fragment containing the remaining coding portion and the 3' noncoding portion of the K14 cDNA (KB-2). The ends of the fragment were filled in with Klenow, and the cDNA was inserted into the *Hinc* II site of plasmid pSP64 (Giudice, G. J., and E. Fuchs, unpublished results). This hybrid plasmid was first cleaved at its unique *Eco* RI site, 3' from the cDNA, and the ends were filled in with Klenow. The plasmid was then cleaved at the *Sac* I site within the cDNA. The fragment containing the pSP64 vector linked 5' to the K14 cDNA (extending from the transcription initiation site of the K14 gene and including the coding sequence of K14 up to the unique *Sac* I site) was isolated and purified for further use. This fragment had a flush 5' end resulting from the residual *Eco* RI site in pSP64 and a *Sac* I 3' end.

Ligation of (a) and (b) Followed by Their Insertion into Plasmid pJ1. The fragments generated in (a) and (b) above were ligated to create a plasmid containing the complete K14 coding sequence (minus five carboxy-terminal amino acid residues) coupled to a sequence encoding 15 amino acid residues encompassing substance P. These sequences were excised from this plasmid using *Hind* III (in the pSP multiple cloning region) and *Sfi* I (in the pAHP.2 sequences 3' from the *hsp70* polyadenylation signal). The fragment was then subcloned into the plasmid pJ1 (Land et al., 1986) at the *Hind* III/*Sma* I sites to produce pJK14-P. The completed construct is shown in Fig. 1 A.

Construction of Deletion Mutants

pJK14-P DNA was cleaved at the unique *Sal* I site located just 5' to the substance P sequence (S; see Fig. 1 A). *Bal* 31 exonuclease was used to generate various length deletions in the cDNA sequence. These fragments were treated with mung bean exonuclease to create blunt-ended DNA. The fragments were then cut at the unique *Kpn* I site in the 5' end of the cDNA (see Fig. 1), and the *Kpn* I-blunt-ended fragments were gel purified on Seaplaque agarose

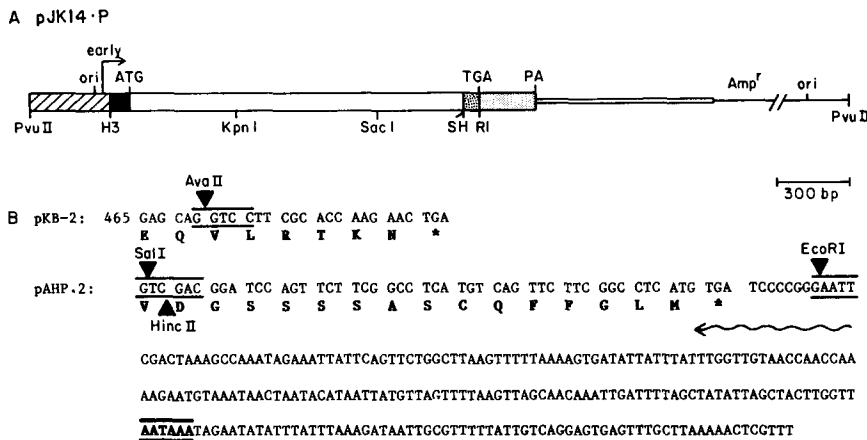


Figure 1. Genetic map of plasmid pJK14·P. (A) The construction of the plasmid pJK14·P is described in Materials and Methods. The components of the plasmid are represented as follows: (Hatched bars) The SV-40 early promoter (and origin of replication) and enhancer sequences that were used to drive the expression of the constructed cDNA. The transcription initiation site is marked by the arrow. (Black box) The 5' untranslated leader sequence of the K14 mRNA. Also in this region, upstream from the K14 sequence, is the Hind III/Hinc II polylinker portion of plasmid pSP64 (Promega Biotec, Madison, WI), which was included as a consequence of subcloning. (White box) The human keratin K14 cDNA. This seg-

ment is missing the sequence encoding the five carboxy-terminal amino acid residues and the TGA stop codon. The Hinc II-Sfi I fragment of plasmid pAHP.2 (Munro and Pelham, 1984) includes the sequences encoding the carboxy-terminal domain of neuropeptide substance P, (heavily stippled box) followed by a TGA stop codon, and the 3' noncoding sequence and polyadenylation signal (PA) of the *Drosophila hsp70* gene (lightly stippled box). (ori) The origin of replication for plasmid pBR322 is indicated as is the ampicillin-resistance gene contained in plasmid pJ1. Important restriction endonuclease sites are marked. H3, Hind III; S, Sal I; H, Hinc II; RI, Eco RI. (B) The relevant nucleotide sequences of the coding strands for plasmids pKB-2 and pAHP.2 are shown so that the reader can examine the junctions between the K14 and substance P sequences and the substance P and *hsp70* sequences. (Squiggled line) The binding site of the primer used for DNA sequencing.

(FMC Corporation, Philadelphia, PA). The fragments encoding various portions of the carboxy end of K14 were then ligated back into pJK14·P at the Kpn I/Sal I site (after filling in the Sal I site with Klenow fragment). Ligated DNA was transformed into bacterial strains MC1061 and DH1.

DNA Sequencing

To determine which Bal 31 deletion clones were in the proper reading frame for substance P expression, plasmid DNA from each deletion clone was isolated and sequenced using the double-stranded sequencing protocol described by Chen and Seeberg (1985).

DNA Transfections

DNA was transfected into PtK2 cells using the calcium phosphate precipitation method (Graham and Van der Eb, 1973) followed by a 15% glycerol shock (Parker and Stark, 1979). SCC 13 cells were transfected by adding plasmid DNA in 150 µg/ml DEAE dextran (Pharmacia, Inc., Piscataway, NJ) and 10 µM chloroquine for 3 h followed by a 10% DMSO shock. Cells were fixed 65 h posttransfection unless otherwise indicated in the text.

Immunofluorescent Labeling

For immunohistochemistry, cells were grown on glass chamber slides (Lab Tek, Miles Scientific Division, Miles Laboratories, Inc., Naperville, IL). After transfection, cells were washed with PBS, fixed in methanol (-20°C) for 10 min, and washed in PBS. For actin labeling, cells were fixed in 3.7% formalin for 10 min at room temperature and then rinsed with PBS.

To detect K14-substance P-tagged proteins, we used the anti-P rat monoclonal antibody NCI/34 (Cuello et al., 1979). As a necessary prerequisite for NCI/34 antibody recognition, protein carboxyl groups were chemically converted to amides, as described by Munro and Pelham (1984).

The mouse monoclonal antibodies LE-41 and LE-61 (Lane, 1982) were used to identify the endogenous PtK2 keratin network. Keratin K14 was detected using a polyclonal rabbit antibody isolated in this laboratory (Fuchs and Marchuk, 1983). Vimentin networks were identified using a rabbit polyclonal antisera made against hamster vimentin (Hynes and Destree, 1978). Tubulin networks were stained using a rabbit polyclonal antibody (Gundersen et al., 1984), and actin was detected using rhodamine-conjugated phalloidin (Molecular Bioprobes, Eugene, OR).

To visualize the primary antibody, we used the following fluorescently labeled secondary antibodies: FITC-conjugated goat anti-rat IgG (Cappel Laboratories, Inc., Cochranville, PA; and Tago, Burlingame, CA). Texas Red-conjugated goat anti-rabbit IgG (Cappel Laboratories, Inc.), and Texas Red-conjugated goat anti-mouse IgG (Tago).

Immunoblot Analysis

Intermediate filament proteins were isolated from transfected PtK2 cells using the procedure of Wu et al. (1982). Proteins were solubilized in 8 M urea and 10% beta-mercaptoethanol, and separated by SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose paper (Towbin et al., 1979). As with the immunolabeling of cells with the substance P antibody, it was necessary to chemically modify the protein bound to the nitrocellulose. After modification, bound proteins were reacted with the substance P antibody, washed in PBS, and then reacted with rabbit anti-rat IgG (Miles-Yeda, Inc., Elkhart, IN) to increase the binding of ¹²⁵I-labeled *Staphylococcus aureus* protein A. The radiolabeled antibody-protein complexes were visualized by autoradiography.

Results

Construction of a Hybrid K14-Substance P cDNA Driven by the SV-40 Early Promoter and Enhancer

Previously, clones containing 93% of the human K14 cDNA (KB-2, Hanukoglu and Fuchs, 1982) and 100% of the human K14 gene sequence (GK-1, Marchuk et al., 1984; 1985) were isolated and sequenced. Since the coding sequence of the cDNA corresponded perfectly with the corresponding portion of the gene, we were able to construct a complete K14 cDNA from the two clones. The details of the construction can be obtained in Materials and Methods. The complete construct is shown in Fig. 1.

There were two major considerations in designing our vector for deletion mutation analyses: (a) We wanted to make certain that the sequences encoding the antigenic determinant of the protein were not lost during the construction of deletion mutants; (b) we wanted to be able to use immunofluorescence to follow the expression of our mutant K14 constructs even when they were transfected into K14-expressing epidermal cells. To accomplish these goals we replaced the sequences encoding the last five amino acid residues of the human epidermal keratin K14 with a small (15 amino acid residue) neuropeptide sequence encoding the carboxy-ter-

minimal end of substance P (Munro and Pelham, 1984). This sequence was retained in all of our deletion analyses (see below). The hybrid K14-substance P sequences were cloned into plasmid pJ1 (Land et al., 1986), immediately 3' to the SV-40 early promoter and enhancer sequence. This hybrid plasmid will be referred to as pJK14·P.

Transfection of PtK2 Cells with pJK14·P

The kidney epithelial cell line PtK2, from kangaroo rat, was chosen for our initial transfection studies. These simple epithelial cells express only two keratins: the type I keratin K18

and the type II keratin K8 (Lane, 1982). Although K14 is not normally coexpressed with K8 in vivo, K8 can utilize K14 as a promiscuous partner for filament assembly in vitro (Hatzfeld and Franke, 1985). In addition, PtK2 cells transfected with the human epidermal keratin gene K14 can readily incorporate K14 into the endogenous keratin filament network (Giudice and Fuchs, 1987).

To make certain that the presence of the neuropeptide sequence in K14·P did not interfere with the ability of the keratin to assemble into filaments, we transfected pJK14·P into PtK2 cells and examined the keratin network 65 h posttransfection. A monoclonal antibody against substance P was

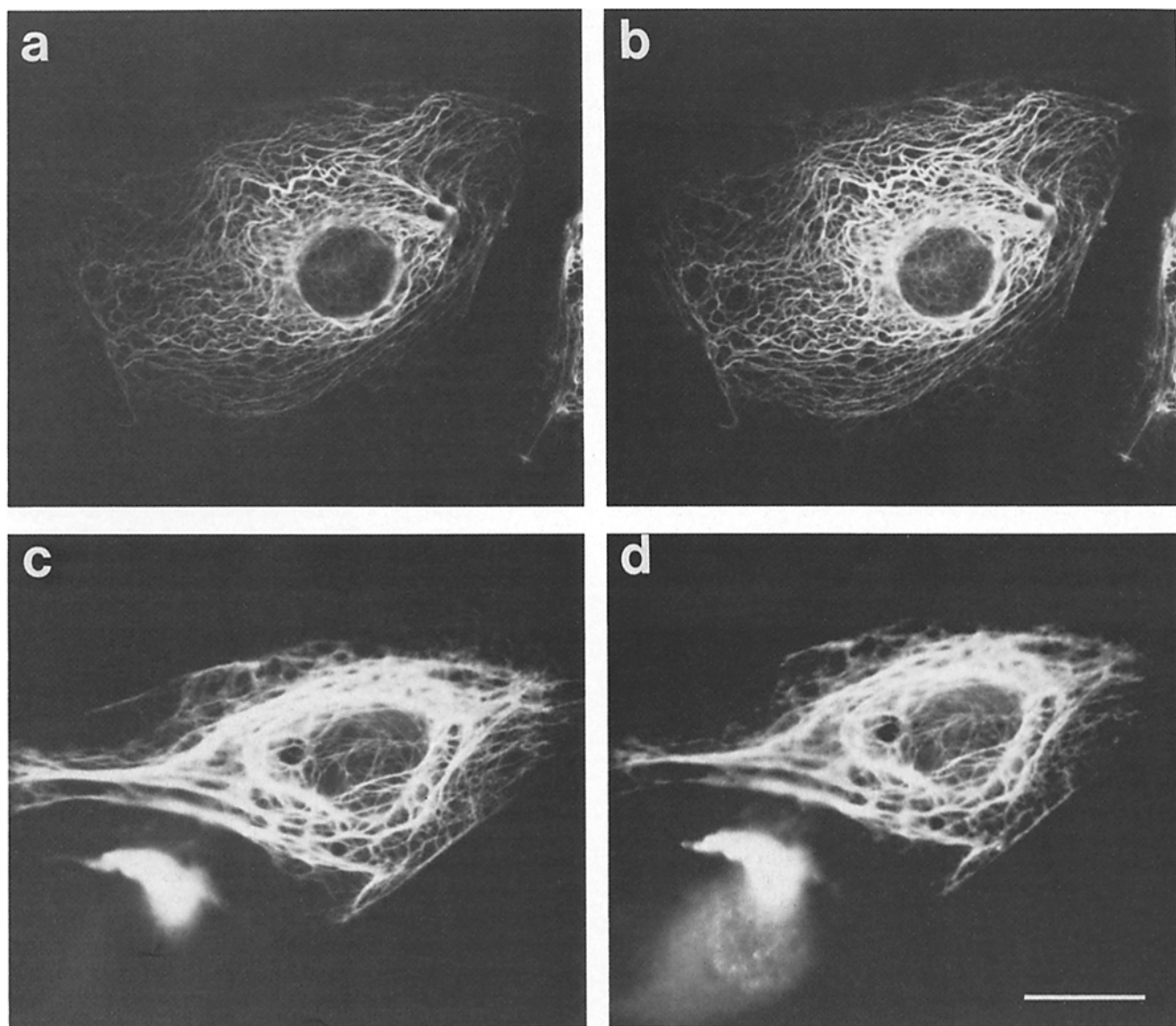


Figure 2. K14·P readily incorporates into the endogenous keratin network of transfected PtK2 cells. PtK2 cells were transfected with plasmid pJK14·P, and the fate of the transfected gene product was examined by double-label immunofluorescence. To visualize the transfected gene product, cells were stained with a rat monoclonal antibody (NCl/34; referred to as anti-P here) recognizing the carboxy-terminal sequence of substance P (a and c). Antibody staining was followed by fluorescein-conjugated goat anti-rat IgG. The endogenous simple epithelial keratin network was detected using a mouse monoclonal antibody (LE41) to identify keratin K8, followed by Texas Red-conjugated goat anti-mouse IgG (b). Both cells in this field were transfected, and thus, express the K14·P protein. To demonstrate that K14·P could be identified specifically with an antiserum specific for epidermal keratins, the double immunofluorescence was repeated, this time using an anti-type I epidermal keratin antiserum, followed by Texas Red-conjugated goat anti-rabbit IgG (d), and anti-P as described above (c). Bar, 20 μ m.

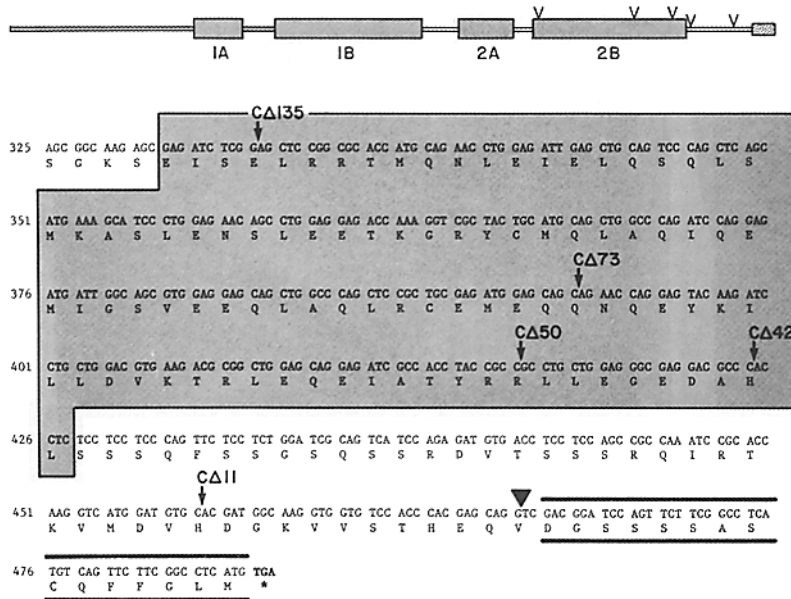


Figure 3. Deletion mutants of pJK14·P missing sequences coding for the carboxy-terminal end of K14. The stick diagram shows the location of the four helical domains (gray boxes; IA, IB, 2A, and 2B) of the K14 keratin and (stippled box) the substance P tag. (V) The sites of the deletions used in our analyses. Beneath the stick diagram is the sequence of the carboxy-terminal region of K14·P. The fourth α -helical domain (2B) is boxed in gray; the substance P tag is identified by the bars above and below the sequence. The exact points of the deletions are identified by arrows, and the names of the deletions are written above each arrow. Each deletion extends from the arrow to the solid triangle immediately preceding the substance P sequence. Construct nomenclature: C, a carboxy-terminal deletion; ΔX , the number of amino acid residues that are missing from K14·P as a result of the deletion.

used to detect the presence of K14·P by immunofluorescence. Monoclonal antibodies against K8 (LE41) and K18 (LE61) were used to identify the endogenous keratin filament network (Lane, 1982). Neither LE41 or LE61 cross-reacted with K14.

Fig. 2a shows that K14·P readily incorporated into the endogenous keratin filament network of the PtK2 cell. Anti-K8 (Fig. 2b) and anti-K18 (not shown) showed complete colocalization with anti-P, indicating that only a single heterogeneous keratin filament network comprised of all three keratins existed in the transfected cells. Fig. 2, c and d demonstrate that K14·P could be identified specifically with either anti-P or with a polyclonal antiserum made against gel-purified human K14. Thus, by all criteria, the construct pJK14·P seemed to be suitable for our proposed mutagenesis experiments.

Construction of Mutant K14-Substance P cDNAs

Plasmid pJK14·P has a unique Sal I site at the junction of the K14 and substance P sequences. It also has a unique Kpn I site within the coding portion of K14. These sites enabled us to generate deletion mutants in the K14 cDNA by: (a) linearizing the plasmid pJK14·P with Sal I; (b) treating the linearized plasmid with the exonuclease Bal 31 for increasing intervals of time; (c) excising the series of K14 deletion fragments with the enzyme Kpn I; and (d) ligating the Kpn I-Bal 31 deletion fragments to the large Kpn I-Sal I fragment of plasmid pJK14·P.

For our transfection studies, the only deletion mutants used were those where the K14 and substance P sequences were both in the same reading frame. To determine the precise location of each deletion, and to select those mutants in the correct reading frame, we prepared a DNA oligonucleotide just 3' from the substance P sequence, and used this as a primer to determine the sequence at the K14-substance P junction of each construct. The deletion constructs which were chosen for study are shown in Fig. 3. These constructs will be referred to as C ΔX pJK14·P, where X represents the

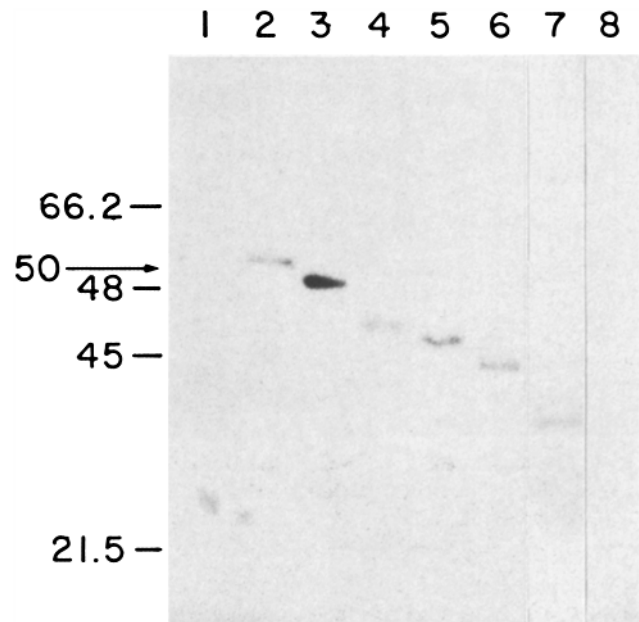


Figure 4. Immunoblot analysis of the mutant keratins expressed in C ΔX pJK14·P transfected PtK2 cells. Intermediate filaments were isolated from C ΔX pJK14·P transfected PtK2 cells. Filaments were dissociated in 8 M urea and 10% β -mercaptoethanol, and the proteins were resolved by SDS-PAGE. Proteins were transferred electrophoretically to nitrocellulose paper, and the paper was exposed to anti-P antibodies, followed by ^{125}I -labeled *S. aureus* protein A (Towbin et al., 1979). Radiolabeled bands containing antibody-protein complexes were visualized by autoradiography. IF extracts were isolated from cells transfected with: lane 1, no DNA; lane 2, pJK14·P; lane 3, C $\Delta 11$ pJK14·P; lane 4, C $\Delta 42$ pJK14·P; lane 5, C $\Delta 50$ pJK14·P; lane 6, C $\Delta 73$ pJK14·P; lane 7, C $\Delta 135$ pJK14·P. The sample in lane 8 is a keratin extract from cultured human epidermal cells. The arrow at the left identifies the migration of the epidermal keratin K14 (as judged by staining with Coomassie Blue). None of the epidermal keratins showed cross-reactivity with anti-P. Molecular mass markers are shown in kilodaltons at the left.

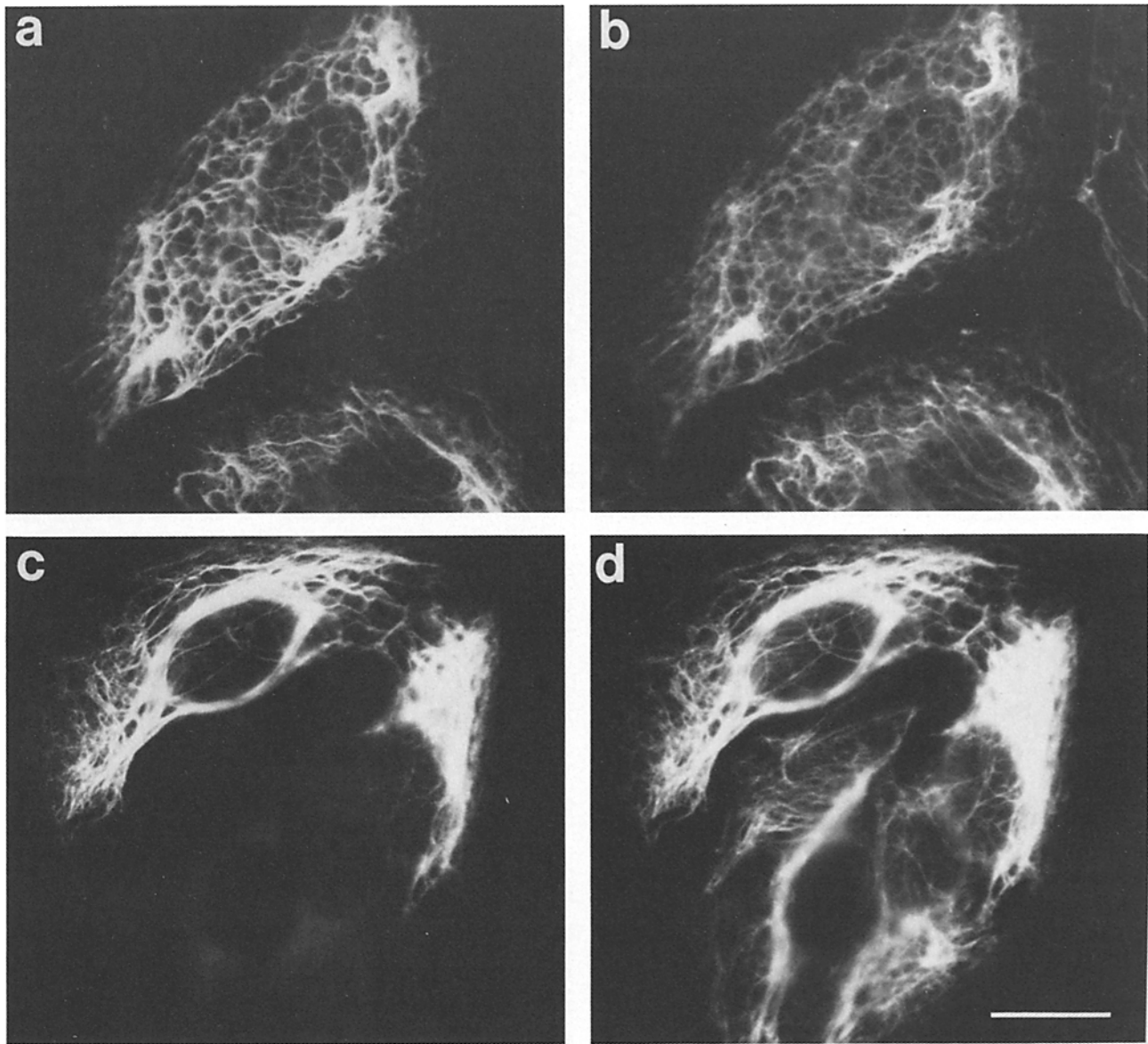


Figure 5. The removal of the nonhelical carboxy terminus of K14 does not interfere with its ability to incorporate into the PtK2 keratin network. PtK2 cells were transfected with C Δ 11 pJK14·P (*a* and *b*) or C Δ 42 pJK14·P (*c* and *d*). The transfected cells were identified by immunofluorescence using anti-P, followed by fluorescein-conjugated goat anti-rat IgG (*a* and *c*). The endogenous keratin network was visualized by staining with LE41, followed by Texas Red-conjugated goat anti-mouse IgG (*b* and *d*). Bar, 20 μ m.

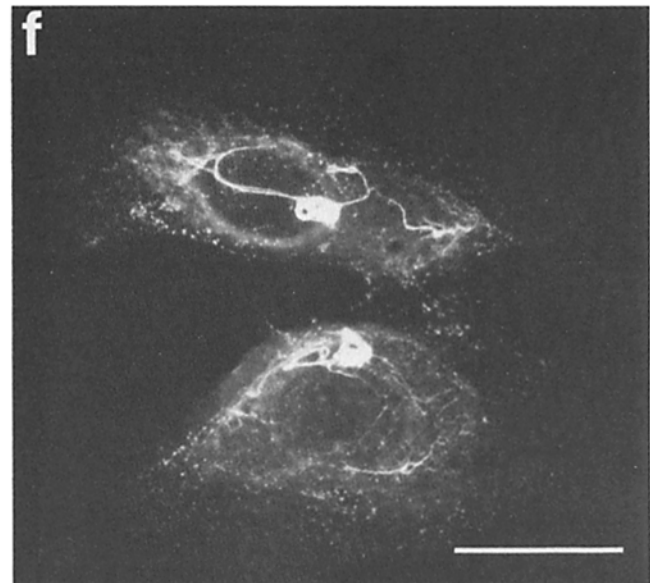
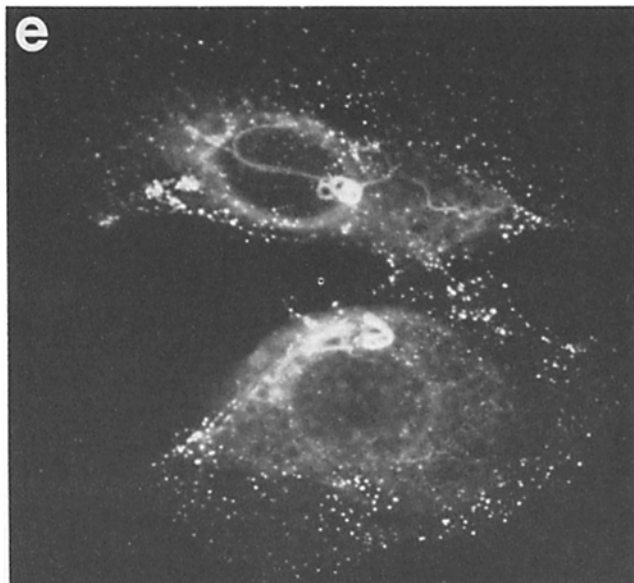
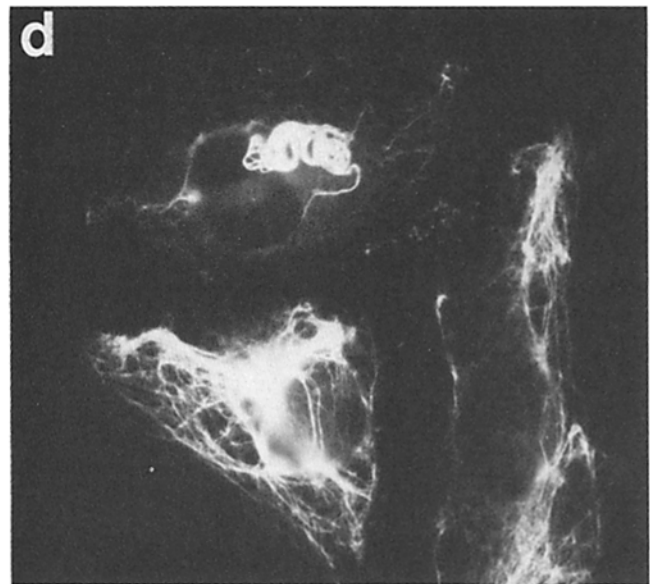
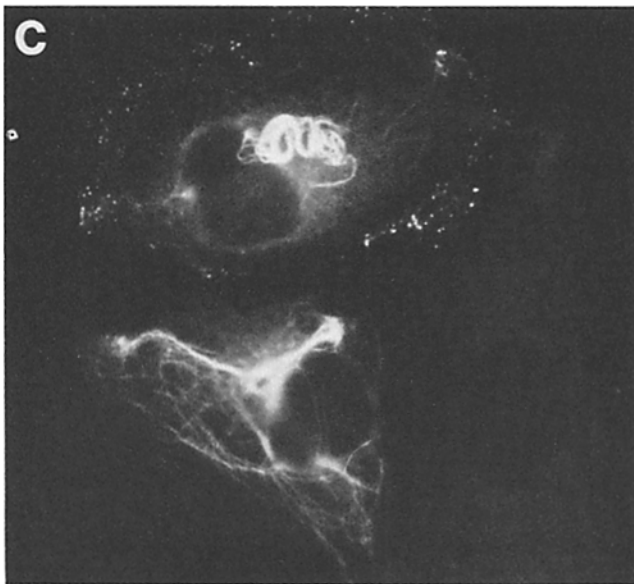
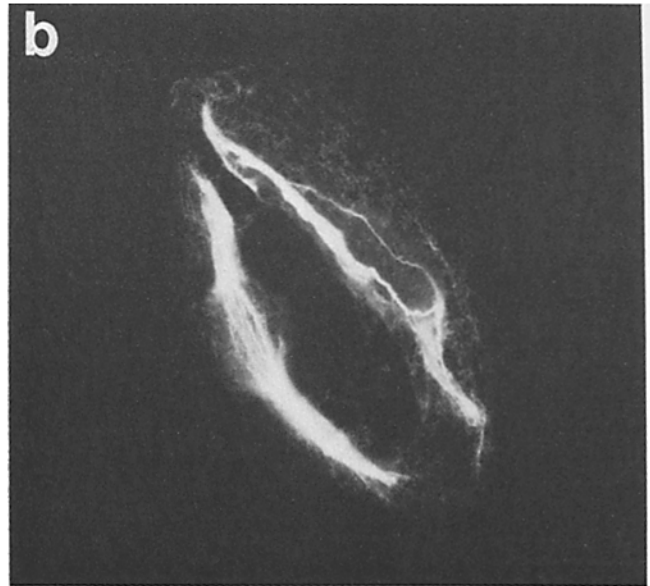
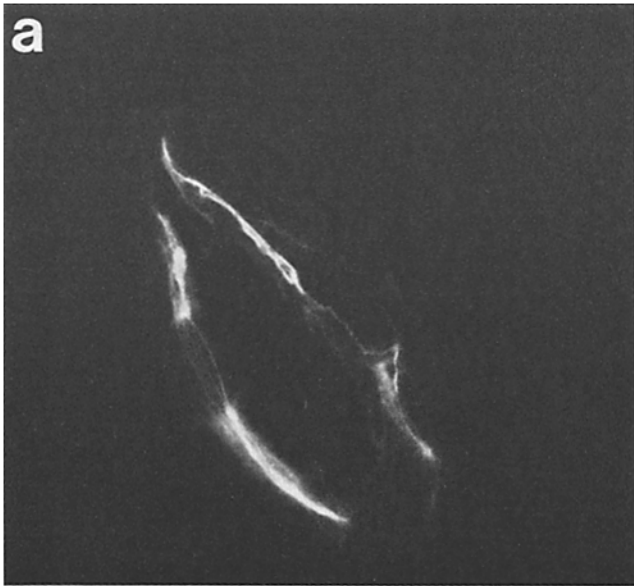
number of amino acid residues removed from the carboxy terminus of the encoded K14 as a consequence of the deletion in the cDNA.

Mutant Keratin Proteins Are Expressed when Deletion Constructs of pJK14·P Are Transfected into PtK2 Cells

Deletion constructs of pJK14·P ranging from C Δ 11 to C Δ 135

were transfected into PtK2 cells, and keratins were extracted 65 h posttransfection. When the isolated keratins were resolved by SDS-PAGE and subjected to immunoblot analyses using an antibody specific for substance P (Fig. 4), a single major discrete protein band was detected in each extract from transfected PtK2 cells. The K14·P band (Fig. 4, lane 2) migrated at slightly slower electrophoretic mobility than

Figure 6. The removal of 10 amino acid residues at the carboxy end of the fourth α -helical domain causes a marked defect in the ability of K14·P to incorporate into the PtK2 keratin network. PtK2 cells were transfected with C Δ 50 pJK14·P and examined 65 h posttransfection. The mutant keratin was detected by staining with anti-P, followed by fluorescein-conjugated goat anti-rat IgG (*a*, *c*, and *e*). The endogenous keratin network of each frame was visualized by staining with LE41, followed by Texas Red-conjugated goat anti-mouse IgG (*b*, *d*, and *f*). At early times (14–30 h) after transfection, most cells showed a morphology similar to that seen in *a* and *b*. By 65 h when the expression of the transfected keratin seemed to be optimal, \sim 85% of the transfected cells showed a collapsed coil network (*c*–*f*). In transfected cells showing either of these distinct keratin networks, spheroid bodies containing mutant and endogenous keratins could often be seen (*c*–*f*). Bar, 20 μ m.



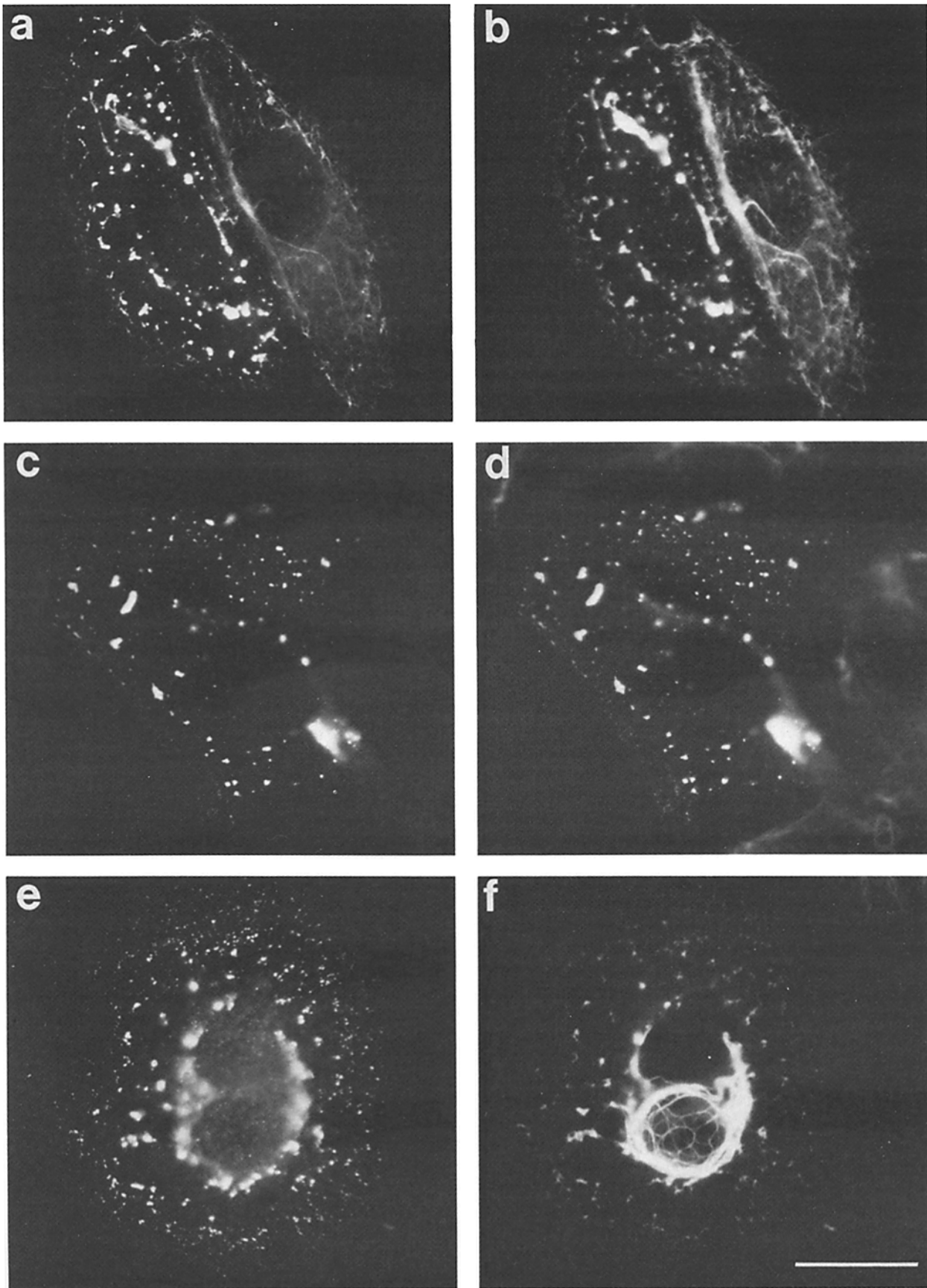


Figure 7. Deletions that progressively lack greater amounts of the α -helical domain of K14·P show more severe perturbations of the endogenous keratin network. PtK2 cells were transfected with either C Δ 73 pJK14·P (*a* and *b*) or C Δ 135 pJK14·P (*c*–*f*) and examined by immunofluorescence. Cells were stained with both anti-P (*a*, *c*, and *e*) and LE41 (*b*, *d*, and *f*) as described in the legend to Fig. 2. (*a*–*d*) Cells examined at 65 h posttransfection; (*e* and *f*) cells examined at 92 h posttransfection. Note that LE41 and anti-K8 staining do not colocalize in the 92-h cells (*e* and *f*). Bar, 20 μ m.

authentic epidermal K14 (arrow marks the migration of human K14 which did not cross react with anti-P). For each progressively larger deletion in K14-P, the detected protein migrated with increasing electrophoretic mobility (Fig. 4, lanes 3-7). For all deletion constructs, the transfected cDNA product appeared to be stable, and its size was comparable to that predicted on the basis of sequence analysis.

The bands detected by immunoblot analyses were not detectable by silver staining. This was expected since all of our experiments involved transient transfections, and 95% of the IF extract represented protein from untransfected cells. Moreover, because the number of plasmid DNAs taken up by each transfected cell was variable, it was impossible to determine the ratio of mutant to endogenous keratin in the transfected cells.

K14-P Mutants Missing Various Portions of the Nonhelical Carboxy-terminal Domain Incorporate Readily into the Keratin Network of PtK2 Cells

To determine whether the removal of a portion of the nonhelical carboxy-terminal domain of K14-P can interfere with its ability to integrate into a keratin filament network, we transfected PtK2 cells with C Δ 11 pJK14-P. Fig. 5, *a* and *b* shows clearly that this mutant, missing 11 additional amino acid residues of K14, incorporated into the PtK2 keratin network without disturbing its morphology. Bundles of keratin filaments containing K8, K18, and C Δ 11 K14-P extended throughout the cytoplasm of each transfected PtK2 cell. Since single keratin filaments were not visualized in these frames, we could not determine whether the incorporation of subunits was restricted to newly synthesized filaments.

When PtK2 cells were transfected with C Δ 42 pJK14-P, the expressed mutant hybrid protein again colocalized with the endogenous keratin network (Fig. 5, *c* and *d*). The mutant K14-P, this time missing 42 additional amino acid residues of K14, incorporated into the PtK2 keratin network without any apparent alterations in morphology. This was striking, because this mutant left just two K14 amino acid residues (plus 15 amino acids from the neuropeptide sequence) after the highly conserved sequence T Y R R L L E G E at the end of the fourth helical domain. Thus, even though the entire nonhelical carboxy-terminal domain of K14 was missing in this construct, it still seemed competent to assemble into keratin filaments.

Mutant K14-P Keratins Missing Portions of the Fourth Helical Domain Disrupt the Keratin Filament Network of PtK2 Cells

In contrast to the behavior of mutants lacking portions of the K14 nonhelical carboxy-terminal domain, mutants missing portions of the α -helical segment caused a marked disruption in the keratin filament network of the PtK2 cells. This could be seen even with a mutant C Δ 50 pJK14-P, missing only the R L L E G E residues at the extreme end of the fourth helical domain (Fig. 6). In 85% of the transfected cells, the mutant keratins incorporated into PtK2 keratin filaments, but in so doing they caused a collapse of the entire keratin filament network. At early times ($t = 18-30$ h) after transfection, the keratin network appeared as a single straightened cable of collapsed filaments (Fig. 6 *a*). Small branches of filaments could often be seen protruding from this macrofibrillar structure. Regardless of the architecture

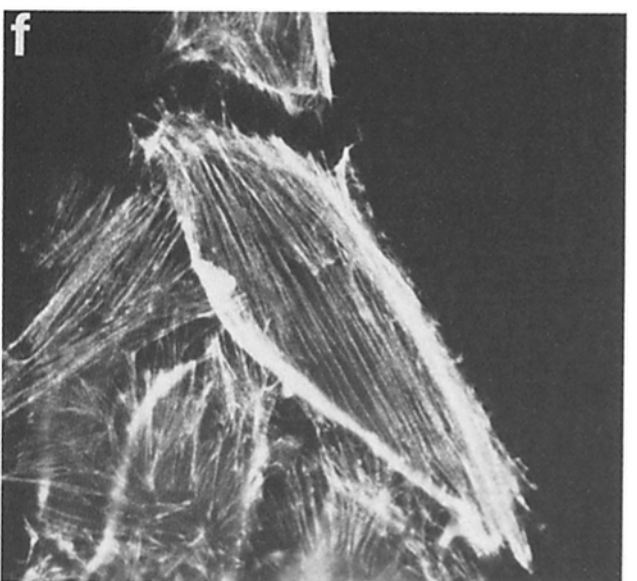
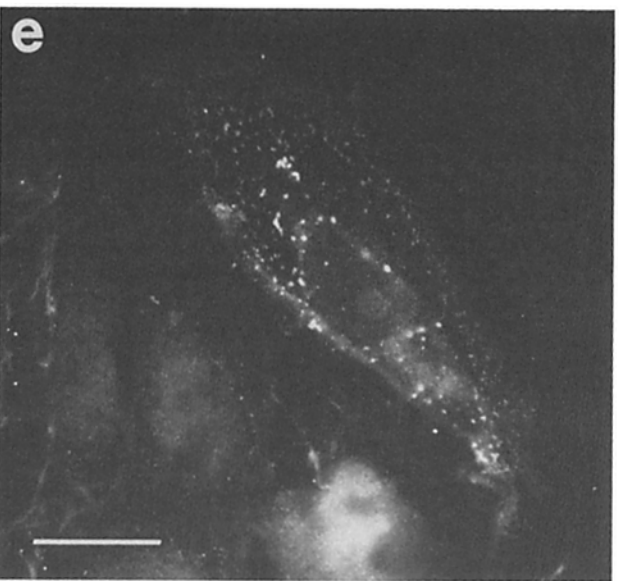
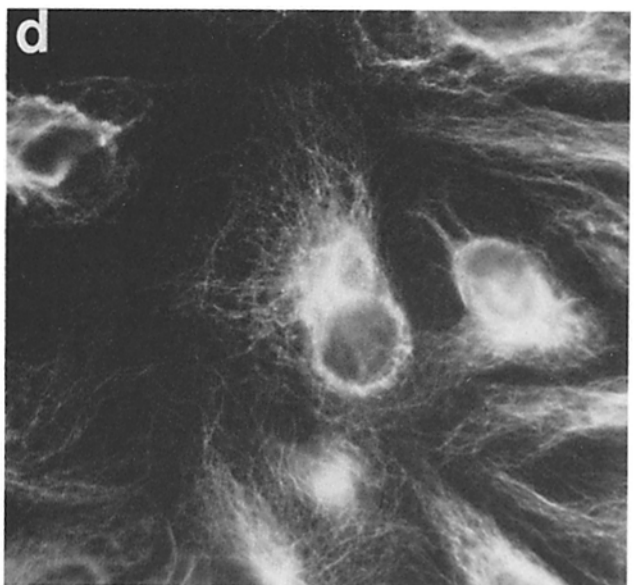
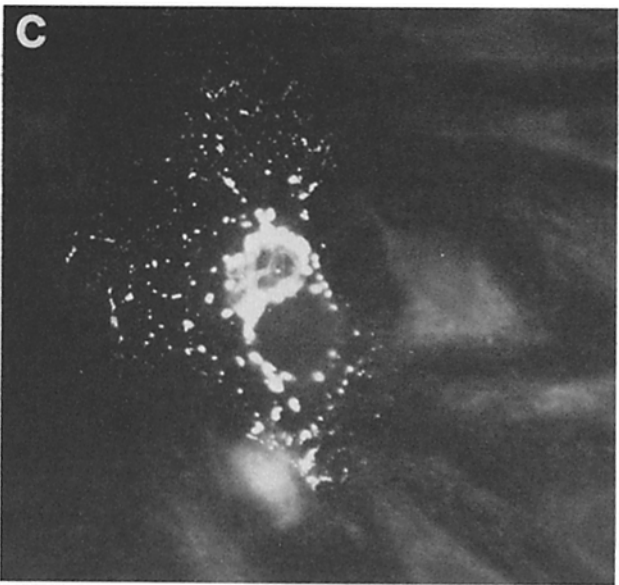
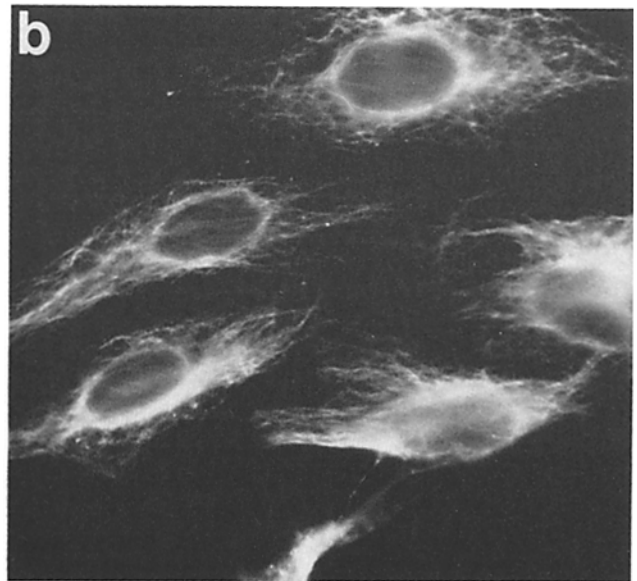
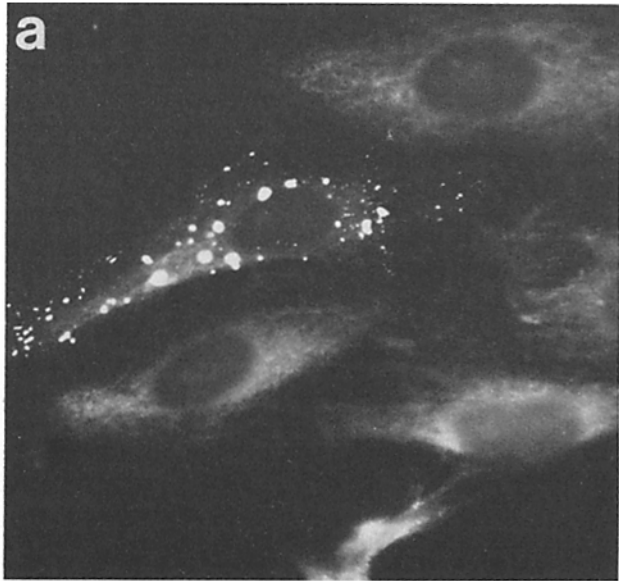
observed, LE41 staining always showed complete colocalization of the endogenous and mutant keratins (Fig. 6 *b*).

At 65-h posttransfection, when the expression of mutant keratin seemed to be optimal, the cables of filaments were frequently seen coiled in a clump near the nuclear envelope (Fig. 6, *c-f*). Most transfected cells also exhibited a few keratin-containing spheroid bodies in their cytoplasm. Whereas at earlier time points many cells were seen with a straightened cable network (Fig. 6, *a* and *b*), now only a few of these cells could be found (not shown). Very rarely, C Δ 50 pJK14-P transfected cells produce a hybrid keratin network that was indistinguishable from that of an untransfected PtK2 cell (lower cell in Fig. 6, *c* and *d*). Collectively, our findings suggest that the collapsed coil structures may require a higher level of mutant protein than the cables, and that the two different abnormal structures may share a precursor-product relation.

Interestingly, at later times (92 h) after transfection, few if any cells had collapsed coils of filaments while many cells had a keratin network which appeared to be almost normal (not shown). At these late times, some cells showed evidence of the filament cables seen very early after transfection, but in these cases the network was more extended and not as thick. These observations raise the possibility that the synthesis of mutant protein may be declining between 65 and 92 h posttransfection.

When PtK2 cells were transfected with C Δ 73 pJK14-P, the resulting network of keratins was dramatically different from that seen in C Δ 50 pJK14-P transfected cells (Fig. 7). While both anti-P (Fig. 7 *a*) and LE41 (*b*) antibodies stained a single network of fibrous structures in the cytoplasm of most transfected cells, the network of filaments was clearly abnormal. At early times after transfection, most cells showed a morphology similar to the right cell in Fig. 7, *a* and *b*. Filament bundles were generally thin and the filaments themselves were often short and discontinuous. Branchlike structures were also observed, giving the filaments a bent or kinked appearance. At 30-65 h posttransfection, cells with cytoplasmic spheroid bodies of keratin could also be seen (e.g., transfected cell shown at left in Fig. 7, *a* and *b*). In these cells keratin did not seem to be involved in filamentous structures. The kinetics of appearance of the nonfilamentous aggregates suggest that they arise when the discontinuous filaments incorporate a critical level of mutant protein. Larger deletions into the fourth helical domain had an even more profound effect on the ability of mutant keratin to disrupt the PtK2 keratin network. The largest deletion that we examined was C Δ 135 pJK14-P, encoding a mutant keratin missing almost its entire fourth helical domain. When expressed in transfected PtK2 cells, this mutant accumulated as protein aggregates (Fig. 7 *c*). The mutant clearly retained its ability to interact with the PtK2 keratins, however, as indicated by the colocalization of LE41 in these aggregates (Fig. 7 *d*).

The spheroid bodies in C Δ 135 pJK14-P-transfected cells were similar in appearance to those seen when antikeratin antibodies were microinjected into simple epithelial cells (Klymkowsky et al., 1983; Tolle et al., 1985). In contrast to the antibody microinjection experiments, our transfection experiments would not a priori be expected to disturb the preexisting keratin network. Nonetheless, even though a network of normal keratin filaments must have existed at the time mutant keratin was first expressed, no filamentous structures containing keratin were found in any of the transfected



cells. Thus, unless the PtK2 keratin filaments are being degraded and resynthesized at an extremely rapid rate, the mutant must have not only interfered with the formation of new keratin filaments, but also disrupted the existing keratin cytoskeleton.

While the size of the aggregates was smaller, the morphology seen in Fig. 7, *c* and *d* was observed in some CA135 pJK14·P-expressing cells at times as early as 18 h posttransfection. Thus, even low levels of this mutant keratin seemed to cause chaos within the endogenous PtK2 network of keratins. Moreover, cell division did not appear to be a necessary prerequisite for the formation of this dramatically altered cytoskeleton, since a number of lone transfected cells could be seen bearing this abnormal morphology.

When CA135 pJK14·P-transfected cells were examined at 92 h posttransfection, anti-P still stained spheroid bodies in the cytoplasm (Fig. 7 *e*), while LE41 staining was markedly reduced in these aggregates (*f*). These results were similar, but more striking than the results obtained with the CA50 mutant. Even more surprising was the appearance of seemingly normal keratin filament networks surrounding the nuclei of the transfected cells. Interestingly, some transfected cells were binucleate, and occasionally in these cells the filamentous network seemed to surround only one of the nuclei (see Fig. 7, *e* and *f*). In all cases these filamentous networks stained with LE41 but not with anti-P, indicating that the mutant keratin was largely if not completely excluded from these filaments. The simplest explanation for this phenomenon is that the expression of the mutant keratin decreased dramatically between 65 and 92 h posttransfection while endogenous keratin synthesis continued, showing a perinuclear initiation of newly formed 8-nm filaments. Failure of the existing mutant keratin to assemble into the newly synthesized keratin filaments suggests that once incorporated into aggregates, the mutant keratins are at least partially inhibited from freely exchanging with the subunits in the filaments. In contrast, the reduction in LE41 fluorescence in the aggregates could mean that K8 and K18 more readily exchange with the newly forming filament network.

Examining the Effects of Mutant-induced Perturbations of the Keratin Network on Other Cytoskeletal Architectures

We wondered whether the mutant-induced disruptions of the endogenous keratin networks might also perturb some of the other cytoskeletal components of the transfected PtK2 cells. To investigate this possibility, we transfected PtK2 cells with CA135 pJK14·P, and examined the endogenous networks of vimentin filaments, microtubules, and actin microfilaments by immunofluorescence (Fig. 8). Our results show clearly that the mutant keratin network did not affect the filamentous networks containing vimentin (Fig. 8, *a* and *b*), tubulin (*c* and *d*), or actin (*e* and *f*). Similar results were obtained for cells transfected with the other mutant keratin constructs

(data not shown). These observations are in agreement with previous studies using microinjection of antikeratin antibodies (Klymkowsky et al., 1983). The data indicate that even when grossly permuted, the keratin filament network of a cell does not seem to influence the structural integrity of the other cytoskeletal components.

Expression of Mutant K14·P Proteins in Squamous Cell Carcinoma Cells of the Skin

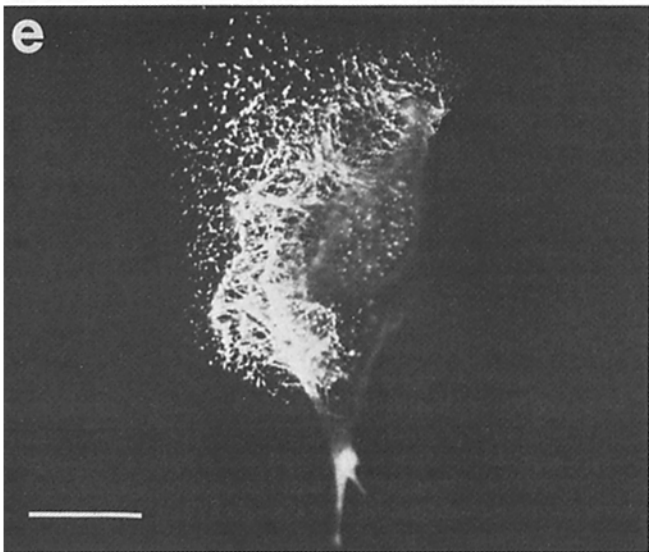
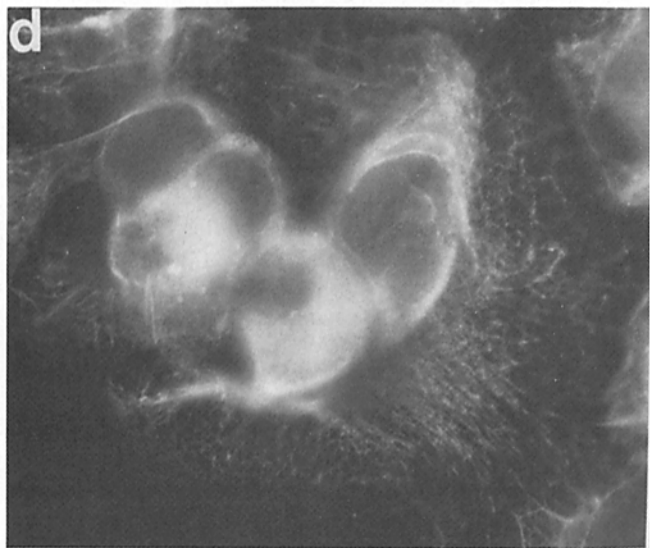
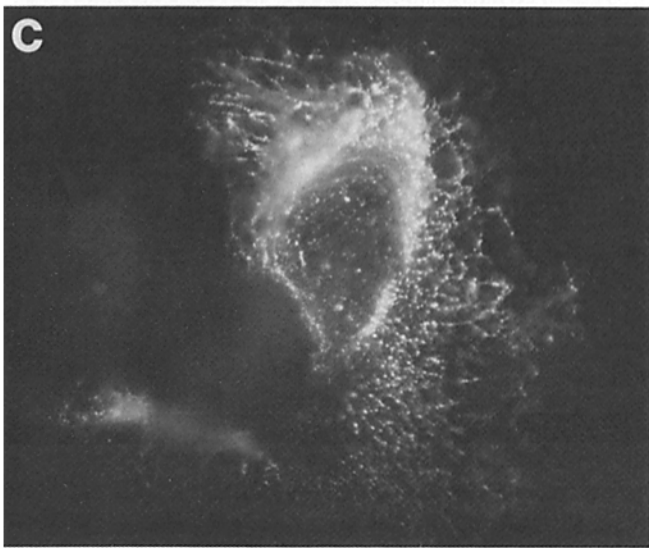
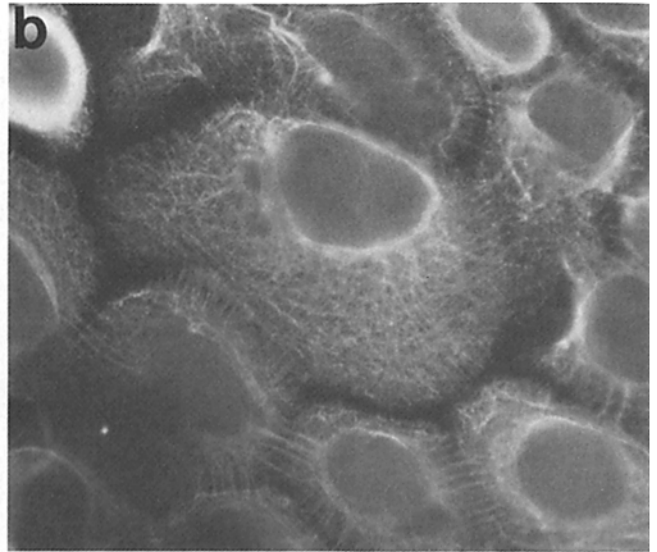
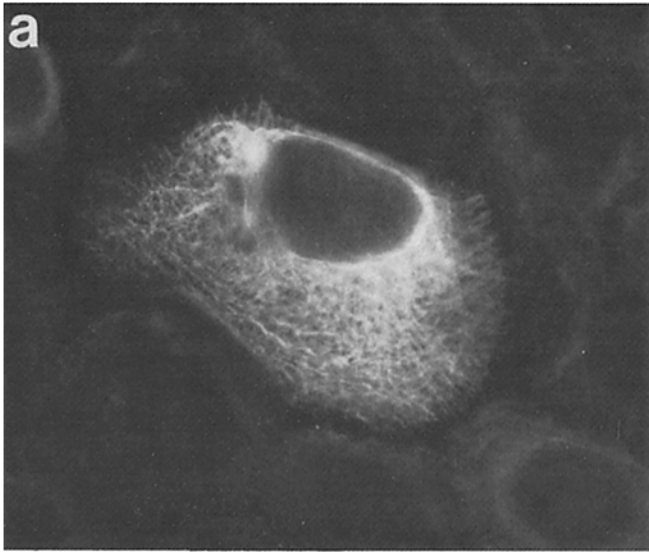
Since both type I and type II keratins seem to be essential for filament assembly (Hatzfeld and Franke, 1985; Eichner et al., 1986), the foreign expression of K14·P and its mutant derivatives in PtK2 cells must have forced them to choose the type II keratin K8 as a promiscuous partner in filament formation. Although studies have shown that K14 and K8 assemble into bona fide 8-nm filaments in vitro (Hatzfeld and Franke, 1985), it was important to determine whether the behavior of K14·P and its derivatives is similar in cells where K5, the true partner of K14 (Nelson and Sun, 1983), is present. For this investigation, we transfected pJK14·P and its derivatives into SCC-13 cells, a line derived from a squamous cell carcinoma of human skin (Wu and Rheinwald, 1981). These cells express seven different keratins: K5, K6, K13, K14, K16, K17, and K19. The overall level of keratins in these cells is approximately five times higher than in PtK2 cells.

The behavior of pJK14·P, CA11 pJK14·P, and CA42 pJK14·P in SCC-13 cells was similar to that observed in the transfected PtK2 cells. In all three cases, the transfected gene product incorporated into the preexisting keratin network of the SCC-13 cells without causing any apparent perturbations. Fig. 9, *a* and *b* shows one example of this, namely a pJK14·P-transfected SCC-13 cell.

Unexpectedly, transfection of CA50, CA73, and CA135 pJK14·P into SCC-13 cells also yielded hybrid keratin networks that were almost always indistinguishable from those of untransfected cells (data not shown). At all times posttransfection, only a small percentage of transfected SCC-13 cells showed abnormal structures resembling those observed for the transfected PtK2 cells (see Fig. 9, *c–e* for rare examples; CA135 pJK14·P). Even these few clearly abnormal cells still retained remnants of a normal filamentous network. These results suggest that either: (*a*) the ratio of mutant to endogenous keratins is too low to elicit the abnormal filament morphology in the SCC-13 cells, or alternatively (*b*) the structure of the keratin network in SCC-13 cells is more stable than it is in PtK2 cells.

Knapp et al. (1983) have observed a reorganization of keratin filaments upon treatment of cultured fetal mouse epidermal cells with a mixture of cytochalasin B (to disrupt actin microfilaments) and colchicine (to disrupt microtubules). A recent study by Kitajima et al. (1986) indicated that cytochalasin B alone is sufficient to induce this perturbation in the keratin networks of human epidermal cells. A priori, the disruption of the microfilament network could induce a per-

Figure 8. Other cytoskeletal architectures of the PtK2 cell are not perturbed even when the keratin filament network is collapsed. PtK2 cells were transfected with CA135 pJK14·P and examined by immunofluorescence at 65 h posttransfection. Cells were stained with anti-P (*a*, *c*, and *e*) as described in the legend to Fig. 2. Cells in *a* were costained with a rabbit antiserum made against hamster vimentin, followed by Texas Red-conjugated goat anti-rabbit IgG (*b*). Cells in *c* were costained with a rabbit antiserum made against porcine tyrosinated tubulin, followed by Texas Red-conjugated goat anti-rabbit IgG (*d*). Cells in *e* were costained with rhodamine-conjugated phalloidin (*f*). Bar, 20 μ m.



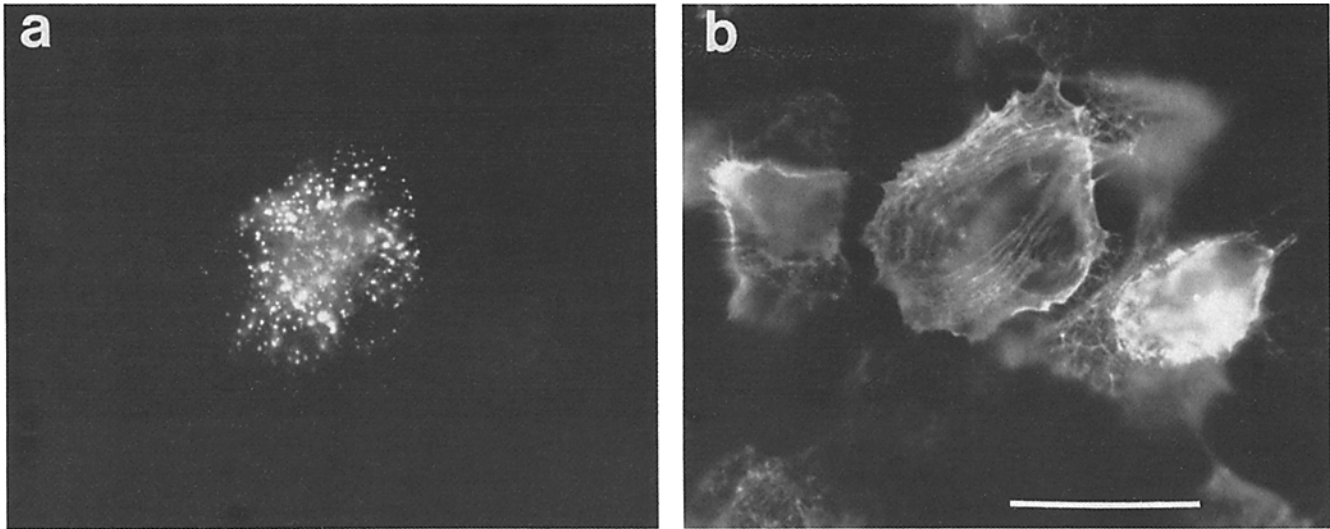


Figure 10. The actin network of SCC-13 cells is not disrupted when the keratin filament network is collapsed. SCC-13 cells were transfected with CA135 pJK14·P and fixed at 80 h posttransfection. Cells in *a* were stained with anti-P as described in the legend to Fig. 2. The actin network of these cells (*b*) was detected by costaining with rhodamine-conjugated phalloidin. Bar, 20 μ m.

turbation in the keratin filament network either because the two networks are physically linked, or because the microfilament network provides an architectural support necessary for the stability of the keratin network. To distinguish between these two possibilities, we disrupted the keratin filament network by transfection with CA135 pJK14·P, and examined the actin microfilament network of SCC-13 cells. Complete disruption of the keratin filaments did not induce any detectable change in the microfilament network (Fig. 10). The microtubule network showed no aberrations either as a consequence of mutant-induced disruption of the keratin filament network (data not shown). Thus, it seems unlikely that either the microfilament or the microtubule networks are covalently interconnected with the keratin filaments in the cultured keratinocyte.

Discussion

In this paper, we began to examine contributions that different portions of a keratin sequence make in defining the overall keratin filament network within a cell. Tagging a K14 cDNA with a small neuropeptide sequence was a prerequisite to tracing the expression of K14 and its mutant derivatives both in simple epithelial cells, where the gene is not normally expressed, and in epidermal cells, where the expression of K14 accounts for $\sim 7\%$ of the total protein of the cell. Although we cannot unequivocally rule out the possibility that the replacement of the last five amino acid residues of the K14 chain with a 15-residue peptide might have caused slight perturbations in the overall keratin filament networks

of the transfected cells, no difference could be detected by the immunofluorescence techniques that we used.

Our rationale behind tagging the carboxy-terminal end of K14 stemmed from three independent studies. In one study, Steinert and co-workers (1983) demonstrated that mild chymotryptic proteolysis of intact mouse epidermal keratin filaments, resulting in the apparent removal of more than half of the nonhelical ends of the keratins, did not disrupt the 8-nm structure. In another study, Kaufmann et al. (1985) showed that removal of almost half of the nonhelical carboxy-terminal residues of desmin produced a protein that was still able to assemble into 10-nm filaments. In contrast, the removal of 67 amino acid residues at the amino-terminal end rendered desmin incompetent for filament assembly on its own. Finally, the recent report of the sequence for the smallest known keratin K19 has revealed that this protein has a 13-amino acid extension of the α -helical rod and no nonhelical tail, and yet it appears to be fully competent in filament assembly (Bader et al., 1986). Collectively, these data indicated that the "safest" place to perturb the K14 sequence might be at the extreme carboxy-terminal end of the chain.

The results of our transfection studies with the mutant keratin CA42 K14·P confirmed and extended earlier findings concerning the role of the nonhelical carboxy-terminal end of IF proteins in filament formation. In fact, this entire domain could be removed without perturbing the ability of K14 to incorporate into keratin filaments. Whether the transfected cells were of simple epithelial or of epidermal origin did not seem to make a difference, indicating that the K14·P could readily accept a promiscuous partner in filament for-

Figure 9. The response of squamous cell carcinoma cells (SCC-13) to transfection with CA135 pJK14·P is distinctly different from that of PtK2 cells. SCC-13 cells were transfected with pJK14·P (*a* and *b*) and CA135 pJK14·P (*c*–*e*) and examined at various times after transfection by immunofluorescence. The transfected cells shown in *a*–*d* were fixed at 26 h posttransfection. The cell in *e* was fixed at 53 h posttransfection. The transfected gene products were detected by staining with anti-P (*a*, *c*, and *e*) as described in the legend to Fig. 2. The endogenous keratin network was visualized by staining with a rabbit antiserum made against type II epidermal keratins, followed by Texas Red-conjugated goat anti-rabbit IgG (*b* and *d*). The cell overlapping the transfected cell shown in *c* and *d* is suprabasal: it appeared as a consequence of stratification in the SCC-13 cultures. Bar, 20 μ m.

mation. Moreover, exchanging the 48 amino acid residue tail of K14 with a foreign peptide sequence did not seem to influence the competence of K14 for filament formation, nor did it alter the gross morphology of the resulting filament network. Thus, unless a carboxy-terminal sequence generates a tertiary structure that in some way blocks the ability of keratin subunits to pack into the 8-nm filament, its sequence per se may be largely irrelevant in the assembly process.

In the experiments that we conducted, normal type I keratins were always present in the transfected cells expressing our mutant type I keratin construct. We do not yet know how the mutant keratins would perform in filament assembly under conditions where other type I keratins are absent. However, we predict that at least some of our mutants, particularly those that have lost helical residues, will not be able to assemble into normal 8-nm filaments when they are the sole type I keratin used in filament formation. This is already forecast from our kinetics studies where the accumulation of a critical level of certain mutants appeared to trigger a collapse of the endogenous keratin filament network. To investigate this issue in more detail, permanent mammalian cell lines or keratin-expressing bacterial lines must be cloned to produce sufficient quantities of mutant proteins for *in vitro* assembly studies.

While certain questions about filament assembly necessitate *in vitro* assembly studies involving mutant keratin polypeptides, other important aspects of filament formation, filament stability, and intracellular interactions involving keratin filaments can only be addressed using a transfection approach. Indeed, one of our most significant findings was the realization that even low levels of a single mutant keratin can be sufficient to cause the collapse of the entire endogenous keratin network. This was particularly evident when PtK2 cells were transfected with the mutant C Δ 135 pJK14-P: within just 18 h transfected cells produced sufficient amounts of mutant protein to disrupt the keratin filament network.

A priori, there are two major reasons why both the preexisting keratin filament network and the newly synthesized keratin filaments should collapse simultaneously: (a) If newly synthesized keratins do not freely exchange with preexisting filaments, then the cytoskeleton of transfected cells would consist of a heterogeneous mixture of normal and abnormal keratin filaments. In this case, abnormal filaments would have to cause a perturbation in the normal keratin filament network, presumably via interfilament interactions. (b) If a dynamic equilibrium exists such that keratin subunits are continuously exchanged along the filament core of new and old filaments alike, then all keratin filaments of transfected cells would contain mutant keratins. If the rate of exchange is significantly high, 100% of the keratin filaments would be destabilized.

While it is plausible that a heterogeneous mixture of normal and mutant filaments might cause a lateral aggregation of filaments similar to that seen in C Δ 50 pJK14-P-transfected cells, it seems unlikely that interactions between normal and mutant filaments could reduce both structures to the partially polymerized aggregates of keratin subunits observed for C Δ 135 pJK14-P-transfected cells. On the other hand, if mutant subunits integrate into both preexisting and newly synthesized filaments, it seems remarkable that the level of exchange between keratin subunits could be so great as to cause the disintegration of the keratin filament network.

Although no indication of a constant dynamic equilibrium has thus far been substantiated for the keratin subunits and filaments, there have been reports of cell cycle-dependent changes in filament organization. In a number of simple epithelial cell lines, radical shape changes accompanied by a reorganization of keratins occur transiently during mitosis (Horwitz et al., 1981). Initially, filament bundles are severed into shorter tufts of fibrils (Aubin et al., 1980). Subsequently, individual keratin filaments are partially unraveled into protofibrillar threads and finally into knotlike dense granules, or spheroidal cytoplasmic bodies (Franke et al., 1982). While globular keratin-containing aggregates have not been seen in mitotic PtK2 cells (Franke et al., 1978; Aubin et al., 1980; Horwitz et al., 1981), some severing of keratin fibrils may take place (Aubin et al., 1980; Franke et al., 1982). It has been suggested that since the bundles of keratin fibrils in PtK2 cells are unusually thick, they may not dissociate as completely as in other mitotic cells (Franke et al., 1982).

Our data indicate that the keratin filament network may be in a dynamic state of flux throughout the cell cycle, even when no gross changes in filament organization can be seen. This might also be true for SCC-13 cells, although it was more difficult to discern because many fewer mutant-transfected SCC-13 cells showed a collapsed keratin filament network.

We were surprised to discover that the "recovery process" of cells transfected with our keratin mutants was not simply the reverse of the initial events following transfection. This was especially true for the C Δ 135 pJK14-transfected cells, where at early times after transfection, newly synthesized mutant keratin incorporated into and caused the disruption of preexisting keratin filaments, whereas at late times after transfection no mutant keratin was incorporated into newly synthesized keratin filaments.

In most transient transfections, the expression of the transfected gene product begins to wane after about 65 h (for review see Gorman, 1985). Therefore, it is likely that most of the mutant keratin seen at 92 h posttransfection is due to protein synthesized at earlier times. This must mean that although newly synthesized mutant keratin can incorporate into a preexisting PtK2 network, it is partially if not fully restricted once it enters the spheroid bodies. Oddly, while the level of mutant keratin in the spheroid bodies remained high, LE41 staining of the spheroid bodies was reduced by 92 h posttransfection. Whether K8 and K18 might be released from the spheroid bodies more effectively than the mutant keratin remains to be determined.

Irrespective of the molecular mechanisms underlying the process, the complexing of endogenous keratins into spheroid bodies enabled us to examine what happens when the existing keratin network is removed from a cell, and is recreated largely *de novo*. Interestingly, newly synthesized keratin filaments formed almost exclusively around the nucleus. This result provides *in vivo* evidence in support of earlier studies by Eckert et al. (1982), who showed that purified keratin, solubilized in 8 M urea and added to Triton X-100-extracted PtK1 cells, associated preferentially with the nuclei of extracted cells when filament assembly was induced. Whether keratin filament assembly is actually initiated on the nuclear envelope is still uncertain.

In summary, a number of discoveries have been made as a consequence of expressing mutant epidermal keratins in epidermal and simple epithelial cells. Most importantly,

these experiments have exposed the existence of a dynamic equilibrium between keratin subunits and the filament network within the cell. Our findings are particularly relevant to the question of whether a single mutant keratin might be sufficient to cause havoc on a keratin filament network in a cell already expressing a number of normal type I and type II keratins. Our results indicate that neither epidermal nor simple epithelial cells have a mechanism that excludes newly synthesized mutant keratins from their filament network. While the effects of the mutants seem to be less severe for epidermal cells than for simple epithelial cells, the appearance of a few transfected cells with grossly abnormal phenotypes suggests that the stability of the epidermal network in mutant-expressing cells may be weakened even if it does not always collapse. Thus, unless a cell develops a mechanism for shutting off the expression of a mutant keratin gene, a major component of its cytoskeletal architecture seems to be destined for perturbation or disruption. Although no human skin diseases have yet to be attributed to a mutation in a keratin gene, our results suggest that a single keratin gene mutation could be a possible candidate for an alteration in the keratin filament network. Further studies will be necessary to determine whether epidermal cells retain their viability and proper growth rates after transfection with our mutants.

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