Expression of Mutant Keratin cDNAs in Epithelial Cells Reveals Possible Mechanisms for Initiation and Assembly of Intermediate Filaments

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Abstract. We have deleted cDNA sequences encoding portions of the amino- and 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 defined the limits of K14 sequence necessary to incorporate into a keratin filament network in vivo without disrupting its architecture. We have also un-covered major differences in the behavior of carboxy- and amino-terminal α-helical mutants which do perturb the cytoskeletal network of IFs: whereas carboxy terminal mutants give rise to aggregates of keratin in the cytoplasm, amino-terminal mutants tend to produce aggregates of keratins which seem to localize at the nuclear surface. An examination of the phenotypes generated by the carboxy and amino-terminal mutants and the behavior of cells at late times after transfection suggests a model whereby initiation of filament assembly occurs at discrete sites on the nuclear envelope and filaments grow from the nucleus toward the cytoplasm.

INTERMEDIATE filament (IF) proteins are expressed in virtually all mammalian cells. Based on their tissue-specific expression, they can be subdivided into five distinct groups: vimentin (cells of mesenchymal origin), glial fibrillary acidic protein (glial cells), desmin (muscle cells), neurofilament proteins (neural cells), and keratins (epithelial cells) (for reviews, see Geisler and Weber, 1982; Steinert et al., 1985; Fuchs et al., 1987). 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.

The keratins constitute the most complex and diverse group of IF proteins. There are >20 keratins (40–70 K) which can be subdivided into two distinct groups: type I keratins are acidic (pKi = 4.5–5.5) and generally small (40.0–56.6 K), whereas type II keratins are basic (pKi = 5.5–7.5) and larger (53–70 K) (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 seem to be expressed as specific pairs, and these pairs are differentially expressed in different epithelia and at various stages of differentiation and development (Moll et al., 1982; Eichner et al., 1984; Sun et al., 1984). While a member of each keratin type is essential for filament formation, almost any combination of type I and type II proteins will lead to filament assembly in vitro (Hatzfeld and Franke, 1985; Eichner et al., 1986). Moreover, when epidermal keratins are expressed transiently in simple epithelial cells after gene transfection, they integrate into the endogenous filament network (Giudice and Fuchs, 1987; Albers and Fuchs, 1987).

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; Dowling et al., 1986; Magin et al., 1986). Throughout these sequences is found a heptad repeat, where the first and fourth residues of every seven are hydrophobic. These repeats are characteristic of α-helical proteins which form coiled-coil structures (Crick, 1953; McLachlan and Stewart, 1975). 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

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

identity; keratins of opposite type share only 25–35% homology in these domains. Across type lines, much of the homology resides at the carboxy 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. Keratin sequences at the amino end of the first helical segment are conserved (T/Q M/I Q/K/R N/T/S L N D/N R/K/Q L/F A S Y/F L/I D K V R A/F L/M E E/Q), but since the homologies are lower, it has been more difficult to assess the importance of these sequences and to assign the amino terminal boundary of the helical domain.

The basic subunit structure of all IFs seems to be a coiled-coil dimer, with two parallel polypeptide chains in register (Woods and Inglis, 1984; Parry et al., 1985; Quinlan et al., 1986). The chains appear to be stabilized by hydrophobic interactions, although electrostatic interactions may also play a role (Parry et al., 1977; McLachlan and Stewart, 1982). In solution, stable tetramers form (Quinlan et al., 1984; Soellner et al., 1985). 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). The ratio of type I/type II proteins in the tetramer is 1:1 and homodimers have been generated in vitro (Quinlan et al., 1986; Hatzfeld et al., 1987). However, it is still unknown whether keratin filaments made in vivo are composed of homodimers, heterodimers or a mixture of the two. For most other IF proteins, e.g., vimentin and desmin, this issue is not relevant, since filaments can assemble from a single polypeptide (Steinert et al., 1981; Geisler et al., 1982, 1985; Kaufmann 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 three to four protofibrils intertwine to form the resulting 8-nm filaments (Aebi et al., 1983; Steven et al., 1983; Ip et al., 1985; Eichner et al., 1986). The sequence of events leading to the assembly of ~5,000–7,500 tetramers into 8-nm filaments remains to be elucidated. In addition, while nucleation of filament assembly can apparently be by-passed in vitro by elevating the concentration of subunits, the process of keratin filament formation in vivo seems to be highly accelerated by (if not dependent upon) a nucleation step involving the nuclear envelope (Albers and Fuchs, 1987). In vitro studies also suggest that an association between the nucleus and IF proteins may play a role in IF assembly (Eckert et al., 1982; Georgatos and Blobel, 1987a; Georgatos et al., 1987).

The details of the sequences essential for filament assembly are largely unknown. Studies by Steinert et al. (1983) showed that mild chymotryptic proteolysis of intact mouse epidermal keratin filaments resulted in the apparent removal of more than half of the nonhelical tails of the keratins, and yet did not disrupt the 8-nm structure. However, removal of additional nonhelical sequences rendered the subunits incompetent for filament assembly. More precise deletion analyses using mutagenesis of cloned keratin cDNAs and gene transfection indicated that a tagged human type I keratin K14 missing the highly conserved L L E G E sequence could not integrate properly into an endogenous keratin filament network, whereas a similar construct retaining this sequence, but missing nearly the entire nonhelical carboxy tail of K14 showed a wild-type phenotype (Albers and Fuchs,

1987). In another study, Kaufmann et al. (1985) showed that proteolytic 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, whereas removal of 67–amino acid residues at the amino-terminal end inhibited assembly of homopolymers, but allowed assembly of heteropolymers between proteolyzed desmin and wild-type desmin. More detailed amino terminal analyses have not yet been reported for any IF protein.

Inside each higher eukaryotic cell, a network of IFs seems to extend from the nuclear envelope at one end to the plasma membrane at the other. Little is known about the nature of the interactions of IFs with either the nuclear or the plasma membrane. Recent recombination studies using purified vimentin and isolated nuclear/plasma membranes from erythrocytes have suggested that the amino terminal tail of vimentin may associate with ankyrin at the plasma membrane, while the carboxy-terminal tail may be involved in interactions with lamin B at the nuclear envelope (Georgatos and Blobel, 1987a,b; Georgatos et al., 1987). However, as yet no in vivo studies have been conducted to confirm these reports. That some interactions must take place between IFs and components of the two membranes seems inevitable. It also seems likely that these interactions may differ among cell types. This is perhaps best exemplified for stratified squamous epithelial cells, where keratin filaments seem to interact with a specialized membrane component, namely the desmosome (Drochmans et al., 1978; Denk et al., 1985).

To define more precisely the limit sequence sufficient for normal integration of an IF protein into an existing IF network, and to examine the roles of nonhelical and helical domains in IF architecture within a cell, we have pursued our deletion analyses which previously focused on the carboxy-terminal domain of human keratin K14 (Albers and Fuchs, 1987). In this report, we describe a series of cDNA deletion mutants at the amino-terminal end of K14. Our results reveal striking differences in the behavior of amino-terminal vs. carboxy-terminal mutations in K14 and provide interesting insights into the possible mechanisms of IF assembly in vivo.

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), and were cultivated as described previously (Wu and Rheinwald, 1981).

Construction of plasmid pJK14·P and Deletion Mutants

pJK14·P

The construction of plasmid pJK14·P has been described previously (Albers and Fuchs, 1987). The insert (K14·P) contains the complete 5' noncoding and coding portion of the cDNA encoded by the gene for the human K14 (Hanukoglu and Fuchs, 1982; Marchuk et al., 1985), with the exception that the sequences encoding the last five carboxy amino acids of the K14 protein have been replaced by a sequence coding for the last five carboxy-terminal residues of the neuropeptide substance P (see Fig. 1). After the TGA stop codon is the 3' noncoding sequence of a Drosophila hsp70 gene (Munro and Pelham, 1984). The insert was cloned in the 5' to 3' direction into the Hind III/Sma I sites of plasmid pJay1 (Land et al., 1986). The plasmid pJay1 contains the SV-40 enhancer and major early promoter to drive the expression of the K14·P cDNA.

NH₂-TERMINAL DELETION MUTANTS. BAL 31 GENERATED:

pJK14·P DNA was cleaved at the unique Hind III site located just 3' to the SV-40 promoter sequence (H3; see Fig. 1). 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 Sac I site in the 3' end of the cDNA (see Fig. 1), and the Sac I–blunt ended fragments were gel purified on Seaplaque agarose (FMC Corp., Rockland, ME). The fragments encoding various portions of the amino end of K14 were then ligated into the vector pGem 5 (Promega Biotec, Madison, WI) at the Nco I/Sac I site, after filling in the Nco I site with Klenow fragment. By filling in the Nco I site of pGem 5, an ATG was retained which then served as an initiation codon for the K14·P inserts.

Clones containing inserts were sequenced as described below. Clones of the proper size and reading frame were then linearized at a unique Eag I site just 5' to the Nco I site. The Eag I ends were filled in with Klenow and the insert was then excised with Sac I. The Sac I/blunt Eag I inserts were then ligated into pJK14·P after the plasmid had been (a) linearized with Hind III, (b) treated with Klenow to fill in the Hind III ends, (c) cut with Sac I, and (d) gel purified to remove the unwanted 5' portion of the K14 gene.

The Bal 31–generated clones will be referred to as NΔXpGem 5, where X corresponds to the number of amino acid residues missing from the K14·P sequence as a consequence of the deletion.

NH₂-TERMINAL DELETIONS. RESTRICTION ENDONUCLEASE GENERATED:

NΔ117. The sequence corresponding to the 5' noncoding portion and the first 117-amino acid residues of K14·P were excised from plasmid pJK14·P by cutting at unique sites with the restriction endonucleases Bste II and Hind III. After filling in the ends with Klenow fragment, the plasmid was resealed with T4 DNA ligase. The ATG sequence at residue 117 of the K14 protein served as the translation initiation codon for the truncated construct. The SV-40 sequences 5' to the Hind III site served as 5' noncoding sequence for the corresponding mRNA.

NΔ169. The starting plasmid was a Bal 31 deletion mutant, NΔ89 pGem5. NΔ89pGem5 was cut at (a) a unique Dra II site present at the sequence corresponding to amino acid residue 169 of K14 and (b) a unique Nco I site present just 3' to the initiation codon site of NΔ89pGem5. After filling in the ends with Klenow fragment, the plasmid (minus the NΔ89 to NΔ169 sequences) was purified and ligated using T4 DNA ligase. The NΔ169 K14 sequence, which now contained a 5' ATG codon, was then excised by (a) linearizing the pGem 5 hybrid with Eag I, (b) filling in with Klenow, and (c) cutting with Sac I. This insert was then ligated into pJK14·P after the plasmid had been (a) linearized with Hind III, (b) filled in with Klenow, and (c) cut with Sac I.

NΔ117/CΔ42 and NΔ117/CΔ50. Plasmid NΔ117pJK14·P was cut with Sac I and Bam HI. The larger vector fragment, missing the carboxy portion of the coding region of K14·P, was gel purified. Sac I/Bam HI inserts of plas-

mids CΔ42 pJK14·P and CΔ50 pJK14·P containing the carboxy portion of the coding region of K14·P (see Albers and Fuchs, 1987) were also gel purified. The NΔ117 fragment was then ligated to the two CΔ42 and CΔ50 fragments to produce, respectively, NΔ117/CΔ42 pJK14·P and NΔ117/CΔ50 pJK14·P plasmids.

NΔ107/CΔ42 and NΔ107/CΔ50. Plasmid NΔ107 pJK14·P was cut with Sac I and Eco RI and the larger vector fragment, missing the carboxy portion of the coding region of K14·P, was gel purified. Sac I/Eco RI inserts from plasmids CΔ42pJK14·P and CΔ50pJK14·P containing the carboxy portion of the coding region of K14·P (see Albers and Fuchs, 1987) were also gel purified. The NΔ107 fragment was then ligated to the CΔ42 and CΔ50 fragments to produce, respectively, NΔ107/CΔ42 pJK14·P and NΔ117/CΔ50 pJK14·P.

All ligated hybrid plasmid DNAs were transformed into the bacterial strain DH5-alpha.

DNA Sequencing

Plasmid DNAs from each deletion clone in pGem 5 were isolated and sequenced using the double stranded sequencing protocol described in the Promega Biotec Sequencing Manual. The commercially available T7 oligonucleotide primer was used for sequence analysis.

DNA Transfections

DNA was transfected into PtK2 and SCC-13 cells using the calcium phosphate precipitation method (Graham and Van der Eb, 1973) followed by a 15% glycerol shock (Parker and Stark, 1979). Cells were fixed 65 h posttransfection unless otherwise indicated in the text.

Immunofluorescent Labeling

For immunohistochemistry, cells were grown on glass chamber slides (Miles Scientific Div., Naperville, IL). After transfection, cells were washed with PBS, fixed in methanol (−20°C) for 10 min, and washed in PBS.

To detect K14-substance P tagged proteins, we used the anti–P rat monoclonal antibody NC1/34 (Cuello et al., 1979). As a necessary prerequisite for NC1/34 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 K6 was detected in cultured SCC-13 cells using a polyclonal rabbit antibody isolated in this laboratory (Fuchs and Marchuk, 1983). Desmosomes were examined with a mouse monoclonal antibody against desmoplakin I (ICN Biochemicals Inc., Irvine, CA). Nuclear lamin networks were visualized using a rabbit antiserum against lamins A, B, and C. Nuclear pore structures were stained using Ascites fluid containing a mouse IgM that reacts specifically with the nuclear pore structure (Snow et al., 1987). Antibodies against both nuclear structures were kindly provided by Dr. Larry Gerace (University of California at San Diego).
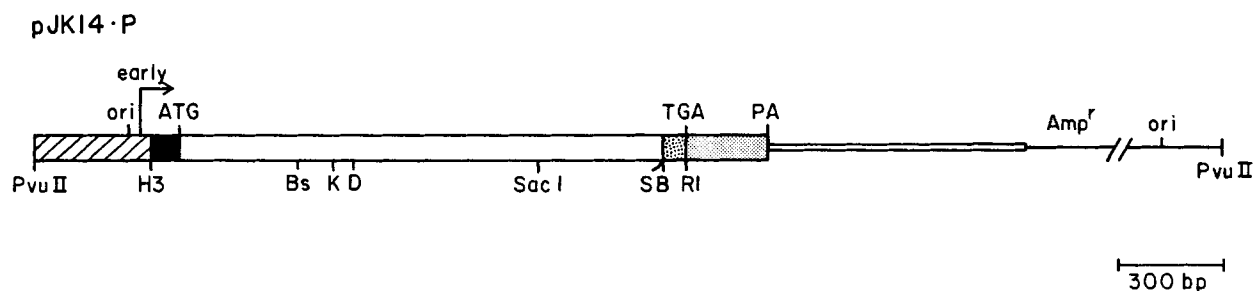
pJK14·P

Figure 1. Genetic map of plasmid pJK14·P. The construction of the plasmid pJK14·P was described previously (Albers and Fuchs, 1987). The components of the plasmid are represented as follows. The hatched bars represent 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. The 5' untranslated leader sequence of the K14 mRNA is shown in black. Also in this region, upstream from the K14 sequence, is the Hind III/Hinc II polylinker portion of plasmid pSP64 (Promega Biotec), which was included as a consequence of subcloning. The white box represents the complete human K14 cDNA. This segment is missing the sequence encoding the five carboxy terminal amino acid residues and the TGA stop codon. The Sal I–Sfi I fragment of plasmid pAHP.2 (Munro and Pelham, 1984) includes 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*). The origin of replication for plasmid pBR322 is indicated (*ori*) as is the ampicillin resistance gene contained in plasmid pJ1. Important restriction endonuclease sites are marked. *H3*, Hind III; *Bs*, Bste II; *K*, Kpn I; *D*, Dra II; *S*, Sal I; *B*, Bam HI; *RI*, Eco RI.

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 Inc., Burlingame, CA), Texas Red-conjugated goat anti-rabbit IgG (Cappel Laboratories, Inc.), Texas Red-conjugated goat anti-mouse IgG (Tago Inc.).

Results

Construction of Amino Terminal Deletion Mutants

In our previous studies, there were two major considerations in designing our vector for deletion mutation analyses: (*a*) We wanted to make certain that sequences encoding the antigenic determinant of the protein were not lost during the construction of deletion mutants, and (*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) sequence encoding the carboxy-terminal end of the neuropeptide substance P (Albers and Fuchs, 1987). In addition, we cloned the hybrid K14-substance P sequences into plasmid pJ1 (Land et al., 1986), immediately 3' to the SV-40 early promoter and enhancer sequence. For our present studies, we have retained the use of this hybrid plasmid, referred to as pJK14·P (Fig. 1).

To construct mutant cDNAs missing varying portions of sequence encoding the amino terminal end of K14·P, we used a number of recombinant approaches, including Bal 31 exonuclease–mediated deletions and restriction enzyme–mediated deletions. In all cases, techniques were employed to ensure retention of a 5' noncoding segment and ATG translation initiation start codon (see Materials and Methods). For each mutant, the precise point of deletion was determined by sequence analysis. Deletions that retained the appropriate keratin reading frame were chosen for further study. The constructs selected for our study are shown in stick diagram form at the top of Fig. 2, with the exact sequence breakpoints illustrated by arrows in the sequence diagram of the
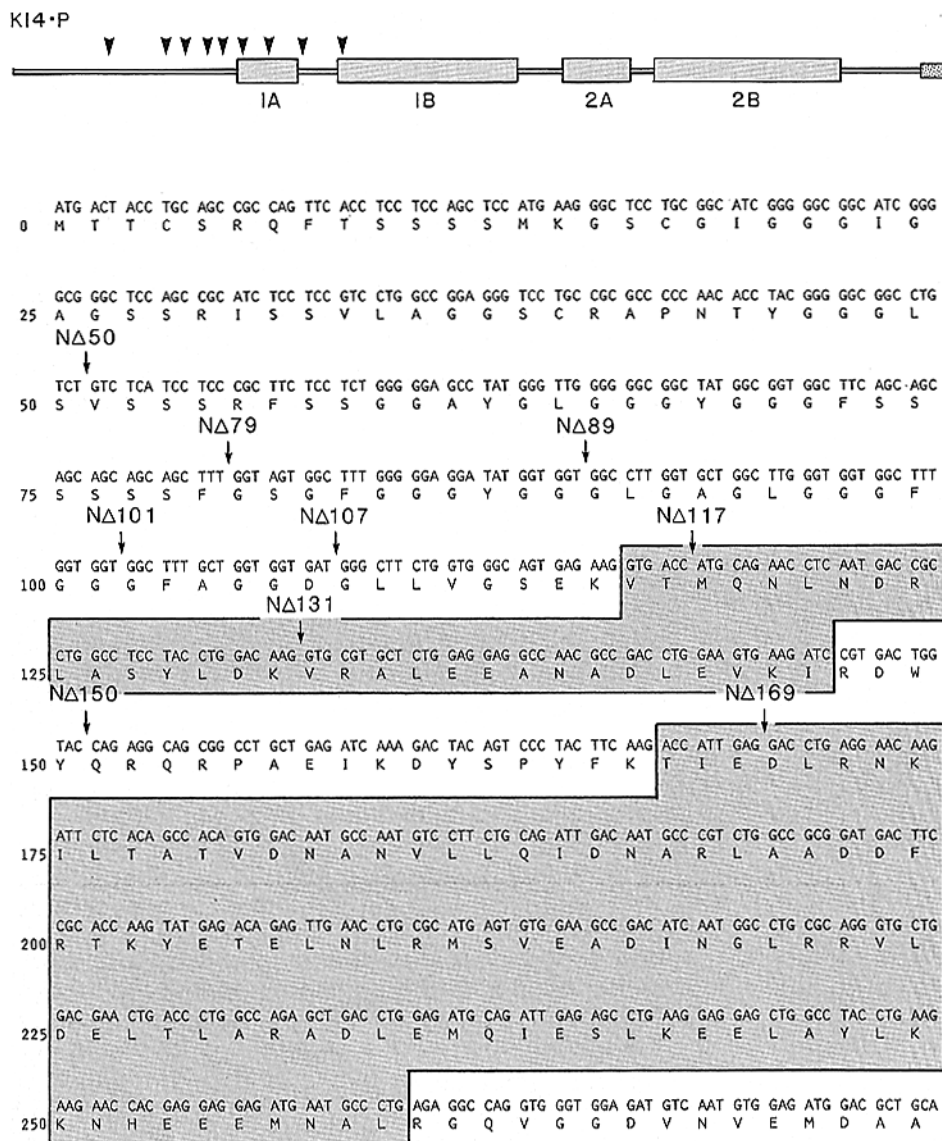

Figure 2. Deletion mutants of pJK14·P missing sequences coding for the amino-terminal end of K14. The overhead stick diagram shows the location of the four helical domains (boxed in gray; *IA*, *IB*, *2A*, and *2B*) of K14·P (substance P tag is represented by the stippled box). Arrowheads mark the sites of the amino deletions used in our analyses. Beneath the stick diagram is the sequence of the amino terminal end of K14·P. The first and second α-helical domains, *1A* and *1B*, are boxed in gray. 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 first amino acid residue to the arrow. Construct nomenclature: NΔX represents an amino terminal deletion; X represents the number of amino acid residues which are missing from K14·P as a result of the deletion.

same figure. In the figure, the gray boxes indicate the α-helical domains of the keratin polypeptide. The mutant constructs are referred to as NΔX pJK14·P (NΔX for short), where X represents the number of amino acid residues removed from the amino 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

The kidney epithelial cell line PtK2, from kangaroo rat, was chosen for our transfection studies. These simple epithelial cells express the type I keratins K18 and K19 and the type II keratins K8 and K7 (Franke et al., 1978; Lane, 1982; Glass and Fuchs, 1988). Although K14 is not coexpressed with these keratins in vivo, the type II simple epithelial keratins can use 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 their endogenous keratin filament network (Giudice and Fuchs, 1987; Albers and Fuchs, 1987).

Deletion constructs of pJK14·P ranging from NΔ50 to NΔ169 were transfected into PtK2 cells, and keratins were extracted 65 h posttransfection. When isolated keratins were resolved by SDS-PAGE and subjected to silver stain and im-

Figure 3. Detection of mutant keratin proteins expressed in transfected PtK2 cells. PtK2 cultures were transfected with the various mutant cDNAs and at 65 h posttransfection, intermediate filament proteins were isolated and resolved by SDS-PAGE. *A* shows a silver-stained gel. *B* is an immunoblot of an analogous gel. The antibody against substance P was used to visualize the transfected gene products. Keratin extract in lane *1* was from cultured human epidermal cells. Other IF extracts are from PtK2 cells transfected with: lane *2*, no DNA; lane *3*, pJK14·P; lane *4*, NΔ50pJK14·P; lane *5*, NΔ79pJK14·P; lane *6*, NΔ89pJK14·P; lane *7*, NΔ101pJK14·P; lane *8*, NΔ107pJK14·P; lane *9*, NΔ117pJK14·P; lane *10*, NΔ131-pJK14·P; lane *11*, NΔ150pJK14·P; lane *12*, NΔ169pJK14·P. Molecular mass markers are shown in kilodaltons at left.

munoblot analyses, a single major discrete protein band was detected in each extract from transfected PtK2 cells (Fig. 3). 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. Since only ∼5% of the cells generating the extracts in Fig. 3 were transfected, the amount of mutant protein relative to wild-type keratins was remarkably high (Fig. 3 *A*). However, it was impossible to quantitate the ratio of mutant to endogenous keratin in the transfected cells, because the number of plasmid DNAs taken up by each transfected cell was variable.

K14·P Mutants Missing Various Portions of the Nonhelical Amino-Terminal Domain Incorporate Readily into the Keratin Network of PtK2 Cells

To determine whether removal of a portion of the nonhelical amino-terminal domain of K14·P can interfere with its ability to integrate into a keratin filament network, we transfected PtK2 cells with NH₂-terminal deletion constructs of pJK14·P ranging from NΔ50 to NΔ10T. At 65 h posttransfection, keratin networks were examined by indirect immunofluorescence. A rat monoclonal antibody (NCI/34) against substance P was used to detect the presence of K14·P (Cuello et al., 1979), and a mouse monoclonal antibody (LE41) against K8 was used to visualize the endogenous keratin filament network (Lane, 1982). These antibodies were specific for their target proteins.

As illustrated in Fig. 4, *a* and *b*, the complete K14·P incorporated into the endogenous keratin filament network of the PtK2 cell without perturbing it and anti-K8 and anti-P showed colocalization (see also Albers and Fuchs, 1987). Moreover, NH₂-terminal mutants of K14·P missing as many as 107 residues (NΔ107 pJK14·P) also incorporated into the PtK2 keratin network with no apparent effect upon cytoskeletal morphology (*c* and *d*). These results are consistent with earlier in vitro studies by Kaufmann et al. (1985), demonstrating that a thrombin-generated NH₂-terminal deletion of desmin lacking 67 residues still coassembled with wild-type desmin into normal 10-nm filaments. Our data not only extend these studies for a second IF protein class, but they also further reduce the limits of NH₂-terminal deletions yielding wild-type IF networks to a mere eight amino acid residues before the apparent start of the first α-helix (see Fig. 2).

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

In contrast to the behavior of mutants lacking portions of the K14 nonhelical amino-terminal domain, mutants missing portions of the α-helical segment caused marked disruptions in the keratin filament network of the PtK2 cells. This could be seen even with a mutant NΔ117 pJK14·P, missing only the first two residues (Val Thr) at the extreme end of the first helical domain (see Fig. 2 for details of the mutants). While ∼15% of the NH₂-terminal mutants showed a seemingly wild-type phenotype, most transfected cells displayed clear abnormalities in their keratin filament network. Interestingly, a wide range of aberrant phenotypes were observed for each NH₂-terminal mutant examined (Figs. 5 and 6). While the percentage of cells showing a particular phenotype varied somewhat among NH₂-terminal mutants, all mutants from
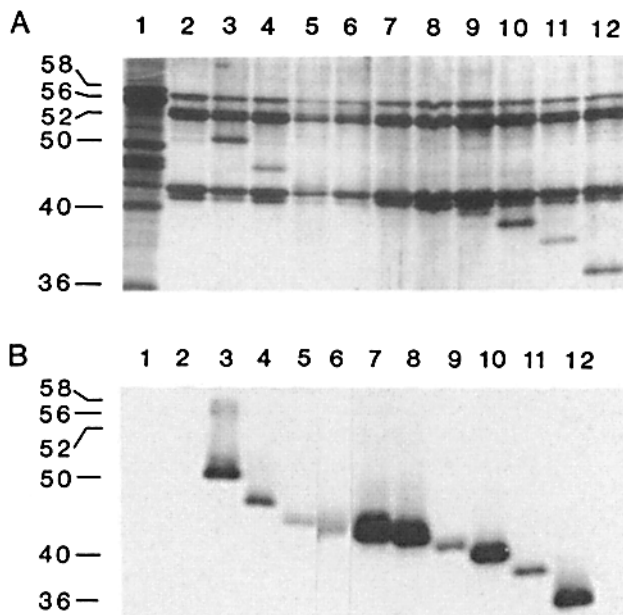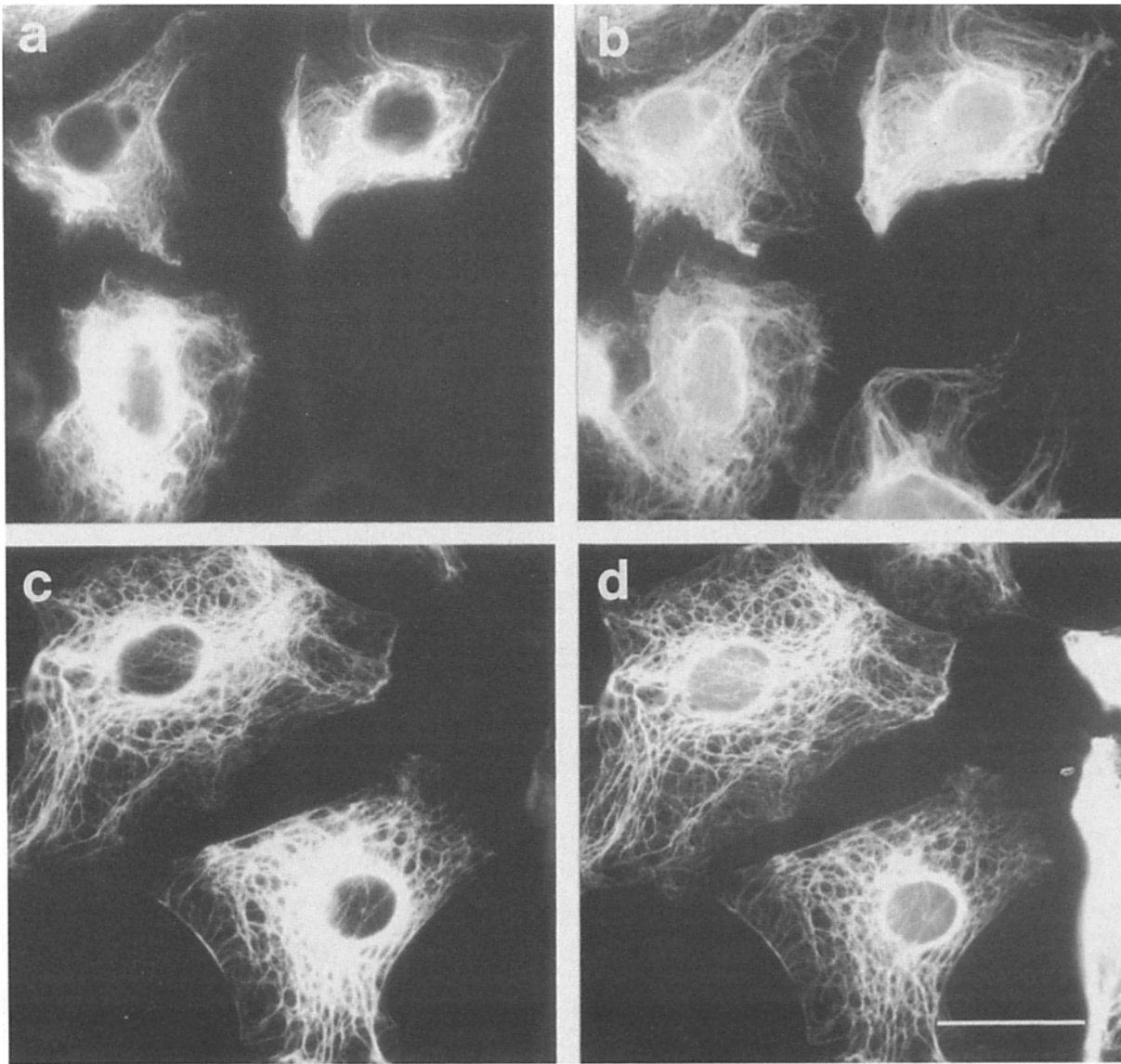
Figure 4. K14·P and amino terminal deletion mutants of K14·P incorporate into the endogenous keratin network of transfected PtK2 cells without disrupting it. PtK2 cells were transfected with plasmid pJK14·P and derivatives of pJK14·P containing various length deletions of the amino-terminal end. Cells were fixed at 65 h posttransfection, 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 (NC1/34; referred to as anti–P) recognizing the carboxy-terminal sequence of substance P (*a* and *c*). Antibody staining was followed by FITC-conjugated goat anti–rat IgG. The endogenous simple epithelial keratin network was detected using a mouse monoclonal antibody (LE41) specific for keratin K8, followed by Texas red–conjugated goat anti–mouse IgG (*b* and *d*). Cells transfected with pJK14·P had a normal keratin filament network as illustrated in *a* and *b*. Cells transfected with NΔ50, NΔ79, NΔ89, NΔ101, and NΔ107pJK14·P derivatives also showed incorporation of the mutants without perturbing the normal filament morphology (*c* and *d*; particular example shown is NΔ107). In all cases, colocalization with the endogenous keratin network was observed. Bar, 20 μm.

NΔ117 to NΔ169 generated a similar range of morphologies. The mildest deviation from a wild-type IF network seemed to be a withdrawal of the network from the plasma membrane (Fig. 5, *a* and *b*). Some cells showed a keratin filament network that appeared as a collapsed coil of filaments (*c* and *d*).

In these cases, the coil of filaments was usually associated with the nuclear membrane, apparently anchored via a discrete attachment site. In other cases, the collapsed filaments appeared as a ring surrounding the nucleus (*e* and *f*). Sometimes, a more severe distortion of the filaments occurred

Figure 5. Mutants lacking various portions of the first and second α-helical domains perturb the PtK2 keratin filament network. PtK2 cells were transfected with NΔ150 pJK14·P (*a* and *b*, *e* and *f*), NΔ117 pJK14·P (*c* and *d*), or NΔ169 pJK14·P (*g* and *h*). After transfection, cells were fixed and costained with anti–P (*a*, *c*, *e* and *g*) and anti–K8 (*b*, *d*, *f*, and *h*) as described in the legend to Fig. 4. Cells were examined at 65 h after DNA addition except for NΔ169 (*g* and *h*) which was fixed at 135 h posttransfection. Bar, 20 μm.
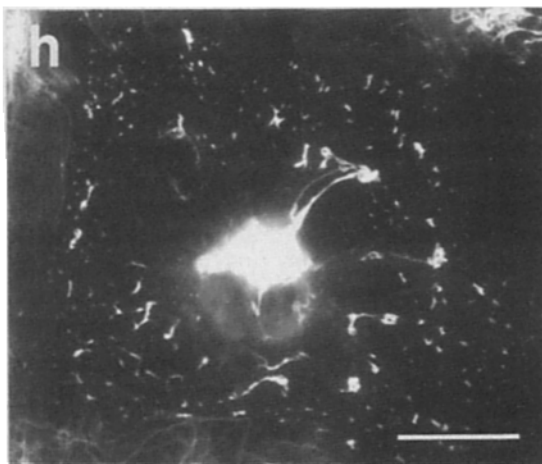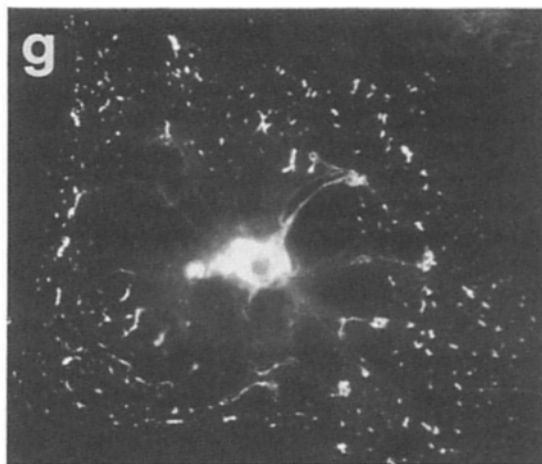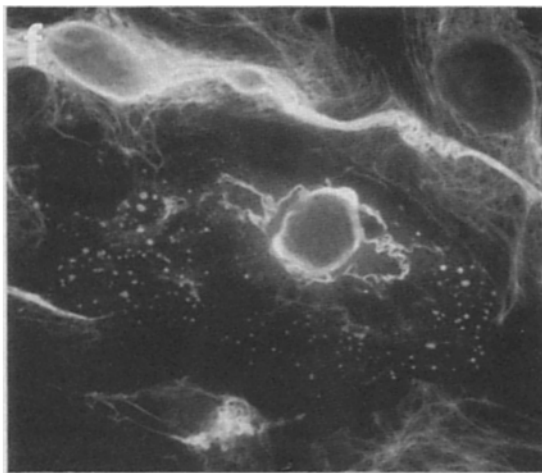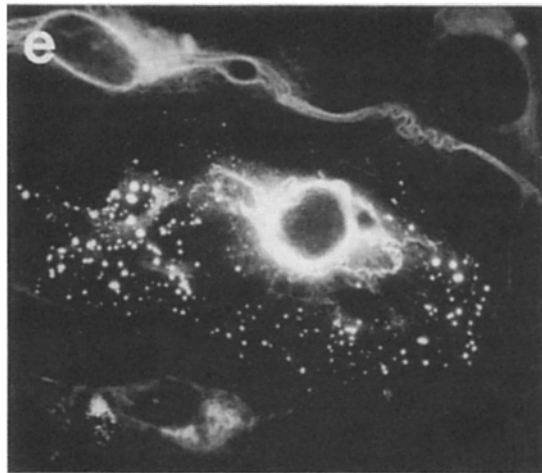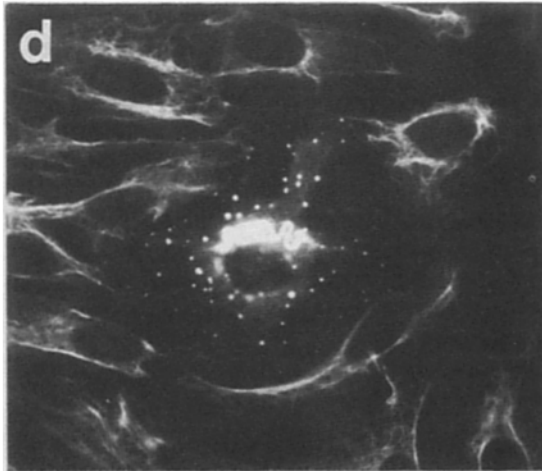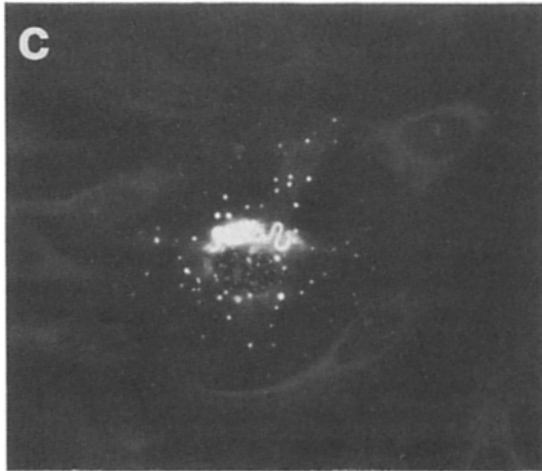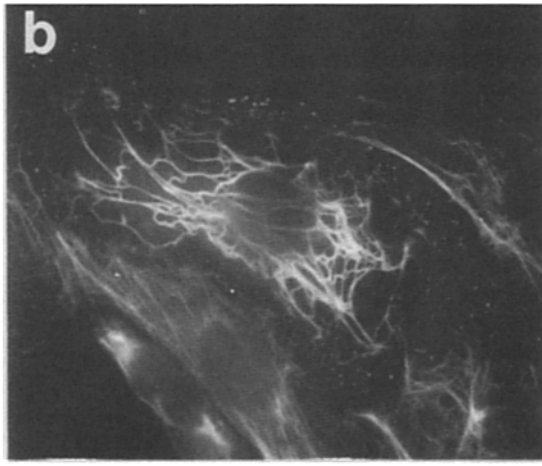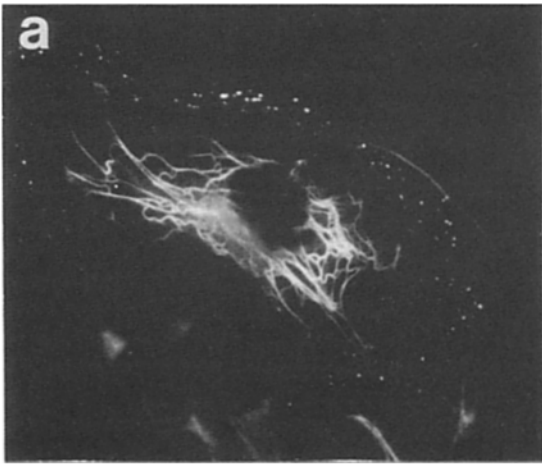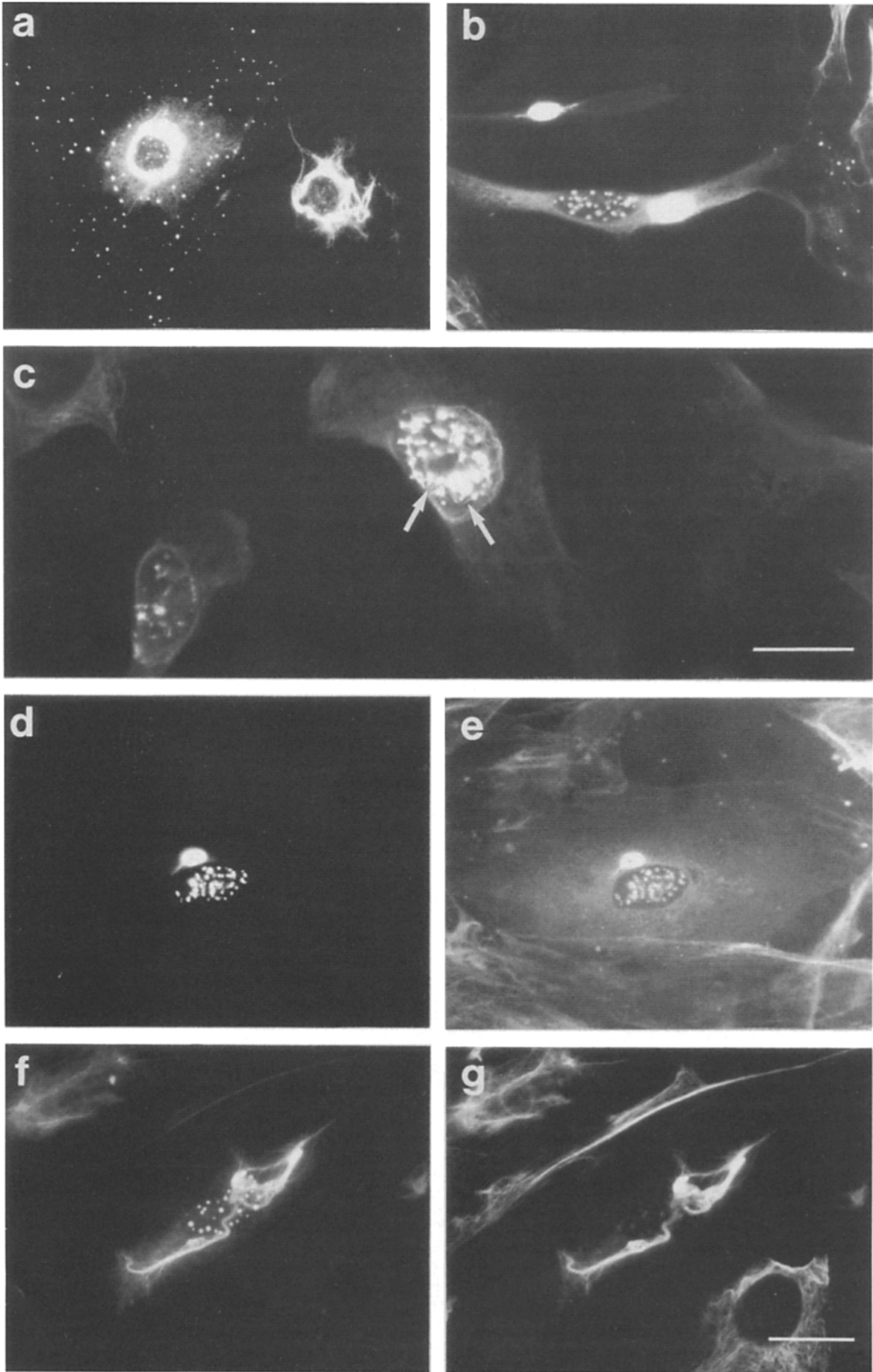
leaving very thin bundles of jagged fibers (e.g., NΔ169; g and h).

The "milder" aberrant keratin filament architectures shown in Fig. 5 were very similar to those observed previously for some carboxy-terminal K14·P mutants (Albers and Fuchs, 1987). This was particularly true for a mutant, CΔ50 K14·P, lacking nine amino acid residues at the end of the fourth alpha-helical domain (Albers and Fuchs, 1987). In fact, for CΔ50 K14·P mutants, almost 85% of the transfected cells showed a phenotype with a collapsed coil of filaments adjacent to the nucleus (similar to that seen in Fig. 5, c and d). In contrast, this morphology as well as other milder perturbations of the keratin filament networks (see example in Fig. 5, a and b) were relatively rare for the NΔ117–NΔ169 mutants.

While the less severe phenotypes were largely similar for both NH₂-terminal and COOH-terminal mutants, the most aberrant phenotypes displayed by these two classes of mutants were strikingly different. Hence, for cells expressing CΔ73 K14·P or CΔ135 K14·P, the keratin network often disintegrated into large cytoplasmic aggregates (Albers and Fuchs, 1987). For cells expressing NΔ117 K14·P or NΔ169 K14·P, however, the most commonly seen severe phenotype was one where anti–P staining occurred in the form of nuclear aggregates (Fig. 6). These often first appeared as small punctate spots in conjunction with a collapsed nuclear network of IFs (a). In cases with little evidence of intact keratin filaments, aggregates were usually larger and more prominent (b). In some cases, small filamentous structures protruded from these aggregates (c).

The punctate aggregates of keratin often costained with both anti–P and anti–K8 antibodies (d and e). However, in some cases, antibodies against the endogenous keratin network did not stain these aggregates (f and g), suggesting the possibility that the mutants may preferentially associate with these nuclear structures. In other cells, very little staining was observed with LE-41 or any other antibodies to the endogenous simple epithelial keratins. Since LE-41 shows pronounced staining in untransfected cells, it seems that in some transfected cells, either extensive masking of antigenic determinants takes place for simple epithelial keratins but not for substance P, or that simple epithelial keratins are preferentially degraded at a faster rate than the NH₂-terminal mutants of K14·P.

Occasionally, cells transfected with carboxy-terminal mutants displayed punctate nuclear staining, and some cells transfected with amino-terminal mutants showed punctate cytoplasmic staining. However, these cases were relatively rare, and the phenotypes were usually less pronounced than the corresponding morphologies exhibited by their mutant counterparts. Thus, whereas the most common pattern of

anti–P staining for COOH-terminal mutants appeared to be punctate and cytoplasmic, a typical pattern of anti–P staining for the NH₂-terminal mutants was punctate and nuclear.

The cytoplasmic aggregates are most likely associated with the inner surface of the plasma membrane (Albers and Fuchs, 1987). Conversely, the absence of recognizable nuclear targeting signals in the keratin sequences, coupled with the cytoplasmic nature of the wild-type keratin network makes it most likely that the nuclear aggregates of keratins accumulate on the surface rather than inside the nucleus. Electron microscopy will be necessary to confirm these assumptions.

Unravelling a Possible Temporal Progression of Mutant Keratin Phenotypes

The collection of NH₂-terminal mutant phenotypes was observed whether the K14·P constructs were transfected into simple epithelial cells, where the endogenous K14 gene is not expressed (Figs. 5 and 6), or epidermal cells, where the endogenous K14 gene is expressed (data not shown). However, in contrast to simple epithelial cells, epidermal cell transfection led to the generation of a higher proportion of phenotypes similar to those shown in Fig. 5, with fewer cases representing the morphologies seen in Fig. 6. Since keratins account for up to 30% of the total cellular protein in epidermal cells and only 1–2% of the total protein in simple epithelial cells, it is likely that the proportion of mutant K14·P protein relative to wild-type keratin is higher in most transfected simple epithelial cells than in transfected epidermal cells. Thus for PtK2 cells, the phenotypes seen in Fig. 5 might occur more frequently at early times after transfection, when the ratio of mutant/wild-type keratin is low, whereas the phenotypes seen in Fig. 6 might occur predominantly at later times, when this ratio is higher. An examination of PtK2 cells at t = 24, 48, 65, 92, and 135 h after transfection with NΔ117 K14·P tended to support this notion. However, cells with plasma membrane–retracted IF networks were still seen as late as 92 h posttransfection and cells with punctate nuclear aggregates were seen as early as 24 h posttransfection. It seems most likely that these exceptions represent transfected cells harboring foreign K14·P plasmids at unusually low or high levels, respectively. Moreover, since the cell cycle of PtK2 cells went from ∼30 h before transfection to ∼48–60 h posttransfection, and since each phenotype could be found in isolated transfected cells, the variation in phenotypes could not be explained by cell cycle differences in the transfected population. This was unlikely in the first place, since the keratin filament network in PtK2 cells has been shown to remain intact even during mitosis (Franke et al., 1978; Aubin et al., 1980).

Figure 6. Amino terminal mutant proteins associate with the nucleus and form punctate aggregates. PtK2 cells were transfected with NΔ117 pJK14·P and fixed at various times after transfection. Cells were examined using double-indirect immunofluorescence with anti–P (a, b, d, and f) and anti–K8 (e and g). a shows small nuclear aggregates of transfected mutant keratins, generally present at early times (14–65 h) posttransfection. At these early times, perinuclear whorls of filaments and small cytoplasmic punctate staining was frequently observed. b shows larger nuclear aggregates of mutant keratins often seen at 65–135 h posttransfection. At these late times, only very few cases of recognizable filaments or cytoplasmic punctate staining were seen. c shows a 65-h posttransfection example of nuclear aggregates stained with anti–P at higher magnification. Arrows denote the presence of short filamentous stubs associated with the aggregates. Nuclear aggregates usually colocalized with endogenous keratins (d and e) though in some cells, colocalization did not occur (f and g). Bars, 10 μm.

Defining the Limit Sequence Necessary for Incorporation of a Keratin into a Wild-Type Network

The largest of our NH$_2$-terminal mutants that incorporated into the endogenous simple epithelial keratin network without disrupting it was NΔ107, while the largest of the COOH-terminal mutants showing the same phenotype was CΔ42 (Albers and Fuchs, 1987). To determine whether a K14·P construct missing sequences coding for 107 NH$_2$-terminal residues and 42 COOH-terminal residues was still capable of incorporating into the endogenous keratin network without disrupting it, we prepared the construct NΔ107/CΔ42 and transfected it into PtK2 cells. Fig. 7 a illustrates the construct. Fig. 8 a shows that this construct is capable of integrating into a simple epithelial keratin network with no apparent effect on the morphology of the transfected cells.

Combinations of headless and tailless mutants involving the removal of even a few amino acid residues from either the fourth alpha-helical domain (NΔ107/CΔ50; Fig. 7 b), the first alpha-helical domain (NΔ117/CΔ42; Fig. 7 c), or both (NΔ117/CΔ50; Fig. 7 d) led to abnormal keratin filament networks. Providing that only a few residues were removed from either end of the helical domain, the differences in phenotypes generated by headless and tailless double mutants were not great. All three of the double mutants described in Fig. 7, b–d, generated phenotypes typical of those seen in Fig. 8, b–d.

In summary, these data indicate that the boundaries of K14 sequence which are essential for normal incorporation into a preexisting simple epithelial keratin network must be within residues 107 and 117 at the amino-terminal end and 415 and 423 at the carboxy-terminal end. Moreover, the fact that the NΔ107/CΔ50 mutant showed a greater percentage of cells with punctate nuclear aggregates than the CΔ50 mutant alone suggests that while the nonhelical amino terminal domain does not seem to play an essential role in enabling a keratin to integrate into an IF network, it may nevertheless influence the overall keratin filament network within a cell.

NH$_2$-Terminal Mutants Do Not Recover, Whereas COOH-Terminal Mutants Do

Previously, we showed that at late times after transfection when mutant keratin gene expression appears to wane (Gorman, 1985), cells that had been expressing COOH-terminal mutants show a restoration to their wild-type phenotype (Albers and Fuchs, 1987). In the first stages of the recovery process, a cage of newly synthesized keratin filaments appeared around the nucleus, while previously synthesized K14·P mutants were only found in the cytoplasm, apparently in a form
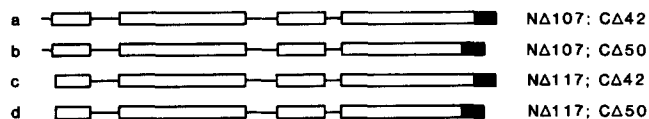
Figure 7. Constructs of K14·P derivatives with amino- and carboxy-terminal deletions. For each construct, white boxes represent the α-helical domains of the K14·P protein while the black box represents the substance P tag. Constructs were prepared as indicated in Materials and Methods.

that was incapable of interfering with assembly at the nucleus.

To determine whether cells transfected with NH$_2$-terminal mutants also showed a recovery phenomenon, we examined their behavior at 92 h posttransfection. Surprisingly, in contrast to cells transfected with COOH-terminal mutants, cells transfected with NH$_2$-terminal mutants showed no evidence of recovery at these late times. Even after 135 h, most cells staining with anti-P still exhibited punctate nuclear staining with no indication of de novo synthesis of a wild-type keratin IF network. Since a priori, it seems that the major phenotypic difference between NH$_2$-terminal and COOH-terminal K14·P-transfected cells is that the NH$_2$-terminal mutants localize at the nucleus while COOH-terminal mutants aggregate in the cytoplasm, the simplest explanation for the failure of NH$_2$-terminal K14·P-transfected cells to regenerate a keratin filament network is that the nuclear location of the mutant prevents the recovery process.

Do NΔX Mutants Affect Desmosome–IF Interactions?

In epidermal cells, where keratins are especially abundant, 8-nm filaments show a strong interaction with the desmosomal plaques at the plasma membrane (Drochmans et al., 1978; Denk et al., 1985). To determine whether desmosomes might be disrupted in epidermal cells transfected with NH$_2$ helical mutants, we introduced these constructs into SCC-13, a squamous cell carcinoma line of epidermal origin (Wu and Rheinwald, 1981). At 65 h posttransfection, cells were stained with both anti-P and a mouse monoclonal antibody against human desmosomal protein. Similar to PtK2 cells, SCC13 cells expressing NH$_2$-helical mutants showed a withdrawal of the keratin filament network away from the plasma membrane (Fig. 9 a, arrow). Nevertheless, the degree of antidesmosomal staining of transfected cells was still prominent (see corresponding region in b). While at first glance the desmosomal plaques in transfected cells often seemed to be more diffuse than those of adjacent untransfected cells, the transfected cells were usually suprabasal, and the larger suprabasal cells of the population often showed such diffuseness. This was presumably because the desmosomal attachments were to cells beneath rather than adjacent to the suprabasal cell. These data are consistent with previous studies (Kartenbeck et al., 1982; Denk et al., 1985), and suggest that the organization of desmosomal structures may be at least partially independent of their association with keratin filaments.

Despite the near complete disruption of the keratin filament network in some NΔX K14·P transfected cells, a few associations between keratin and desmosomes remained (c and d, arrows). Interestingly, these associations were frequently in regions where evidence of tight desmosomal plaques was lacking (see example in c and d). Even more striking was the observation that the keratin networks in neighboring untransfected cells sometimes appeared to be disordered as a consequence of mutant keratin expression in an adjacent cell (f, arrows). This disruption was readily apparent, since cells within a normal (untransfected) population of keratinocytes typically show networks of 8-nm filaments which are juxtaposed in a precise mirror image fashion to the 8-nm filaments of neighboring keratinocytes. The fact that desmosomes and mutant keratins do associate, coupled
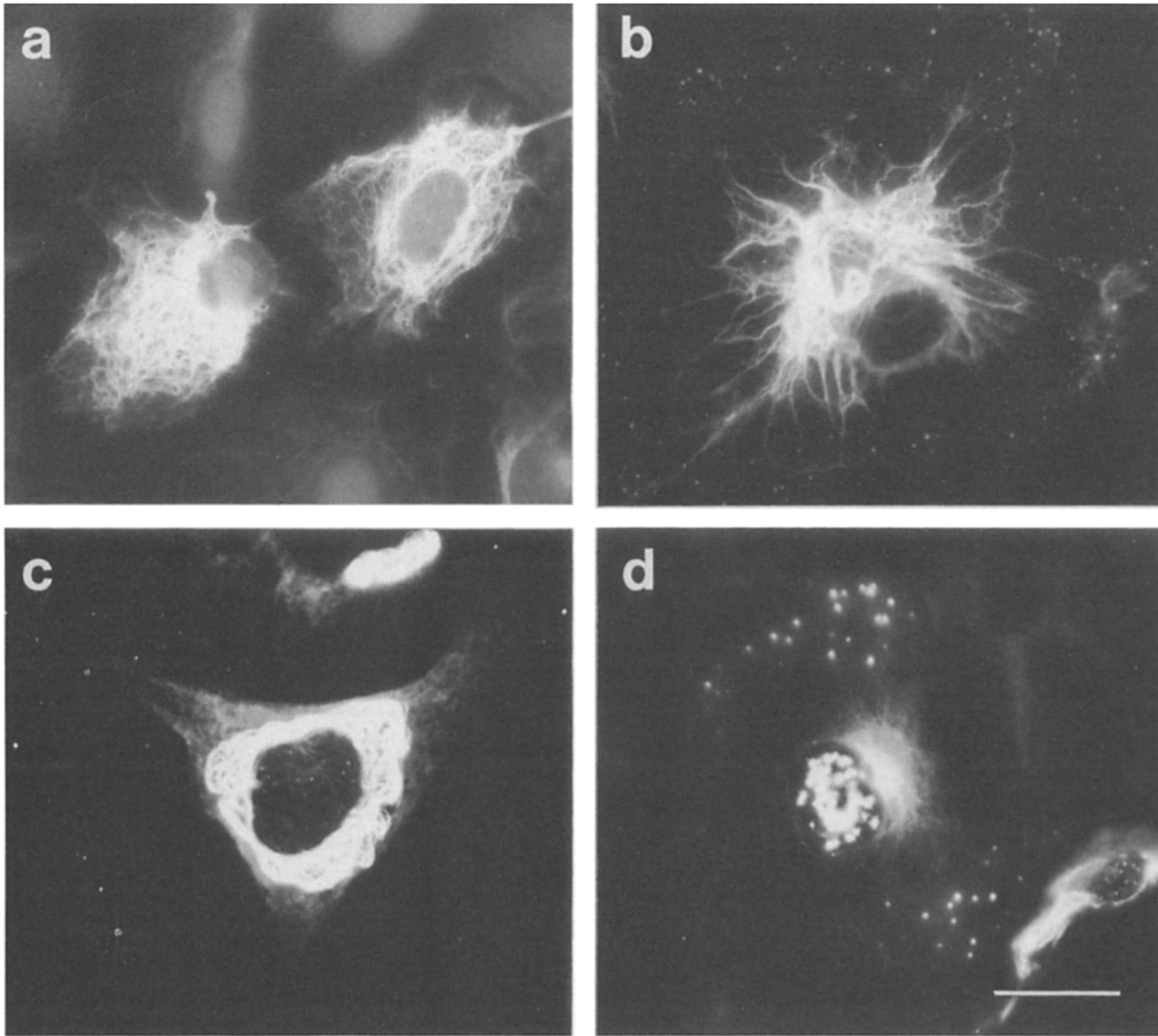
Figure 8. Transfection of plasmids lacking both amino and carboxy K14·P sequences defines the limit domain necessary for formation of normal keratin multimer subunits. (*a*) Anti-P staining of PtK2 cells transfected with NΔ107/CΔ42 pJK14·P. (*b–d*) A range of representative phenotypes observed for PtK2 cells transfected with the headless and tailless α-helical mutants described in Fig. 7 (*b–d*) and stained with anti-P. Specific examples shown are from cells transfected with (*b*) NΔ107/CΔ50 pJK14·P, (*c*) NΔ117/CΔ50 pJK14·P, and (*d*) NΔ117/CΔ42 pJK14·P. All three phenotypes in *b–d* were seen irrespective of which of the three α-helical mutants was used for transfection. Bar, 20 μm.

with the observation that an intermediate filament network in an adjacent cell can be altered by disrupting the IF network in a neighboring cell, mutant keratin is suggestive that some intercellular sensing device was triggered upon expression. Since the major integrators of keratin networks within a sheet of epidermal cells are the desmosomes, it seems inevitable that such a sensory device would involve a component of the desmosome. Hence, while our immunohistochemical methods did not detect gross alterations in the desmosomes as a consequence of mutant keratin expression, aggregates of mutant protein retain their association with the desmosome and may cause subtle perturbations that are transmitted to adjacent cells. Additional investigations will be necessary to examine the nature of these changes.

Do NΔ117 Mutants Affect the Nuclear Lamin Structure or the Nuclear Pore Networks?

The formation of keratin aggregates at the nuclear surface of NΔ117 transfected cells led us to wonder whether their presence might perturb other nuclear structures; e.g., the meshwork of lamin filaments at the inner surface of the nucleus, or the nuclear pores. To examine these possibilities, we transfected PtK2 cells with NΔ117 K14·P and then costained with anti-P and a general anti-lamin antiserum or a monoclonal antibody to nuclear pore complexes. No changes in the pattern of anti-lamin staining or anti-nuclear pore staining were observed in cells transfected with NΔ117 K14·P. In cells where anti-P revealed a nuclear punctate staining for
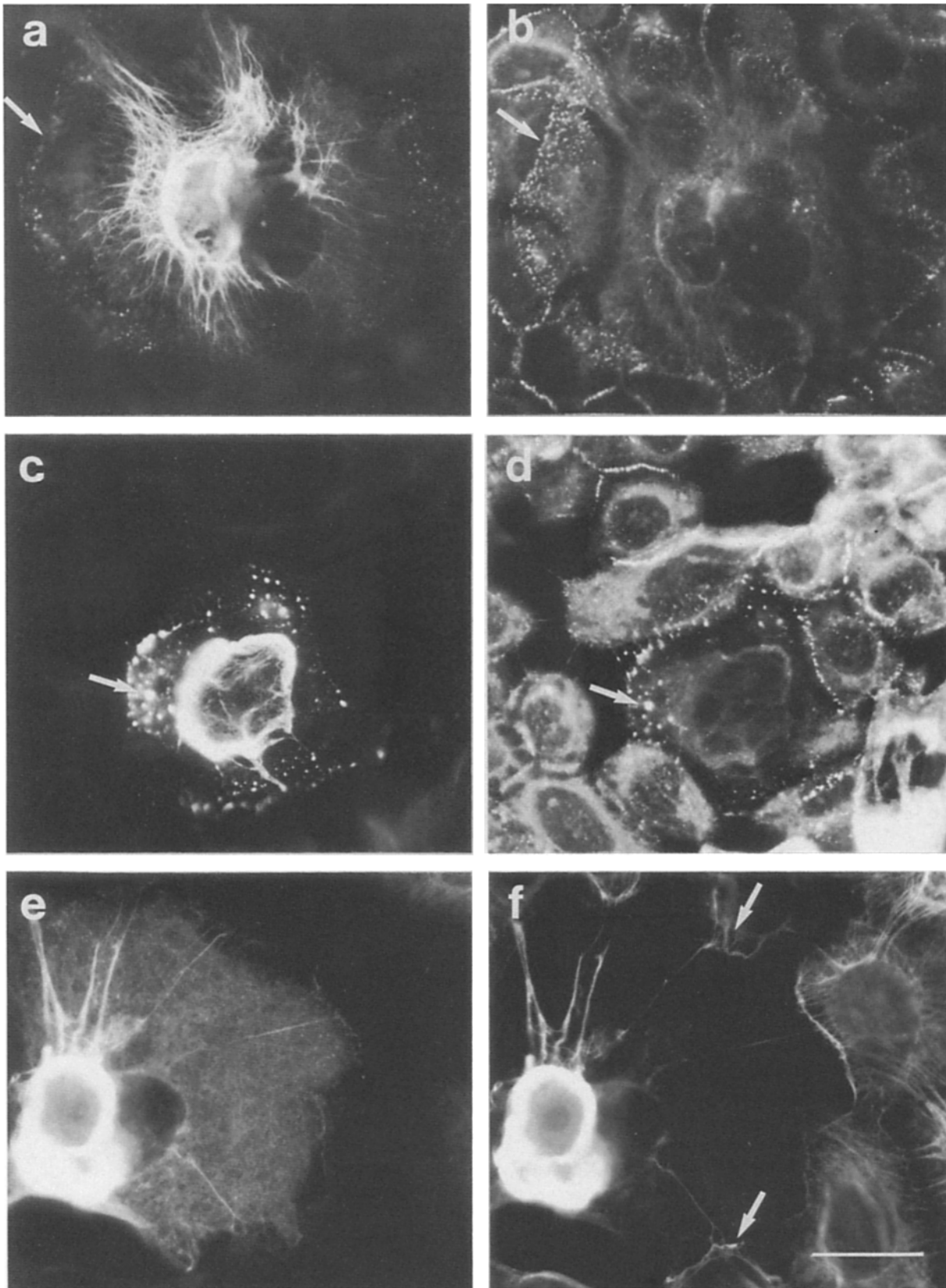
Figure 9. Altered keratin filament networks in squamous cell carcinoma cells transfected with amino-terminal α-helical mutants of pJK14·P. SCC-13 cells were transfected with either NΔ117/CΔ42 pJK14·P (*a* and *b*), NΔ150 pJK14·P (*c* and *d*), or NΔ117/CΔ50 pJK14·P (*e* and *f*). At 65 h posttransfection, cells were fixed and costained with anti–P (*a, c,* and *e*), and either anti–desmosome antibody (*b* and *d*) or antiserum against epidermal type II keratins (*f*). Arrow in *a* shows depletion of keratin filaments near the cytoplasmic membrane of a transfected suprabasal cell, while desmosomal proteins still appear in abundance at the periphery of this cell (*b, corresponding arrow*).
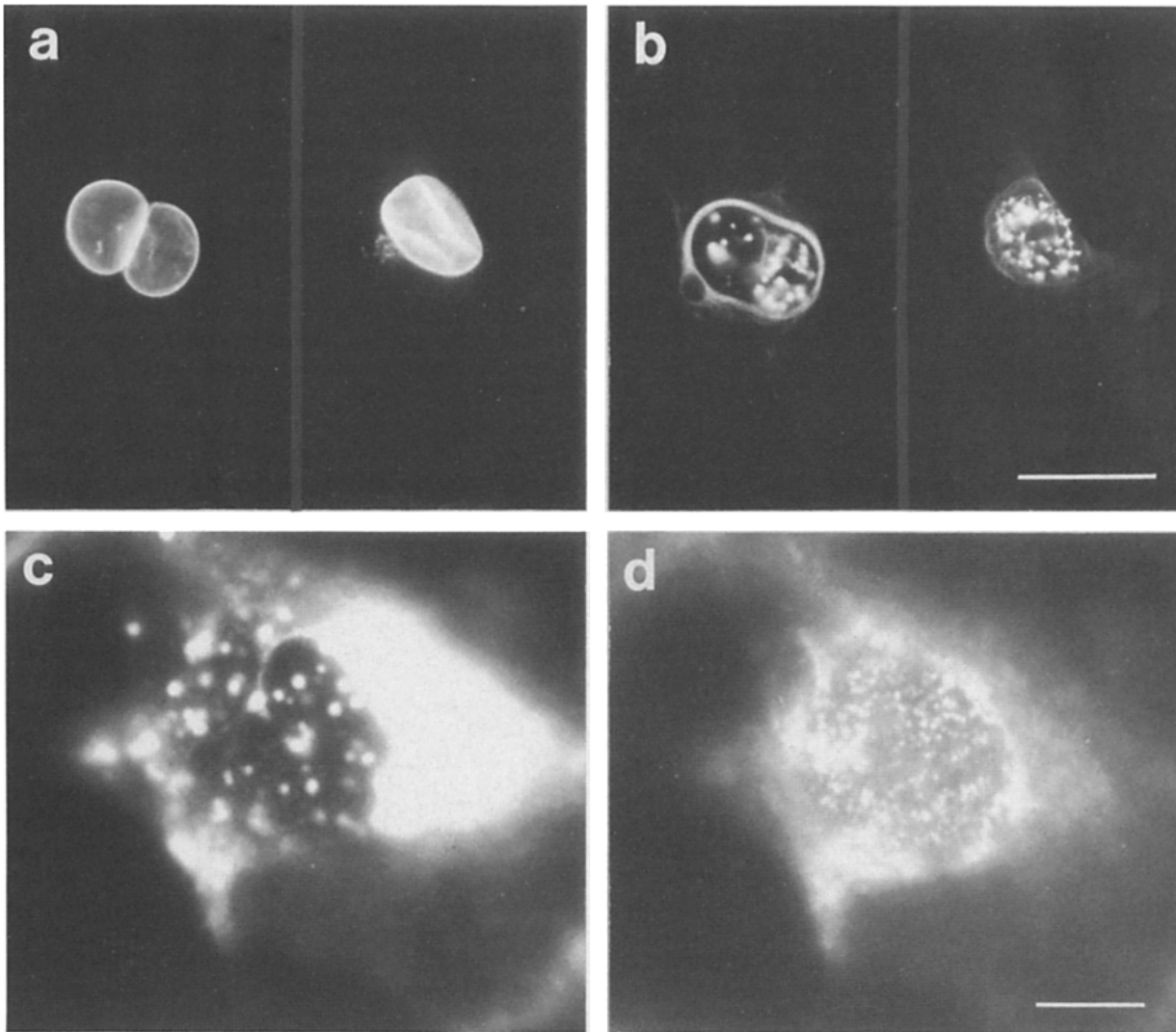
Figure 10. Gross alterations in nuclear structures do not occur as a consequence of forming nuclear aggregates of mutant keratins. (*a* and *b*) PtK2 cells were transfected with NΔ117/CΔ42 pJK14·P. At 135 h posttransfection, cells were fixed and stained with a general anti–lamin antibody (*a*) and anti–P (*b*). (*c* and *d*) PtK2 cells were transfected with NΔ117/CΔ50 pJK14·P. At 65 h posttransfection, cells were fixed and stained with anti–P (*c*) and anti–nuclear pore antibody (*d*). Bars: (*a* and *b*) 15 μm; (*c* and *d*) 5 μm.

keratins, the anti–lamin staining still appeared diffuse (Fig. 10, *a* and *b*). Similarly, the punctate pattern of keratin aggregation did not appear to influence the punctate pattern of nuclear pore distribution (Fig. 10, *c* and *d*). The failure to see unequivocal colocalization of keratin aggregates with either nuclear lamin filaments or pore complexes precluded our ability to determine whether these aggregates associate with either of the two nuclear structures. However, we can say that obliteration of the keratin network and the accumulation of nuclear aggregates of keratin did not interfere in a gross fashion with the organization of either the lamin meshwork or the nuclear pores.

Discussion

Helical Sequences Seem to be Sufficient for Multimer Subunit Formation

In this study, we examined contributions that the amino- and carboxy-terminal portions of a keratin sequence make in defining the overall keratin filament network within a cell. Previously, in an examination of carboxy-terminal mutations, we had shown that tagging a K14 cDNA with a small neuropeptide sequence enabled us to trace the expression of K14 and its mutant derivatives both in simple epithelial cells, where the gene is not normally expressed, and in epidermal

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Arrows in *c* and *d* depict colocalization with anti–P and anti–desmosomal antibodies, respectively. In this transfected suprabasal cell, regions that costained did not show evidence of tight desmosomal junctions. Arrows in *f* illustrate untransfected cells whose intermediate filament networks were altered apparently as a consequence of mutant keratin expression in the adjacent transfected cell. Bar, 20 μm.

cells, where the expression of K14 accounts for ~7% of the total protein of the cell (Albers and Fuchs, 1987). 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.

The results of our transfection studies with the mutant keratin NΔ107 K14·P confirmed and extended earlier findings concerning the role of the nonhelical amino-terminal end of IF proteins in filament formation. In fact, nearly the entire amino-terminal domain could be removed without perturbing the ability of K14 to incorporate into keratin filaments of both simple epithelial and epidermal origin. Moreover, we discovered that a mutant K14·P construct, NΔ107/CΔ42 K14·P, missing both the amino and the carboxy nonhelical domains, was still competent to integrate into a keratin filament network without any gross perturbation on the network.

The finding that a headless and tailless type I keratin protein can integrate into a preexisting keratin network suggests strongly that the structure of the largest multimer subunit which typically incorporates as a unit into a growing keratin filament must be very nearly wild type even in cells where this mutant comprises a part of the multimer unit. With regards to the formation of coiled-coil dimers, this result may have been expected, since the sequences predicted (by secondary structure analyses) to be involved in the coiled-coil are not affected in the NΔ107/CΔ42 mutant. However, our results further predict that the interactions necessary to form tetramers and even higher ordered subunits must also have occurred normally even though some of the keratin polypeptides in these structures were headless and tailless. Hence our findings suggest that the α-helical domains of IF proteins may be largely responsible not only for coiled-coil interactions, but also for higher ordered subunit interactions within the intermediate filament. This notion is consistent with in vitro binding studies demonstrating that under conditions favoring dimer formation, proteolytically prepared type I helical segments can interact with nitrocellulose-immobilized type II helical segments to form apparent heterotypic tetramers (Hatzfeld et al., 1987). This finding is also reasonable considering that the end domains of IF proteins tend to be hypervariable in sequence, making it unlikely that they play a role in multimeric subunit formation.

Even though the helical domains of K14·P may be sufficient for formation of higher ordered subunit structures, our results do not necessarily imply that the nonhelical sequences are irrelevant in the assembly process. Indeed, it could be that the presence of endogenous type I and type II keratins during the assembly process obscured a role for the end domains in, e.g., stabilizing lateral and/or end-to-end interactions necessary to pack the subunits into an 8-nm filament. Indeed, the fact that an NΔ67 desmin fragment coassembles with wild-type desmin in vitro, but does not form IFs on its own is suggestive that the nonhelical NH2-terminus may be involved in such interactions (Kaufmann et al., 1985). Similarly, using enzymatic methods, Steinert et al. (1983) have observed disassembly of keratin filaments whose subunits have lost much of their nonhelical sequences through chymotryptic digestion. To evaluate more precisely the role of nonhelical end domains in filament stabilization, it will be necessary to conduct in vitro filament assembly studies with purified mutant and wild-type keratins.

What Is the Molecular Nature of Associations between Keratins and Other Cellular Elements?

Previous morphological and biochemical studies have suggested that keratins interact with desmosomal plaques in the plasma membranes of keratinocytes (see for example Drochmans et al., 1978). In addition, Kartenbeck et al. (1982) and Denk et al. (1985) have demonstrated that the organization of desmosomal structures may be independent of their association with keratin filaments. Our studies confirm these earlier observations, but in addition suggest that the keratin filament networks among cells of an epidermal sheet may be interconnected, since mutant keratin-triggered disruption of an IF network in one cell can result in altered filament networks within a neighboring cell. Presumably, this linkage is an indirect one, and is mediated via the desmosomal plaques. While such a connection has always been implied by the observation that keratin filaments in one cell seem to line up in perfect mirror image arrays to those filaments in an adjacent cell, our experiments provide a direct demonstration of this penetration.

Keratin filaments also interact with the nuclear envelope (Eckert et al., 1982; Albers and Fuchs, 1987; Georgatos and Blobel, 1987a,b; Georgatos et al., 1987). Recent in vitro studies have revealed IF-nuclear associations between type III IF proteins (desmin and vimentin) and lamin B (Georgatos and Blobel, 1987a,b; Georgatos et al., 1987). A priori it might seem unlikely that the IF-nuclear associations that we have observed with our NH2-terminal mutants are analogous to those observed by Georgatos et al. since (a) nuclear staining with a general lamin antibody remained diffuse in transfected cells displaying anti-P staining nuclear aggregates, and (b) associations with lamin B and vimentin/desmin were via the nonhelical carboxy domain of the IF protein (Georgatos and Blobel, 1987a,b; Georgatos et al., 1987), whereas our headless and tailless K14·P mutant still showed nuclear localization. However, in our transfected PtK2 cells, interactions clearly took place between mutant keratins and wild-type keratins, and this precludes our ability to determine whether the nuclear associations with NH2-terminal mutants were direct or indirect in nature. Hence additional in vivo studies will be necessary to determine whether lamin B–IF interactions are physiologically relevant, and whether they include the keratin filament family.

Initiation of Keratin Filament Assembly May Be Blocked by NH2-Terminal Mutant Expression

While certain questions about filament assembly necessitate in vitro studies involving mutant keratin polypeptides, other important aspects of filament formation, such as nucleation of assembly and intracellular interactions involving keratin filaments can only be addressed using a gene transfection approach. Indeed, one of our most significant findings was the realization that amino- and carboxy-terminal helical mutants of K14·P exhibit very different phenotypes when they are expressed in either simple or stratified squamous epithelial cells: Whereas carboxy terminal mutants missing most of the fourth α-helical domain dissociate the endogenous keratin

filament network into cytoplasmic aggregates, amino-terminal mutants missing portions of the first α-helical domain often generate aggregates of keratin that appear to localize on the nuclear surface. Moreover, unlike cells transfected with carboxy-terminal mutants, which are able to recover when mutant keratin synthesis wanes 92–135 h after transfection, amino-terminal mutants seem to cause a change in the keratin filament network which is irreversible during this time frame. We have not determined the basis for the difference in cells transfected with COOH- vs. NH₂-terminal mutants. It could be that the two classes of mutants have different effects on intracellular associations between IFs and other cytoplasmic proteins or organelles. Alternatively, it may be that RNA or protein generated by one class of mutant is more stable, and that the observed differences are due to variation in mutant protein concentration within transfected cells.

Regardless of the explanation, the correlation between cytoplasmic aggregates and IF recovery versus nuclear aggregates and failure to reinitiate filament formation provides considerable insight into the process of in vivo IF assembly. Previously, we found that cells whose keratin network has been obliterated by COOH-terminal helical mutants regenerate a network of endogenous IFs around the nucleus, suggesting that the nucleation of IF assembly may occur at the nuclear surface (Albers and Fuchs, 1987). In these studies, de novo IF assembly occurred at seemingly discrete nuclear sites in cells that still contained cytoplasmic aggregates of mutant protein. Hence, the cytoplasmic aggregates must not have interfered with the reinitiation process. The fact that we did not observe recovery in cells showing nuclear aggregates of mutant protein is intriguing: if the localization of the NH₂-terminal mutants at the nucleus interferes with the initiation process, this could explain why cells transfected with NH₂-terminal mutants do not easily recover.

Keratin Mutant Phenotypes Suggest a Model for In Vivo Filament Assembly

To conduct a detailed investigation of the kinetics of filament assembly, it would be necessary to examine newly synthesized keratin subunits in a cell line that had been stably transfected with a mutant driven by an inducible promoter. Thus far, our attempts to produce such lines with a hybrid glucocorticoid-inducible promoter- (mouse mammary tumor virus LTR) keratin mutant gene have been unsuccessful, due to constitutive expression of the construct even in the absence of glucocorticoids (Giudice, G. J., and E. Fuchs, unpublished data). However, an examination of our transiently transfected cells at early and late times after transfection have provided us with some insights as to the possible sequence of events that take place after mutant keratin expression. At early times after transfection, a number of cells showed a keratin filament network which was withdrawn from the cytoplasmic membrane, with no apparent disruption in the filaments per se. In contrast at late times after transfection, a greater percentage of cells showed little or no evidence of a discernable keratin filament network, but did show nuclear aggregates of keratin. These nuclear aggregates were often present in isolated, mononucleate transfected cells, suggesting that a round of cell division was not essential to generate the phenotype. Moreover, at the earliest times that we were able to detect mutant keratin protein in a transfected cell, we

found relatively uniform staining of the keratin filament network at the level of immunofluorescence light microscopy. The simplest interpretation of these data is that the keratin filament network is a dynamic one, and that there is a progression of events that takes place within a single cell upon NH₂-terminal mutant keratin expression: a withdrawal of the keratin filament network from the cytoplasmic membrane seems to precede the disappearance of the filament network and the localization of mutant protein into nuclear aggregates.

There are few explanations that can account for the generation of a mutant phenotype with a seemingly normal keratin filament network withdrawn from the plasma membrane. The most likely possibility would seem to be that keratin filaments elongate from the nuclear membrane towards the plasma membrane, and that addition of NH₂-terminal mutants to the ends of growing filaments might block further elongation and cause a regression of the filament network towards the nucleus. Previous in vitro and in vivo studies have suggested that initiation of intermediate filament assembly occurs at the nuclear envelope (Eckert et al., 1982; Albers and Fuchs, 1987; Georgatos and Blobel, 1987a,b; Georgatos et al., 1987), and hence by whatever means of subunit addition, keratin filaments would be expected to grow in a nuclear to cytoplasmic fashion. COOH-terminal mutants might also block elongation, since some of these mutants cause the keratin network to collapse in a coil of filaments with no apparent plasma membrane affiliation (Albers and Fuchs, 1987). Interestingly, Georgatos et al. (1987) recently demonstrated an interaction between vimentin/desmin and ankyrin, a cytoskeletal protein at the inner surface of the plasma membrane. They have proposed that ankyrin might serve a natural "capping" function to block IF elongation in erythrocytes and to anchor the vimentin filaments at the plasma membrane. In our in vivo studies of NH₂-mutant transfected epidermal cells (see Fig. 9, c and d), the remains of a few keratin filaments at desmosomal junctions might suggest a similar function for desmosomes. Correspondingly, the NH₂-terminal mutants may have acted as an unnatural capping agent to block IF elongation.

If the ends of most keratin filaments were anchored (and not growing) at the plasma membrane, it then seems difficult to visualize how they could be so dramatically affected by the mutant keratins. On the contrary, the fact that many keratin filaments appear to withdraw from the plasma membrane when NH₂-terminal mutants are expressed suggests that only a few filaments may be tightly anchored, while most filaments seem to be in a continual state of growth. Clearly if this is the case, then a dynamic equilibrium must exist between filament assembly and disassembly. Hence, both the mutant keratin phenotypes and the large scale immunofluorescent labeling of filaments in cells observed at early times after transfection are supportive of the notion that the keratin filament network is not a static one in cells.

In summary, our data is consistent with the hypothesis that keratin filament assembly is initiated at the nuclear membrane at a limited number of discrete sites or organizing centers. Once initiated, filaments seem to be elongated by a dynamic process which proceeds towards the plasma membrane. While this model clearly needs to be tested in more detail by additional in vivo and in vitro studies, it serves as a base for further in vivo and in vitro experiments.

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