Assembly of Amino-Terminally Deleted Desmin in Vimentin-free Cells

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Abstract. To study the role of the amino-terminal domain of the desmin subunit in intermediate filament (IF) formation, several deletions in the sequence encoding this domain were made. The deleted hamster desmin genes were fused to the RSV promoter. Expression of such constructs in vimentin-free MCF-7 cells as well as in vimentin-containing HeLa cells, resulted in the synthesis of mutant proteins of the expected size. Single- and double-label immunofluorescence assays of transfected cells showed that in the absence of vimentin, desmin subunits missing amino acids 4–13 are still capable of filament formation, although in addition to filaments large numbers of desmin dots are present. Mutant desmin subunits missing larger portions of their amino terminus cannot form filaments on their own. It may be concluded that the amino-terminal region comprising amino acids 7–17 contains residues indispensable for desmin fila-

ment formation in vivo. Furthermore it was shown that the endogenous vimentin IF network in HeLa cells masks the effects of mutant desmin on IF assembly. Intact and mutant desmin colocalized completely with endogenous vimentin in HeLa cells. Surprisingly, in these cells endogenous keratin also seemed to colocalize with endogenous vimentin, even if the endogenous vimentin filaments were disturbed after expression of some of the mutant desmin proteins. In MCF-7 cells some overlap between endogenous keratin and intact exogenous desmin filaments was also observed, but mutant desmin proteins did not affect the keratin IF structures. In the absence of vimentin networks (MCF-7 cells), the initiation of desmin filament formation seems to start on the preexisting keratin filaments. However, in the presence of vimentin (HeLa cells) a gradual integration of desmin in the preexisting vimentin filaments apparently takes place.

I NTERMEDIATE filaments (IFs),[1] along with microtubules and microfilaments, are part of the cytoskeleton of most eukaryotic cells. The different classes of IF subunits are expressed in a more or less tissue-specific manner in the adult organ (9, 70, 71, 74). Sequence data revealed that IFs can be subdivided into six types including the lamins (type V) (21, 70) and nestin (type VI) (48).

All IF proteins share an α-helical "rod" domain of conserved secondary structure and size. This central rod, which is flanked by nonhelical end domains of variable size, sequence, and chemical characteristics (9, 21, 23, 24, 48, 70, 72, 74, 77), comprises ~310 amino acid residues for type I to IV as well as type VI, and 356 amino acids for lamins. The α-helical rod plays an important role in filament formation. Albeit several proposals have been made to explain IF

assembly (1, 9, 70, 72), the exact mechanism of filament formation is not yet fully understood.

The IF end domains are the major sites of postsynthetic modifications such as phosphorylation and limited proteolysis (9, 22, 26, 42, 55, 74). Reassembly experiments with intact and proteolytically digested IF subunits revealed that the amino-terminal domains of desmin and vimentin are involved in in vitro filament formation (30, 41, 75). Next to limited proteolysis of the amino-terminal parts of desmin and vimentin, phosphorylation of these domains might be another mechanism involved in the regulation of filament rearrangement (4, 13, 15, 17–19, 25, 26, 39, 40, 42, 47, 69). Furthermore, it has been suggested that the amino-terminal domain is involved in binding of IFs to the plasma membrane (29, 31).

Removal of the last 27 carboxy-terminal amino acids did not affect the in vitro filament-forming capacity of the desmin subunits (41). The existence of a tailless keratin (cytokeratin 19) (6) as well as transfection studies with mutant keratin cDNAs (2, 3) and modified desmin genes (37) allow the assumption that the tail domain is not an indispensable part of IF proteins. On the other hand, the carboxy-

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

terminal domain has also been suggested to be involved in binding of IFs to the nuclear lamina (29–31). Since it displays the highest variability in both length and sequence (74), it might also have some subunit-specific function.

To study expression, assembly, and possible function of IFs inside the living cell various approaches have already been undertaken. For instance IF subunits were expressed in tissue culture cells by means of IF mRNA injection (20), by transfection with IF cDNAs or genes (2, 3, 16, 32, 33, 37, 46, 54, 60), and by expressing IF genes in transgenic mice (11, 12, 45, 57). Because modifications can easily be achieved at the DNA level, transfection of modified cDNAs or genes into tissue culture cells is an excellent tool to study the role of the different IF protein domains in vivo. A number of such studies has already been published (2, 3, 37, 46, 51). However, we assume that in the transfection experiments described previously (2, 3, 37), the effects on filament formation caused by deletions in the IF subunits were masked by the presence of intact endogenous IFs. To avoid this we established a vimentin-free in vivo test system for the expression of intact and mutant desmin genes. In this expression system, the mutant desmin subunits form homopolymeric IFs. No copolymerization can occur with endogenous IF subunits. Our in vivo assembly study with normal and amino-terminally deleted desmin subunits shows a number of differences with the previous in vivo IF assembly study in cells containing intact IFs (3). The use of a vimentin-free expression system also allowed us to study the initiation of (type III) IF assembly in the absence of preexisting IFs into which the newly synthesized subunits can be incorporated.

Materials and Methods

Plasmid Construction

In general, the procedures suggested by Maniatis et al. (52) were followed for construction of plasmids and preparation of plasmid DNA.

The complete hamster desmin gene without 5' regulatory sequences, pDes (57) was used for expressing the complete desmin protein in MCF-7 and HeLa cells and for preparation of the amino-terminal deletion constructs of the hamster desmin gene. In this construct 57 bp of 5' and 775 bp of 3' untranslated sequences, including the poly A signal, are present.

For the preparation of the amino-terminal deletion constructs of the hamster desmin gene (NΔXpRSVDes) listed in Table I, the 0.7-kbp Hpa II–Bam HI fragment of the hamster desmin gene (60) (from +25 to +737 bp relative to the CAP site) was subcloned into an Acc I–Bam HI-digested pUC19 plasmid. Excision of DNA fragments with Stu I (+91) and Bal I (+385), or with Stu I (+91) and Sma I (+526) resulted, after religation of the remaining plasmid, in a deletion of 294 (Stu I–Bal I) and 435 bp (Stu I–Sma I) in the first exon of the desmin gene. Both plasmids were cleaved with Bam HI (+737) and Hind III (pUC19 polylinker). The deletion-containing fragments were ligated in pDes from which the 0.7-kbp Bam HI (+737)–Hind III (pUC19 polylinker) fragment was removed. This resulted in the plasmids NΔ98pRSVDes and NΔ145pRSVDes. For the other amino-terminal deletion constructs, a pUC19 plasmid, containing the Hpa II (+25)–Bam HI (+737) fragment, was cleaved at the unique Stu I site at position +91. Bal31 exonuclease was used to generate deletions of various length in the DNA sequence. The plasmids were treated with T4 DNA polymerase to create blunt ended DNA cleaved at the unique Bam HI site at position +737. A pUC19 plasmid containing the Hpa II (+25)–Bam HI (+737) desmin fragment was cleaved with Stu I (+91)–Bam HI (+737). The Bam HI blunt desmin fragments were ligated in this plasmid. The resulting plasmids were cleaved with Bam HI (+737) and Hind III (pUC19 polylinker) and the deletion-containing fragments were ligated in the pDes plasmid from which the 0.7-kbp Hind III (pUC19 polylinker)–Bam HI (+737) fragment was removed.

The RSV promoter was isolated by linearizing the pRSV-CAT plasmid (34) by cleaving it at the unique Nde I site. T4 polymerase was used to create

blunt-ended DNA. Hind III linkers were ligated to this plasmid. The RSV promoter was excised with Hind III. This resulted in a 0.58-kbp Hind III–Hind III fragment which was ligated in the 5'-3' orientation in the Hind III (pUC19 polylinker) site of all desmin deletion constructs. The resulting constructs contain some additional base pairs from the pUC19 polylinker between the Hind III and Acc I sites. The DNA constructs were transformed into bacterial strain Escherichia coli HB101 or JM109.

All DNA preparations to be used in transfection experiments were purified on two successive CsCl gradients.

To determine which deletion constructs were in the proper reading frame for desmin protein expression, plasmid DNA from each deletion clone was isolated and sequenced using the double-stranded sequencing protocol described by Chen and Seeberg (14, 35).

Cell Culture

MCF-7 cells (50, 53, 67) were maintained in Eagle's modified MEM (EMEM; Flow Laboratories, Maclean, VA) supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY) and 6 ng/ml insulin (bovine). HeLa cells were cultivated in DMEM (Gibco Laboratories) supplemented with 10% FCS. Stably transfected cell lines of MCF-7 and HeLa cells were maintained in EMEM supplemented with 10% FCS, 6 ng/ml insulin, and 300 μg/ml Geneticin (G418; Gibco Laboratories), and in DMEM supplemented with 10% FCS and 300 μg/ml G418, respectively.

DNA Transfections

Cells were transfected by the calcium phosphate precipitation method, essentially as described by Wigler et al. (78). Cells were plates in 100- or 35-mm culture dishes (Costar Corp., Boston, MA) 24–48 h before transfection and grown in DMEM supplemented with 10% FCS. Transfections were carried out on cell cultures that had reached ∼40% confluency. 20 μg/100 mm or 5 μg/35 mm plasmid DNA was added to each culture dish as a calcium phosphate precipitate, 20 min later 5 ml/100 mm or 2 ml/35 mm culture medium containing 5 μg/ml chloroquine (Sigma Chemical Co., St. Louis, MO) was added. After 5 h of incubation the cells were glycerol shocked for 2.5 min, and incubated in normal growth medium for 48 h. Thereafter immunofluorescence assays, immunoprecipitation, and Western blotting procedures were performed. For preparation of stably transfected cell lines, cells were plated in 100-mm dishes and cotransfected with 1 μg pSV-neo (68) and 19 μg of a construct. 24 h after transfection the cells were brought under G418 selection (300 μg/ml). After ±14 d, colonies of stable transformants were transferred to 96-microwell dishes with a sterile pipette tip. After wells reached confluency, these cells were analyzed for desmin expression by indirect immunostaining and Western blotting.

Antibodies and Indirect Immunofluorescence Assay

Single- and double-label indirect immunofluorescence staining procedures were performed on cultured cells as described previously (45). We used the

Table I. Deletion Mutants of Hamster Desmin

Construct	Size of deletion	Number of amino acids deleted	
pRSVDes	None	None	
NΔ3	3 AA	AA 5–7*	
NΔ10	10 AA	AA 4–13	(AA 14 Y→ D)‡
NΔ13	13 AA	AA 5–17*	
NΔ60	60 AA	AA 4–63	
NΔ91	91 AA	AA 4–94	
NΔ94	94 AA	AA 4–97	(AA 98 Q → E)‡
NΔ98	98 AA	AA 4–101	
NΔ125	125 AA	AA 4–128	
NΔ145	145 AA	AA 4–148	(AA 149 R → G)‡

The deletion constructions are named NΔXpRSVDes (NΔX) where N stands for amino-terminal deletion, whereas ΔX indicates the number of amino acids removed as a result of the deletions made in the hamster desmin gene. AA, amino acids.
* After ligation AA 5 of desmin is restored.
‡ Amino acid substitutions caused by ligation.

following polyclonal and monoclonal antibodies: (a) A polyclonal rabbit antibody (poly-des) to chicken gizzard muscle desmin (65); (b) the mAb RD301 to chicken desmin (60); (c) the affinity-purified polyclonal antibody (poly-vim) to bovine lens vimentin (64); (d) the mAb RV202 to bovine lens vimentin (66); (e) the affinity-purified polyclonal antibody (poly-ker) to human skin keratins (64); and (f) the mAbs RCK102 to human keratins 5 and 8 (10), RCK106 (66), CK 18-2 (10), and RGE 53 (63), all three to keratin 18, and RCK105 to keratin 7 (66).

One-dimensional PAGE and Western Blotting

The preparation of cytoskeletal extracts of cultured cells as well as one-dimensional SDS-gel electrophoresis and immunoblotting procedures have been described previously (10). Western blots were incubated either with the mAb RD301 to desmin, followed by ^{125}I-labeled goat anti–mouse antibody, or with the polyclonal rabbit antibody (poly-des) to desmin, followed by a swine anti–rabbit antibody, and subsequently with ^{125}I-labeled *Staphylococcus aureus* protein A. The radiolabeled bands were visualized by autoradiography.

Cell Labeling, Extraction, and Immunoprecipitation of IF Proteins

For cell labeling, tissue culture cells were grown in 100-mm dishes in 6 ml of methionine-free EMEM for 2 h. Thereafter, 200 μCi [^{35}S]methionine (>1,000 Ci/mmol sp act, Amersham Corp., Arlington Heights, IL) and 0.36 ml dialyzed FCS (final concentration 6%) was added. After a 24-h incubation at 37°C the cells were rinsed three times with PBS. Cells were harvested by scraping with a cell lifter (Costar Corp.) in PBS and pelleting.

IF proteins were isolated as described previously (8). In all solutions 1 mM of the protease inhibitor PMSF was included.

Radiolabeled IF proteins were obtained selectively by immunoprecipitation with specific monoclonal and polyclonal antibodies as described (8). An aliquot containing 5 × 10^6 cpm of the [^{35}S]methionine-labeled cell extract was used for each precipitation. A volume containing 2,000 cpm of the precipitate was used for gel analysis. After separation the gels were fluorographed, dried, and exposed to Kodak X-Omat AR-5 film (Eastman Kodak Co., Rochester, NY).

Results

Selection of the Desmin Expression System

The hamster desmin gene and modifications thereof can be expressed after transfer into different types of nonmuscle cells (37, 57, 60). To study desmin filament formation in vivo, vimentin-containing cells have previously been transfected with different types of desmin deletion constructs (37). Since desmin and vimentin can form heteropolymers in vivo (62, 73), it could not be excluded that the endogenous vimentin compensates for the effects caused by deletions in the desmin gene. To avoid this, desmin filament formation was studied in cells lacking vimentin. The human breast adenocarcinoma epithelial cell line MCF-7 we used for our transfection studies does express keratins 7, 8, and 18, but neither desmin nor vimentin (38, 53). The effect of endogenous vimentin on mutant desmin assembly was determined by transfection of mutant desmin genes into HeLa cells, a human epithelial cell line expressing both vimentin and keratins 7, 8, 17, and 18 but no desmin (53).

Both the hamster desmin promoter (unpublished results) as well as the hamster vimentin promoter (38) show little or no activity in MCF-7 cells. For this reason we replaced the 5' upstream region of the hamster desmin gene by the 0.58-kbp promoter fragment (34), yielding the pRSVDes plasmid (Fig. 1 A). The RSV promoter is capable of directing high level expression of different types of constructs in both MCF-7 and HeLa cells (unpublished results). To test whether ex-
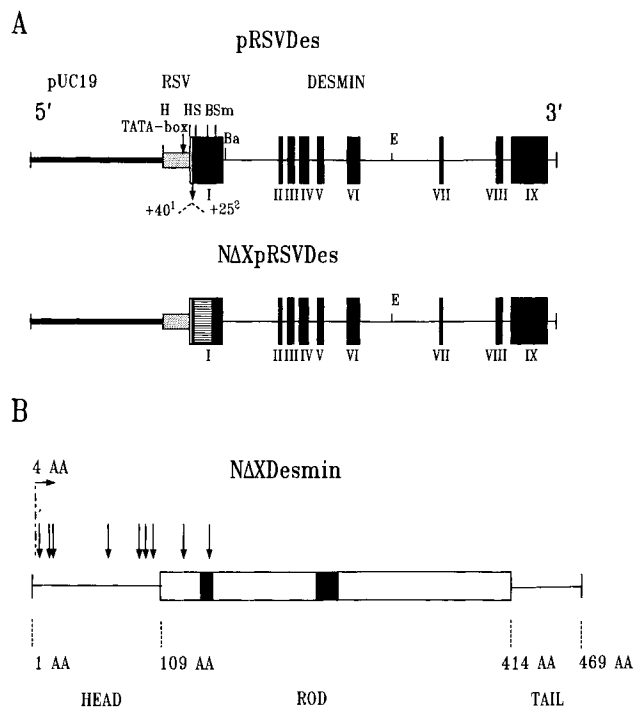

Figure 1. (A) Schematic representation of the expression vectors for hamster desmin (pRSVDes) and mutations thereof (NΔXpRSVDes [NΔX]). N indicates amino terminal deletion, whereas ΔX indicates the number of amino acids (AA) deleted as a result of the modifications made in the hamster desmin gene. RSV indicates that the desmin gene and mutations thereof are under control of the RSV promoter. ▬▬▬, pUC19; ■, RSV sequence; □, noncoding area RSV promoter; ▤, deleted area of desmin exon 1; –, noncoding sequences; ■, desmin exons. *H*, Hind III; *S*, Stu I; *B*, Bal I; *Sm*, Sma I; *Ba*, Bam HI; *E*, Eco RI. 1, relative to the CAP-site of RSV; 2, relative to the CAP-site of the desmin gene. (B) Schematic representation of deleted desmin subunits. All deletion constructs contain AA 1–4. The size of each deletion is indicated by vertical arrows.

pression of pRSVDes resulted in desmin filament formation in MCF-7 and HeLa cells, both cell types were transfected with this construct. In a typical transient transfection assay ±30% of the HeLa cells and ±15% of the MCF-7 cells reacted positively with desmin antibodies 48 h after transfection. The desmin filament network in MCF-7 cells showed no morphological abnormalities and was similar to that of HeLa cells transfected with pRSVdes (compare Figs. 3 *a* and 4 *a*) or with the complete hamster desmin gene (not shown) (37, 60).

Construction of Mutant Desmin Genes

Various mutant genes were constructed by removing sequences of the hamster desmin gene encoding the amino-terminal region of the protein (Materials and Methods, Fig. 1, and Table I). In all deletion constructs the original desmin ATG start codon, as well as the surrounding region starting from +25 up to +91 bp relative to the CAP site were retained. Therefore, in all amino-terminal deletion constructs the first three amino acids of desmin are maintained.

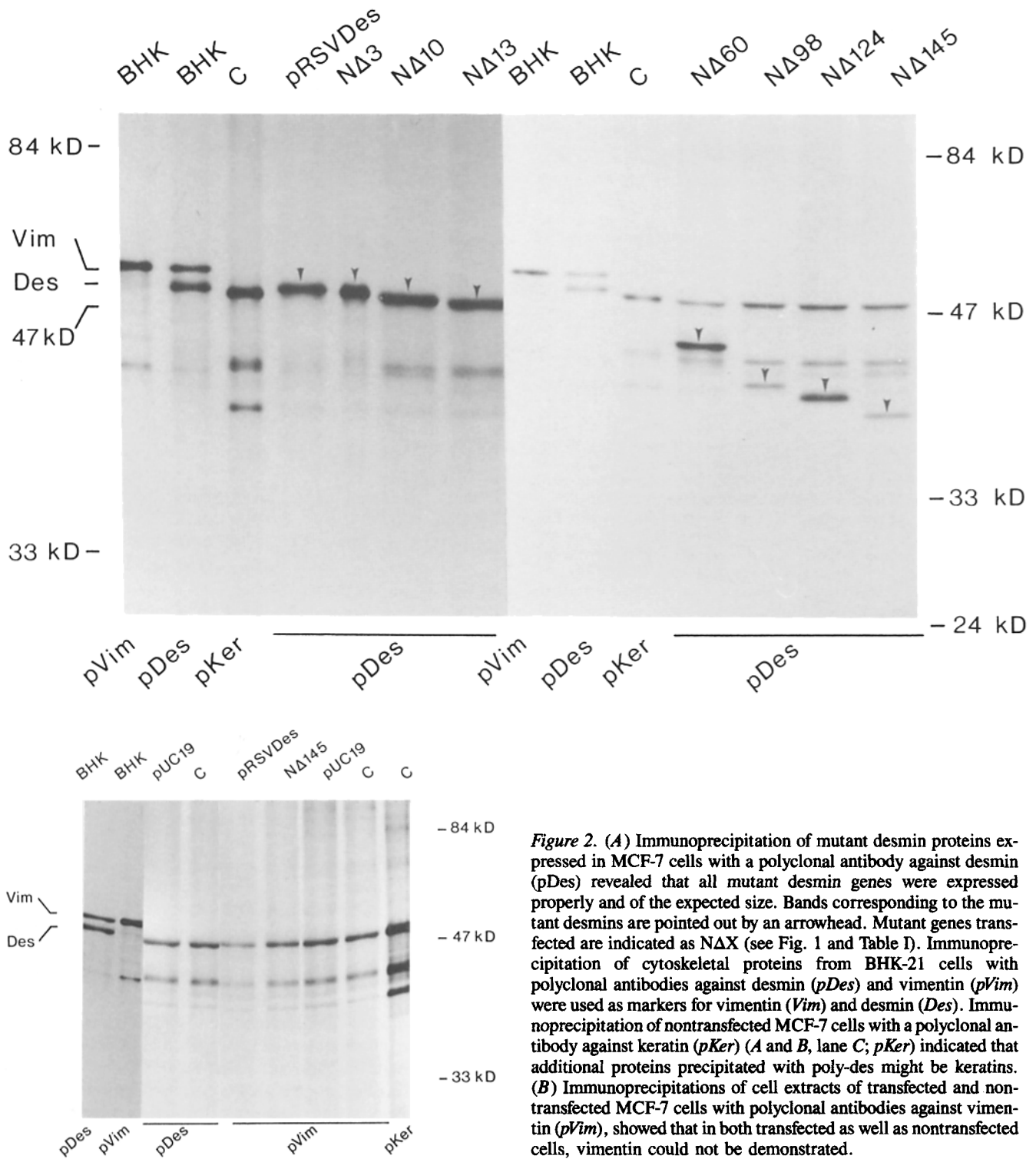Expression of the deletion constructs NΔXpRSVDes

Figure 2. (A) Immunoprecipitation of mutant desmin proteins expressed in MCF-7 cells with a polyclonal antibody against desmin (pDes) revealed that all mutant desmin genes were expressed properly and of the expected size. Bands corresponding to the mutant desmins are pointed out by an arrowhead. Mutant genes transfected are indicated as NΔX (see Fig. 1 and Table I). Immunoprecipitation of cytoskeletal proteins from BHK-21 cells with polyclonal antibodies against desmin (pDes) and vimentin (pVim) were used as markers for vimentin (Vim) and desmin (Des). Immunoprecipitation of nontransfected MCF-7 cells with a polyclonal antibody against keratin (pKer) (A and B, lane C; pKer) indicated that additional proteins precipitated with poly-des might be keratins. (B) Immunoprecipitations of cell extracts of transfected and nontransfected MCF-7 cells with polyclonal antibodies against vimentin (pVim), showed that in both transfected as well as nontransfected cells, vimentin could not be demonstrated.

should result in amino-terminally deleted desmin subunits from which 3–145 amino-terminal residues are missing (Fig. 1 B).

Expression of Mutant Desmin

MCF-7 cells were transfected with the different deletion constructs, labeled with [³⁵S]methionine, and analyzed for mutant desmin protein expression 48 h after transfection. IF proteins were isolated and immunoprecipitated with polyclonal antibodies directed against desmin (poly-des), vimentin (poly-vim), and keratins (poly-ker). Immunoprecipitation of the mutant desmin proteins with poly-des demonstrated that all mutant genes were expressed properly and that the mutant IF proteins were of the expected size (Fig. 2 A). In addition to the mutant desmin bands four other protein bands were observed in each sample. These bands were also de-

Table II. Indirect Immunofluorescence Desmin Staining Patterns Observed in Transiently Transfected Vimentin-free MCF-7 and Vimentin-containing HeLa Cells

Transfected constructs	Immunofluorescence pattern in transfected MCF-7	Immunofluorescence pattern in transfected HeLa*
pRSVDes	F	F
NΔ3	F	F
NΔ10	F, Do	F, Do
NΔ13	Do, Di	F, Do, Di
NΔ60	Di	F, Di
NΔ91	Di	F, Di
NΔ94	Di	F, Di
NΔ98	Di	F, Di
NΔ125	Di	F, Di
NΔ145	Di	F, Di

F, filamentous staining pattern; Do, dotted staining pattern; Di, diffuse staining pattern.
* Staining pattern depends on expression level of mutant desmin. If expression level was low, most cells showed intact desmin filaments; if the expression level was high, most cells showed staining patterns identical to those seen in MCF-7 cells.

tected in nontransfected MCF-7 cells (Fig. 2 *B*, lane *C*; *pDes*). Immunoprecipitation of transfected and nontransfected MCF-7 cells with poly-ker indicated that these bands probably represent endogenous keratins (Fig. 2 *B*, compare lanes *C*; *pVim*, *pDes*, and *pKer*). MCF-7 cells are completely devoid of vimentin, as demonstrated by the absence of vimentin after immunoprecipitation with poly-vim, both before and after transfection (Fig. 2 *B*).

The mutant desmin genes were also expressed properly after transfection into HeLa cells, as demonstrated by Western blotting with polyclonal antibodies against vimentin and desmin (results not shown).

The Effect of Amino-Terminal Deletions on the Filament-forming Capacity of Desmin in MCF-7 Cells

Transient transfection of the amino-terminal desmin deletion constructs into MCF-7 cells resulted in four, morphologically distinct immunofluorescence patterns after staining with poly-des (Table II and Fig. 3). Expression of pRSVDes and the deletion construct NΔ3 yielded normal filamentous desmin staining patterns (Fig. 3 *a*). Expression of the deletion construct NΔ10 resulted in a deviating picture. In addition to desmin filaments, in nearly all transfected cells small fluorescent desmin dots were seen (Fig. 3 *b*). While in some cells the dotted pattern was most pronounced, others showed a predominantly filamentous feature. The filaments were mainly observed in the nuclear area, whereas the dots seemed to be situated more closely to the plasma membrane. Upon transfection of the deletion construct NΔ13 no normal desmin filaments were observed. The transfected cells displayed a diffuse cytoplasmic staining together with strongly fluorescent dots (Fig. 3 *c*). These dots were not seen in close proximity of the nucleus. In few transfected cells, hazy filamentous structures were observed together with the diffuse cytoplasmic and dotted staining pattern. Although after transfection of NΔ10 and NΔ13, the observed desmin dots did not show any particular interaction with the nuclear envelope, very large dots often clustered around the nucleus.

To ensure that these dots were not caused by overexpression of the mutant desmin proteins, MCF-7 cells were transfected with smaller quantities of the respective constructs (2–200 times less than described in Materials and Methods). The total amount of DNA used to transfect cells was kept constant by adding pUC plasmid DNA. These experiments showed that the dots were not caused by overexpression, since they remained at each concentration, although they became very small when lower amounts of the construct were transfected.

Expression of the deletion constructs NΔ60 to NΔ145 did not result in the formation of desmin filaments. Only a diffuse cytoplasmic staining reaction was seen in these MCF-7 cells (Fig. 3 *d*).

The Effect of Amino-Terminal Deletions on the Filament-forming Capacity of Desmin in HeLa Cells

The effects of amino-terminal deletions on filament formation in HeLa cells were much less pronounced as compared with the effect seen in MCF-7 cells (Table II and Fig. 4). Transfection of the mutant desmin genes generally yielded normal filaments (not shown). However, for each construct, at least some transfected HeLa cells displayed desmin patterns, similar to those observed in MCF-7 cells (Fig. 4, *a–d*). Transfection of smaller quantities of construct DNA into HeLa cells (200 times less than described in Materials and Methods), resulted in intact desmin filaments only. Presumably, only when mutant desmins are expressed at high levels, IF formation is disturbed and the staining patterns are similar to those observed in transfected MCF-7 cells.

Interaction of Intact and Mutant Desmin with the Endogenous Keratin Network in MCF-7 Cells

Double immunofluorescence labeling of transiently transfected MCF-7 cells revealed that the endogenous keratin filaments are morphologically different from the exogenous desmin networks formed by intact desmin subunits (Fig. 5, *a* and *b*), although in some areas of the cell colocalization of both filament systems could be observed.

Transfection of all deletion constructs on MCF-7 cells resulted in desmin staining patterns as described above. Double and single labeling with keratin antibodies revealed that the keratin filament organization was never affected by the mutant desmin proteins (Fig. 5, *b*, *d*, *f*, and *h*). The desmin dots, seen after transfection of NΔ10 and NΔ13 were situated in the darn of the keratin network (Fig. 5, *c–f*).

Interaction of Intact and Mutant Desmin with Endogenous Keratin and Vimentin Filaments in HeLa Cells

After expression of intact desmin subunits double immunofluorescence staining showed that the newly synthesized desmin did colocalize completely with the endogenous vimentin filaments (see also references 37, 57, 60). Moreover, after expression of the different desmin constructs, vimentin staining patterns were identical to those of desmin, even if desmin filament formation was disturbed (Fig. 6, *c*, *d*, *g*, *h*, *k*, and *l*). These results strongly suggest that in HeLa cells vimentin and desmin form heteropolymers.

Unexpectedly in HeLa cells the exogenous desmin filaments, formed by intact desmin subunits, colocalized nearly completely with the pre-existing endogenous keratin filaments
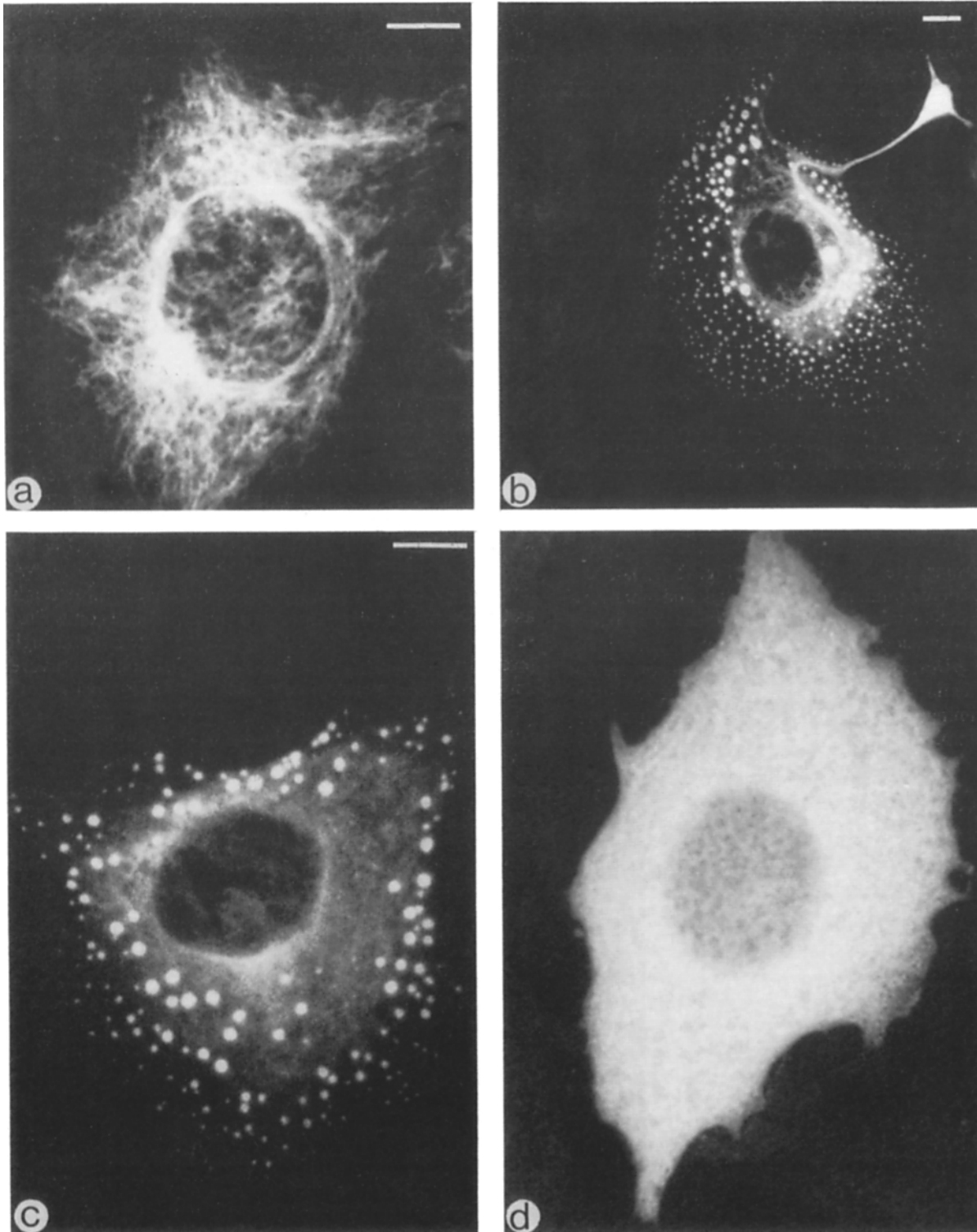
Figure 3. Single-label indirect immunofluorescence assay of MCF-7 cells transfected with (*a*) pRSVDes, (*b*) NΔ10, (*c*) NΔ13, (*d*) NΔ145, and incubated with a polyclonal antibody against desmin (*poly-des*). Abnormal desmin staining patterns already occur after deletion of 10 amino-terminal residues. Bars, 10 μm.

(Fig. 6, *a* and *b*). In contrast to MCF-7 cells, a certain number of cells transfected with NΔ10 or NΔ13 and displaying the desmin dots as described above, showed the same pattern after staining with five different keratin antibodies (Fig. 6, *e* and *f*). Expression of the other deletion constructs did not affect endogenous keratin filament networks (Fig. 6, *i* and *j*).

Initiation of Desmin Filament Formation in MCF-7 Cells

To study the initiation site of intact desmin subunits in the absence of vimentin filaments, MCF-7 cells were transfected with pRSVDes. At subsequent time intervals after transfec-
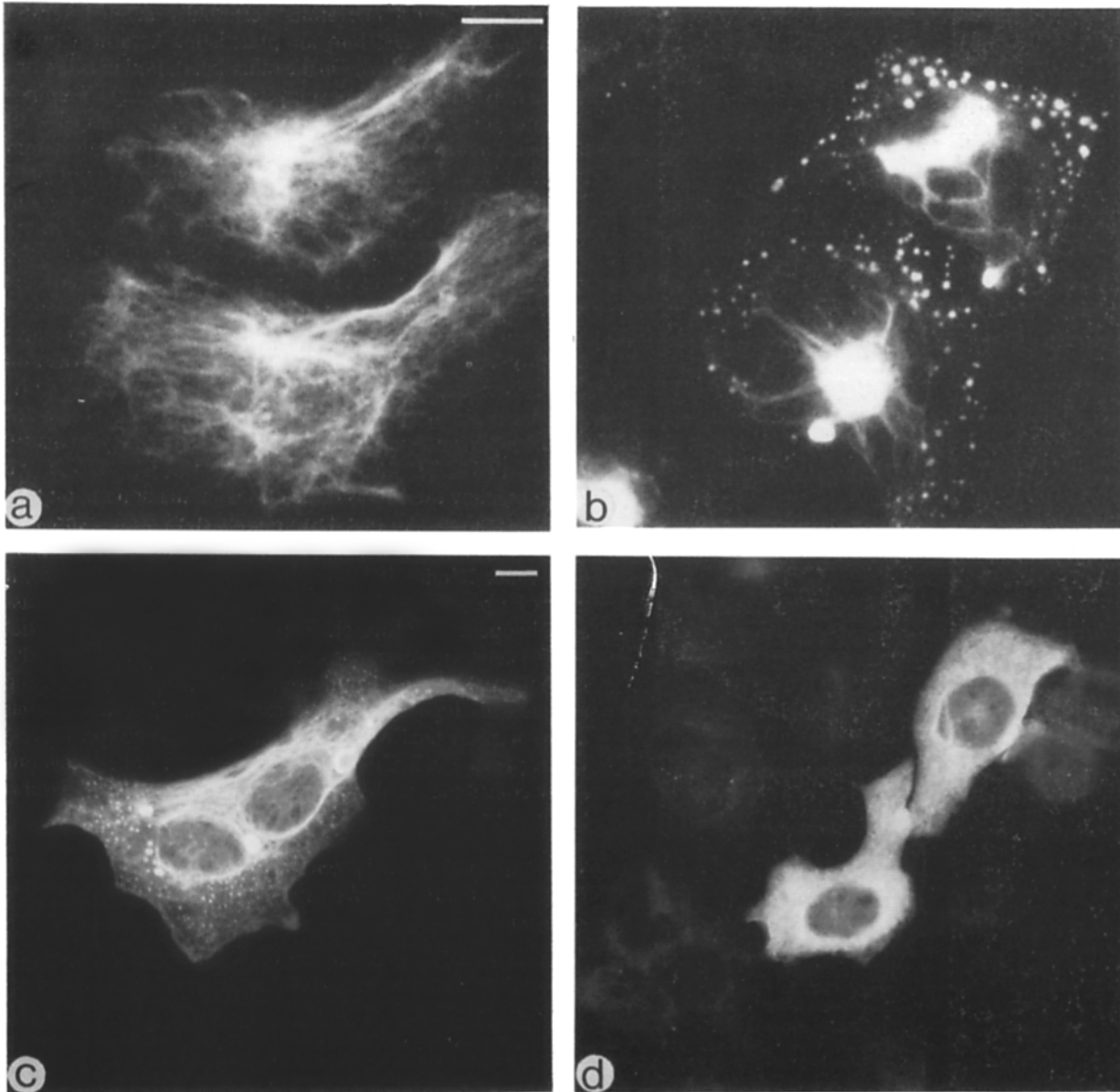
Figure 4. Single-label indirect immunofluorescence assay of HeLa cells transfected with (*a*) pRSVDes, (*b*) NΔ10, (*c*) NΔ13, (*d*) NΔ124, and incubated with a polyclonal antibody against desmin (*poly-des*). Staining patterns identical to those observed in MCF-7 cells are present in HeLa cells expressing high levels of mutant desmin. Bars, 10 μm.

tion (7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 24, 48, 72, and 173 h) cells were analyzed by the indirect immunofluorescence assay with the polyclonal desmin antiserum. In contrast to previous "testtube" observations (29), desmin filament formation in MCF-7 cells did not start at the nuclear envelope. Desmin filament formation appeared to initiate in the cytoplasmic area. Some transfected cells displayed a dotted staining pattern at the beginning of the time course (Fig. 7 *a*). The dots developed into short tiny desmin filaments randomly situated throughout the cytoplasm (Fig. 7, *b* and *c*). These rodlike structures then elongated and formed an extensive IF structure (Fig. 7 *d*). To study this phenomenon in more detail, MCF-7 cells were stably transfected with pRSVDes and lines expressing low levels of desmin were selected. Double labeling immunofluorescence with keratin and desmin antibodies suggested that the scarce desmin fila-

ment structures partially colocalized with the endogenous keratin filament network (Fig. 8). Therefore, at least in MCF-7 cells, desmin filament formation seems to start on the endogenous keratin filament network rather than on initiation sites at the nuclear or plasma membrane. In MCF-7 cells the initial process following transfection was reversed late (173 h) after transfection. Intact filaments were reduced to tiny, short, rodlike structures spread throughout the cytoplasm (Fig. 9, *a* and *c*). Remarkably, in some cells the rodlike structures appeared to cluster at the cell membrane (Fig. 9, *a* and *b*).

Discussion

A variety of studies has already been devoted to the elucidation of the role of the nonhelical IF end domains in filament
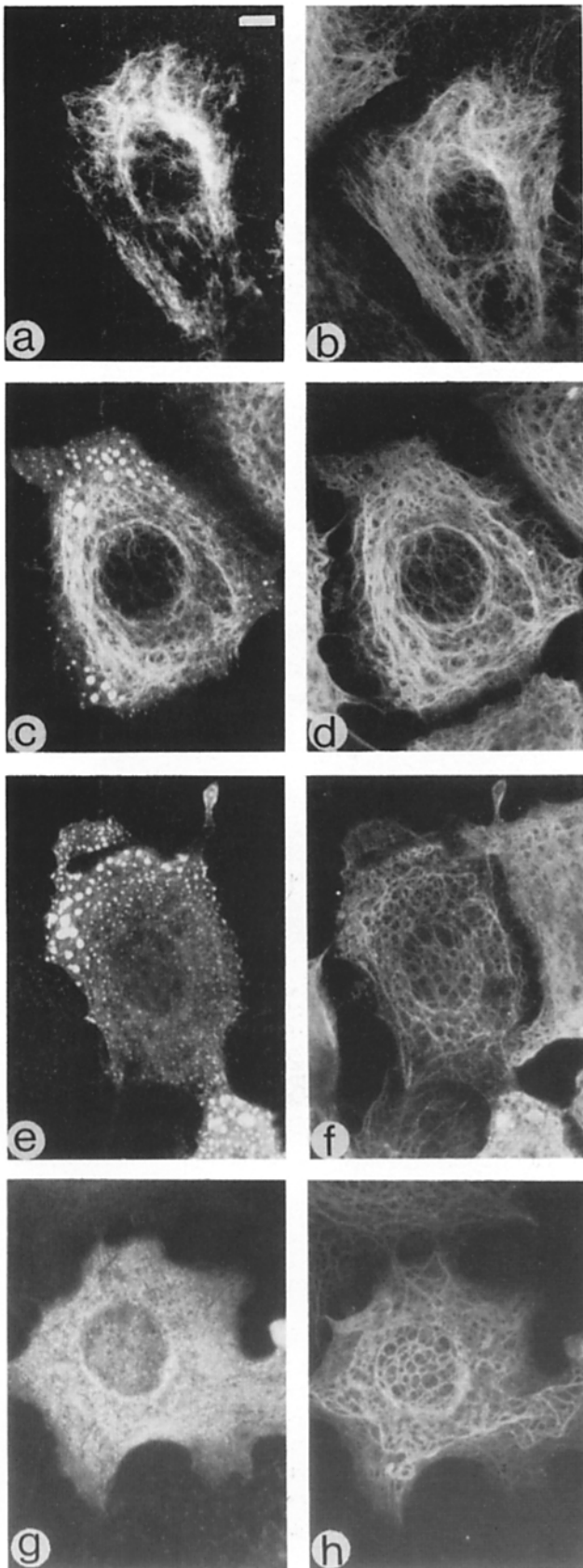
Figure 5. Double-label indirect immunofluorescence assay of MCF-7 cells transfected with (*a* and *b*) pRSVDes, (*c* and *d*) NΔ10, (*e* and *f*) NΔ13, (*g* and *h*) NΔ98, and incubated with a polyclonal antibody against desmin (*poly-des*). (*a*, *c*, *e*, and *g*) and a monoclonal anti-

formation. For instance amino-terminally deleted keratin subunits incorporated into the preexisting keratin network without disturbing it (3). On the other hand, previous in vitro reassembly studies with desmin subunits missing 67 amino-terminal residues showed that these subunits, incapable of filament formation on their own, could form normal desmin filaments when mixed with wild-type desmin subunit (41). The mechanism of IF assembly might be different for keratin and type III IF subunit. However, we do suspect that in the in vivo reassociation study with mutant keratin cDNAs, the presence of intact endogenous keratin subunits may mask the effects of deletions in the nonhelical end domains on filament formation. Our vimentin-free expression system eliminates this problem. No IF subunits, capable of copolymerization with mutant desmin subunits, are present in the MCF-7 cells. As the amino-terminal part of the IF subunit seems to be of major importance in regulating filament rearrangements (9, 13, 15, 17–19, 22, 26, 30, 40–42, 47, 55, 69, 74, 75), we chose to study the effects of deletions in this domain on fila-ment formation. All our desmin constructs were expressed properly in MCF-7 and HeLa cells after transient transfec-tion. Since IFs are expressed in a tissue-specific manner, it might be possible that control mechanisms for desmin as-sembly operative in muscle cells are not present in epithelial MCF-7 cells. However, considering the impossibility of study-ing homopolymeric desmin assembly in vimentin-containing muscle cells together with the fact that normal desmin fila-ments were formed when the nonmodified gene (pRSVDes) was expressed in MCF-7 and HeLa cells, we believe that our study contributes to the understanding of type III IF assem-bly in vivo.

The Amino-Terminal Domain of Desmin Is Indispensable for Filament Formation In Vivo

In both vimentin and desmin a highly conserved nonapeptide SSYRRXFGG (X being isoleucine or methionine in vimen-tin, and threonine in desmin) is present closely behind the initiation methionine (position 1) (36, 49, 58, 60). This nona-peptide contains a phosphorylation site for protein kinase C at position 13 of desmin (42). In construct NΔ10 amino acid residues 4–13 are removed, including the two serine residues at positions 12 and 13, the tyrosine at position 14 is sub-stituted for aspartic acid. Transfection of this construct in MCF-7 cells resulted already in abnormal desmin organiza-tion. The observed staining pattern suggests that these mu-tant subunits are still capable of assembling into filaments but probably lost the capability to bind to the plasma mem-brane, presumably via interaction with ankyrin which is sup-posed to serve as a natural capping factor (27, 28, 31). The lack of capping, which probably blocks IF elongation, might cause curling of the desmin filaments at their amino-terminal ends resulting in the dotted staining pattern. Whether the disturbed staining pattern is caused by removal of the phos-

body against keratin (*RCK106*) (*b*, *d*, *f*, and *h*). Weak immunofluo-rescent dots visible in *d* and *f* are caused by the very strong fluores-cent desmin dots. Single labeling with mono-ker never showed dotted keratin staining patterns similar to those observed for desmin. Note intact keratin filaments in all transfected cells. Bars, 10 μm.
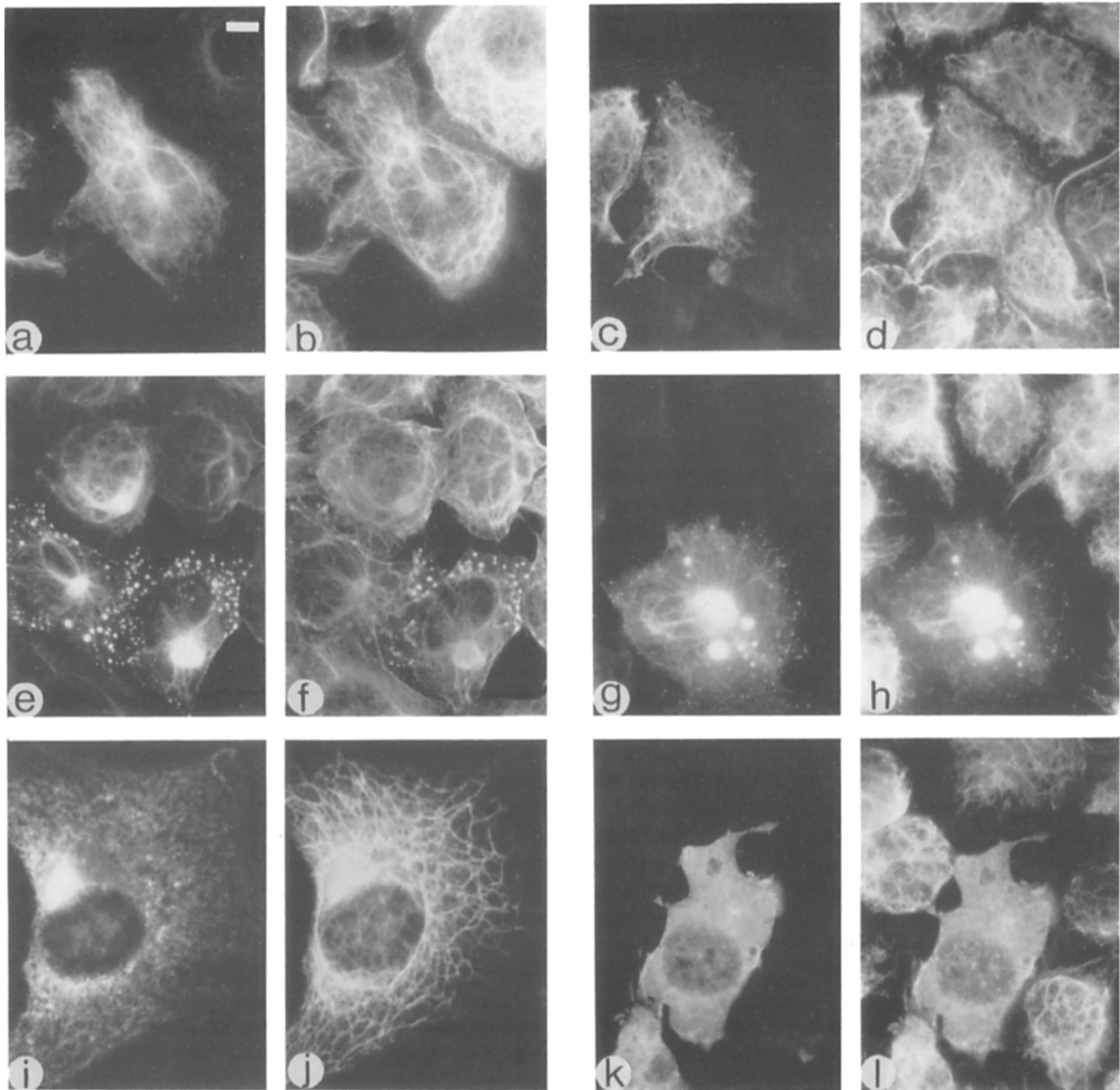
Figure 6. Double-label indirect immunofluorescence assay of HeLa cells transfected with pRSVDes (*a–d*), NΔ10 (*e–h*), NΔ145 (*i–l*), and incubated with a polyclonal antibody against desmin (*poly-des*) (*a, c, e, g, i,* and *k*), an mAb against keratin (*RCK102*) (*b, f,* and *j*) and an mAb against vimentin (RV202) (*d, h,* and *l*). Note complete colocalization of desmin and vimentin staining. Large colocalizing areas are also present for desmin and keratin staining in cells transfected with pRSVDes and NΔ10. In cells transfected with NΔ145, no colocalization of desmin and keratin patterns is observed. Bars, 10 μm.

phorylation site or merely by disturbance of the conserved sequence is not yet clear. Removal of amino acids 5–17 (NΔ13) including the residues SSYRRT from the nonapeptide causes the loss of filament forming capacity of the mutant subunit. These data indicate that this conserved sequence is involved in filament formation in vivo. The presence of desmin dots after expression of NΔ13 revealed that the mutant subunits retain the possibility to interact with other cellular components (probably the endogenous keratin filaments). Whether the remaining residues of the nonapeptide (FGG) are involved in this interaction or other residues

situated between position 17 and 63 is not yet understood. Expression of desmin constructs containing larger deletions than NΔ13 (NΔ60–NΔ145, Table I) resulted in desmin subunits incapable of forming desmin filaments or dots. The diffuse cytoplasmic staining observed, indicates that these mutant subunits remained in the soluble phase.

Intact Vimentin Subunits Capable of Copolymerization with Mutant Desmin Subunits Mask the Effect of Mutations on IF Formation

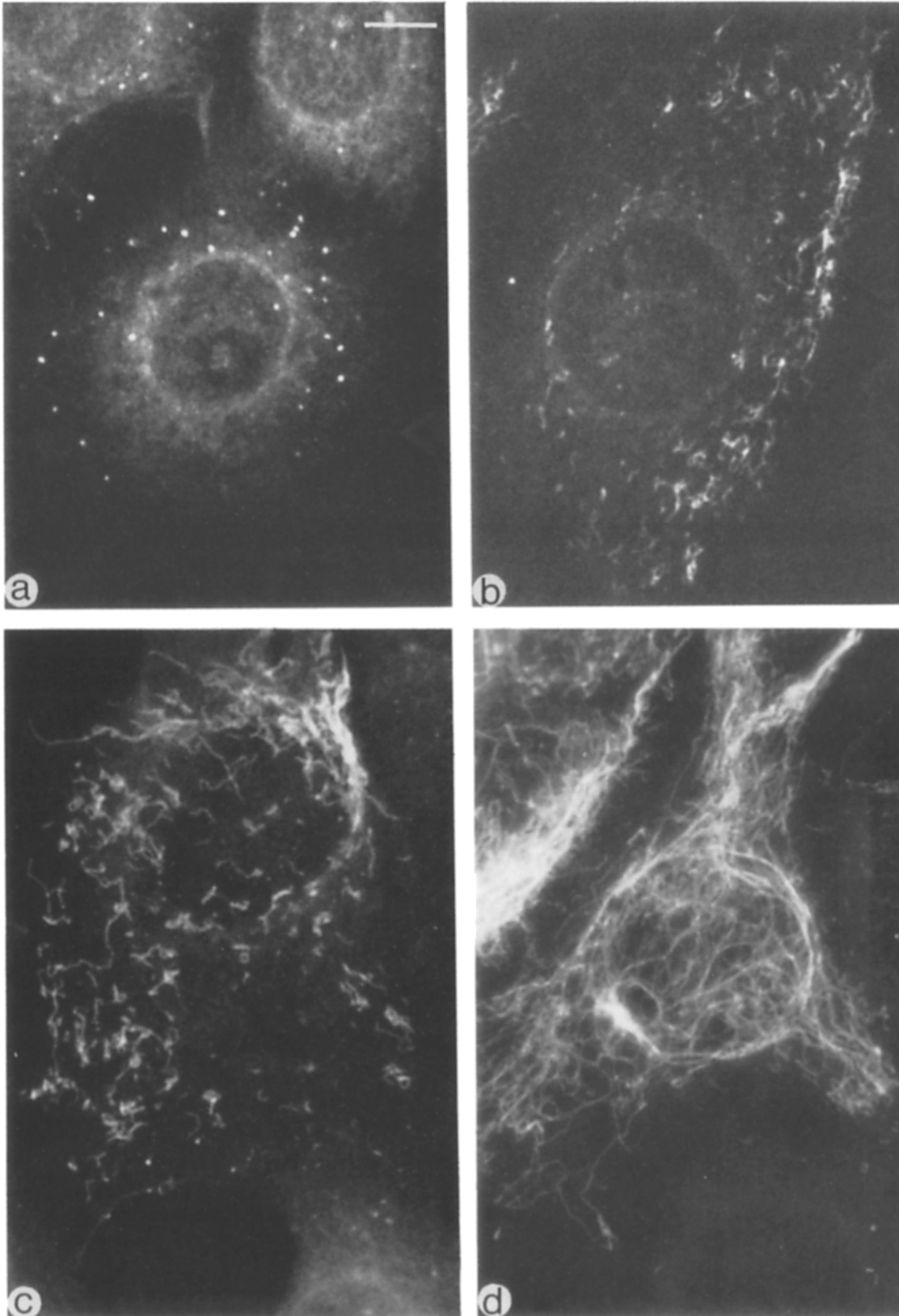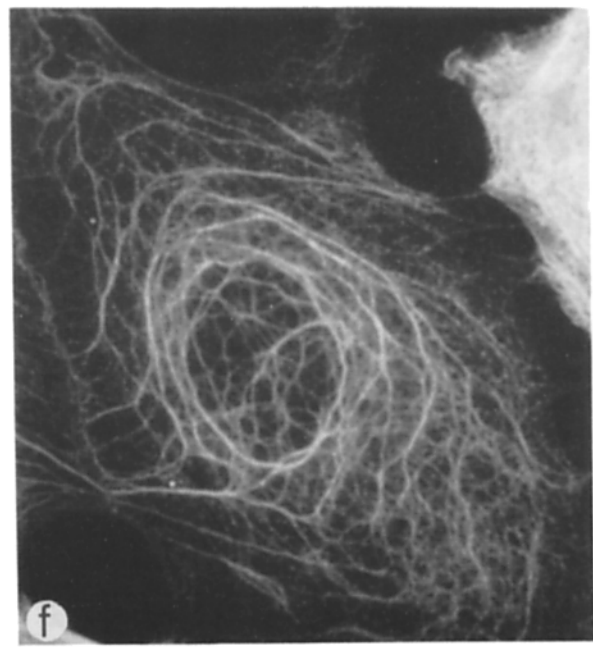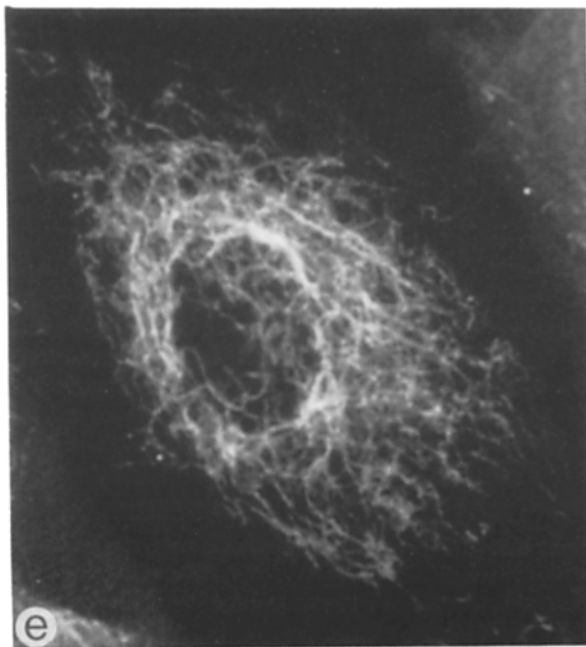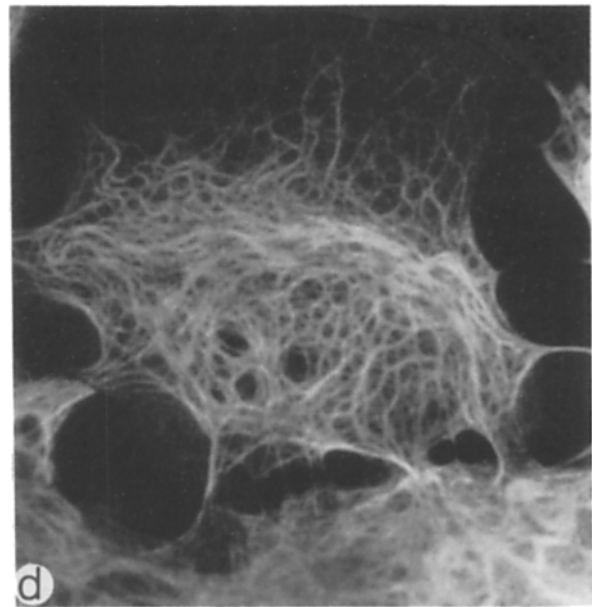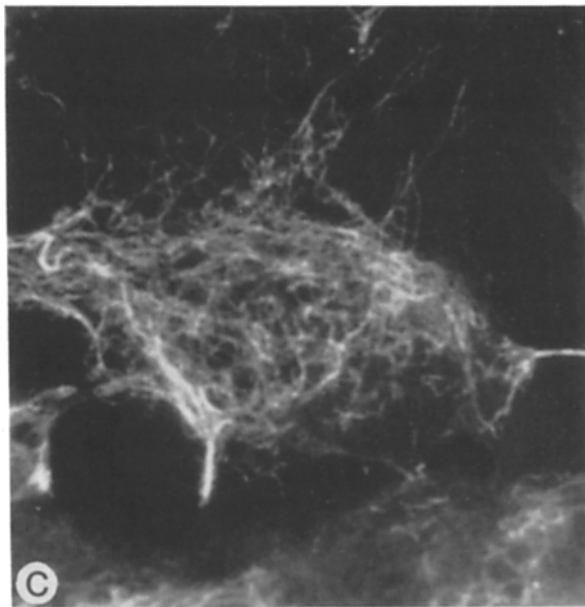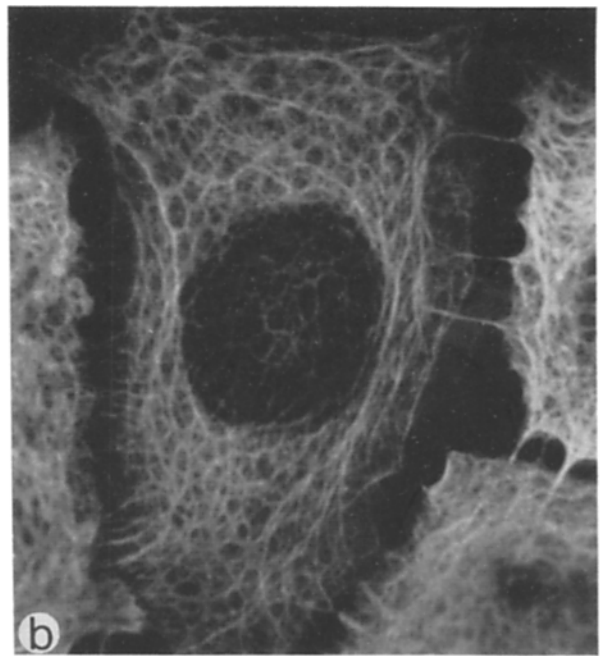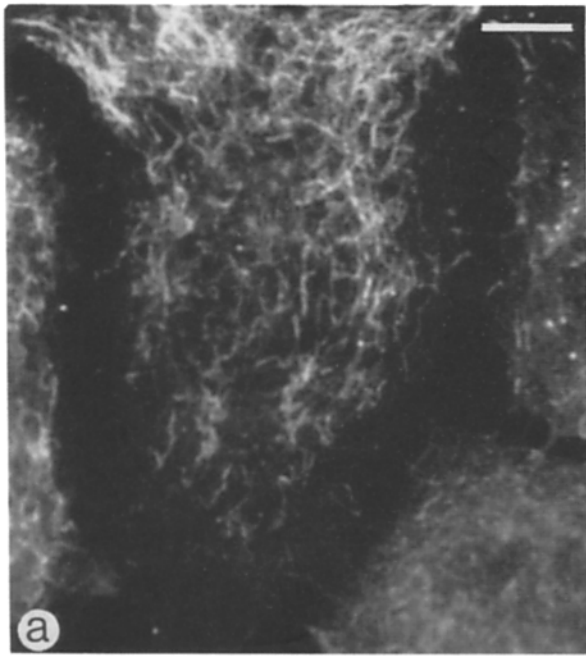All cells transfected with the mutant desmin constructs, dis-

Figure 7. Single-label indirect immunofluorescence assay of MCF-7 cells transfected with pRSVDes, and incubated with a polyclonal antibody against desmin (*poly-des*). Cells were fixed (*a*) 16, (*b* and *c*) 48, and (*d*) 72 h after transfection. Note that initiation of desmin filament formation starts in the cytoplasmic area. Bars, 10 μm.

Figure 8. Double-label indirect immunofluorescence assay of MCF-7 cells stably transfected with pRSVDes and expressing only low levels of desmin. Cells are stained with a polyclonal antibody against desmin (*poly-des*) (*a*, *c*, and *e*) and a monoclonal antibody against keratin (*RCK102*) (*b*, *d*, and *f*). Note large colocalizing areas of desmin and keratin staining, especially when desmin filaments are scarce. Bars, 10 μm.
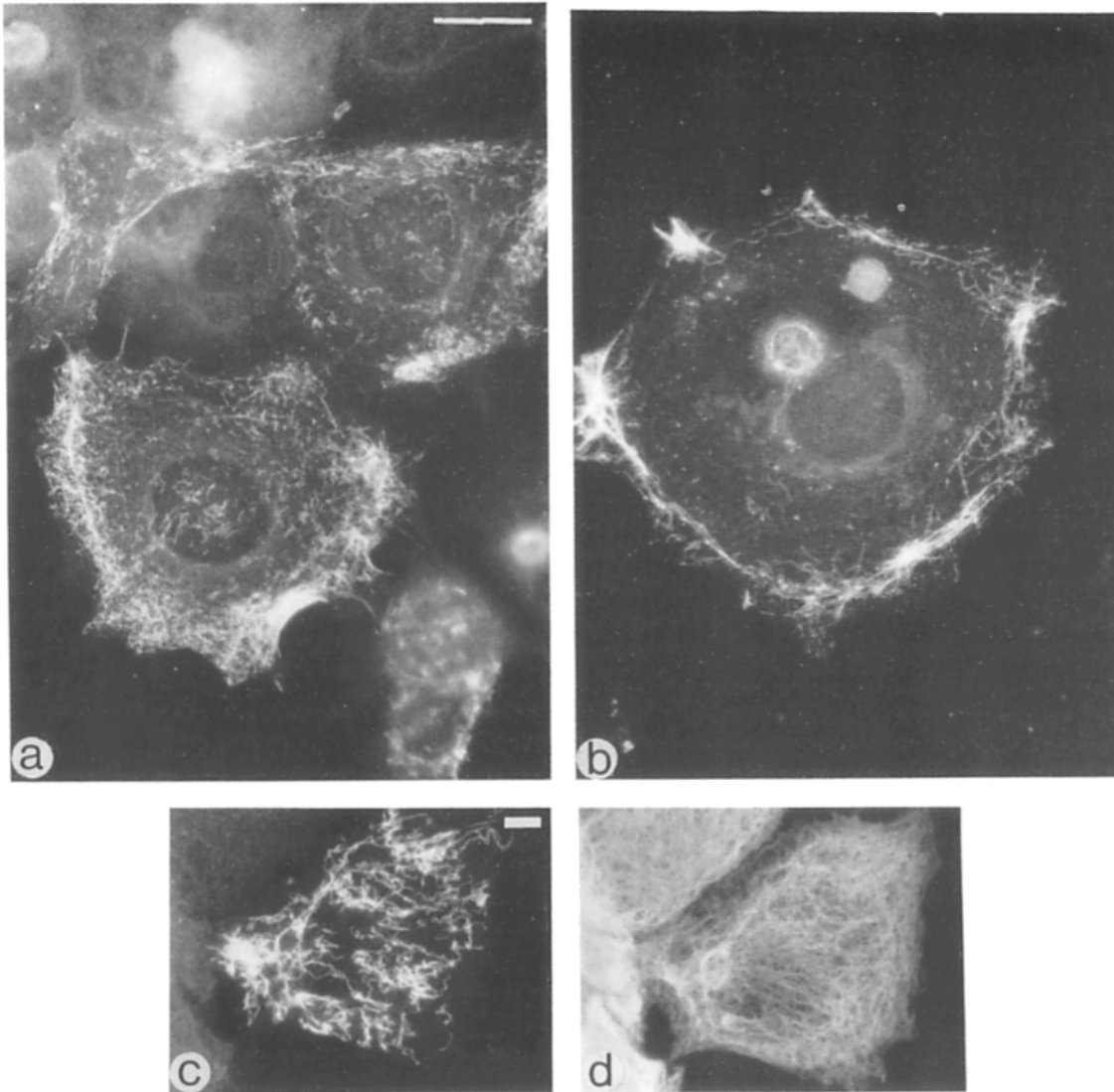
Figure 9. Single- and double-label indirect immunofluorescence assay of MCF-7 cells transfected with pRSVDes. Cells were fixed and stained with a polyclonal antibody against desmin (*poly-des*) (*a–c*) and an mAb against keratin (*RCK106*) (*d*) 173 h after transfection. Note short, cytoplasmic filamentous desmin structures which show large areas of colocalization with keratin filaments. Some transfected cells showed a clustering of the rodlike desmin structures near the plasma membrane at late times after transfection. Bars, 10 μm.

played identical desmin and vimentin staining patterns, confirming that desmin and vimentin form heteropolymers in transfected HeLa cells (62). In most transfected HeLa cells expression of the mutant desmin subunits resulted in normal filamentous desmin staining, although for all mutant desmin subunits, patterns identical to those observed in MCF-7 cells were seen in a number of cells. Most likely, only a high ratio of mutant desmin subunits in the desmin/vimentin heteropolymers resulted in the distorted desmin (and vimentin) patterns identical to those seen in MCF-7 cells. This view is sustained by preliminary results obtained with stably transfected HeLa cells expressing various amounts of mutant desmin. Comparison of the results obtained by the in vivo assembly study with amino terminally deleted keratin subunits (3) and our data revealed a number of differences. Desmin subunits missing as little as amino acid residues 5–17 were, when expressed at high levels in

HeLa cells, already capable of disrupting the preexisting vimentin network. Only deletions in the α-helical domain of keratin subunits affected filament morphology. Furthermore, expression of desmin subunits missing amino terminal residues 4–64 (NΔ60) showed the same desmin staining pattern as observed after expression of desmin subunits missing their complete amino-terminal domain in addition to coil 1B (NΔ145, Fig. 1 *B*). These differences might be caused by different assembly mechanisms of keratin and type III IF subunits. However, we do suspect that the endogenous keratin network present in cells used for transfection of mutant keratin cDNAs (3) is largely responsible for the discrepancy.

Effect of Mutant Desmin on Other IF Networks

In MCF-7 cells expression of mutant desmin never affected endogenous keratin networks. However, in some areas of the

cell intact desmin filaments seemed to colocalize with keratin filaments.

In HeLa cells only expression of NΔ10 and NΔ13 did affect endogenous keratin filaments. Preliminary results with stably transfected HeLa cells expressing various levels of mutant desmin reveal that keratin IF are only affected at high mutant desmin expression, indicating that the desmin region between amino acid residues 17 and 64 is necessary for interaction. Whether the effect of mutant desmin on keratin filaments is mediated by endogenous vimentin, or represents an interaction between desmin and a particular keratin type not present in MCF-7 cells, or is caused by an IF associated protein, remains unsolved. In HeLa cells intact desmin/vimentin and keratin filaments seemed to colocalize completely. Although rather seldom, colocalization of vimentin and keratin filaments has been reported previously (7, 43, 44).

In MCF-7 Cells the Initiation of Desmin Filament Assembly Does Not Take Place at the Nuclear Envelope

Our experiments, as well as preliminary results obtained with MCF-7 cells stably transfected with pRSVDes and expressing only low levels of intact desmin, revealed that desmin filament assembly in MCF-7 cells does not start at the nuclear envelope as suggested previously for vimentin (29, 74). In contrast, the initiation of desmin filament assembly seemed to occur in the cytoplasm. Hence we may conclude that in MCF-7 cells no vectorial assembly of desmin filaments takes place. Moreover we noticed that the desmin and preexisting keratin networks seemed to colocalize largely when desmin filaments were scarce. However, abundant desmin filaments, showed a different distribution as compared to the endogenous keratin networks, although some overlap was still observed.

As vimentin and desmin are closely related (58-61), the mechanism of filament assembly was expected to be alike for both subunits. However, neither cytoplasmic rodlike structures as observed in MCF-7 cells, nor a nuclear IF cap as described for injected vimentin in BHK-21 cells (76) were observed upon desmin expression in HeLa cells. Only a gradual increase of desmin fluorescence intensity, which colocalized completely with the preexisting vimentin filaments, was seen. This is in agreement with the recent observation that chicken vimentin does incorporate in preexisting mouse vimentin filaments at assembly sites on the vimentin network (56). However, no discontinuous localization of desmin on the preexisting vimentin filaments at early times after transfection was observed. Uniform distribution of newly formed IF subunits within preexisting filaments has been reported for NF-L subunits too (5).

Conclusions

When searching for the function of IF domains in in vivo filament formation by means of transfection studies with modified genes, it is necessary to avoid cell systems in which intact IF subunits capable of copolymerization with mutant subunits are present. Our in vivo studies are in line with the in vitro finding that tetramers containing less than four mutant subunits with intact rod domains are still capable of normal filament formation (41), thereby masking the effects caused by mutant IF subunits. By using vimentin-free MCF-7 cells

to express the various desmin constructs, we were able to localize the amino-terminal region necessary for desmin filament formation in vivo at amino acid residues 7-17. Most likely the conserved nonapeptide SSYRRTFGG present in desmin at position 12-20 is involved in desmin filament formation in vivo. In MCF-7 cells desmin filament assembly starts in the cytoplasmic area, probably on endogenous keratin filaments. In HeLa cells newly formed desmin filaments incorporate uniformly into the preexisting vimentin filaments. In both cell types no initiation at the nuclear membrane was observed. Further studies with stably transfected cells hopefully will reveal more details about certain features of the mutant IF subunits, such as protein stability, solubility, and the interaction with other cellular components.

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