

Keratin intermediate filament dynamics in cell heterokaryons reveals diverse behaviour of different keratins

Jesús M. Paramio^{1,*}, M. Llanos Casanova¹, Angel Alonso² and José L. Jorcano¹

¹Department of Cell and Molecular Biology, CIEMAT, Av. Complutense 22, E-28040 Madrid, Spain

²DKFZ-ATV, Im Neuenheimer Feld, 242, 61920 Heidelberg, Germany

*Author for correspondence (e-mail: chus@ciemat.es)

SUMMARY

To study the dynamics of keratin intermediate filaments, we fused two different types of epithelial cells (PtK₂ and BMGE+H) and studied how the keratins from the parental cells recombine and copolymerize to form the heterokaryon cytoskeleton. The behaviour of the keratins during this process was followed by immunofluorescence using specific antibodies. After fusion, the parental cytoskeletons undergo a depolymerization process most apparent in the region adjacent to the fusion area. The depolymerized subunits spread throughout the heterokaryon and copolymerize into a new hybrid cytoskeleton. The complete process is very rapid, occurring in 3-4 hours, thus demonstrating the highly dynamic nature of the keratin cytoskeleton. Although newly synthesised subunits contribute to the formation of the hybrid cytoskeleton, the process takes place with similar kinetics in the absence of protein synthesis, showing the dynamic nature of the keratins from pre-existing cytoskeletons. During this process, specific keratins behave differently. Keratins K8, K18, K5 and K10 are mobilised from the parental cytoskeletons and reassemble rapidly into the hybrid cytoskeleton (3-6 hours), whereas K14 requires a substantially longer period (9-24 hours). Thus, different keratins, even when they form part of the same heterodimeric/tetrameric complexes, as is the case for K5 and K14, exhibit different dynamics. This

suggests that individual polypeptides or homopolymeric complexes rather than exclusively heterodimeric/tetrameric subunits, as is currently thought, can also take part in keratin intermediate filament assembly and dynamics. Biochemical analysis performed in the absence of protein synthesis revealed greater amounts of K5 than of K14 in the soluble pool of BMGE+H cells. Crosslinking and immunoprecipitation experiments indicated an excess of monomeric K5, as well as of K5/K14 heterodimers and K5 homodimers in the soluble pool. These results are in agreement with the different dynamic behaviour of these keratins observed in immunofluorescence. On the contrary, the phosphorylation levels of K5 and K14 are similar in both the soluble pool and the polymerized fraction, suggesting that phosphorylation does not play an important role in the different dynamics displayed by these two proteins. In summary, our results demonstrate that, following fusion, the keratin intermediate filament network reshapes rather rapidly and that keratins are highly dynamic proteins, although this mobility depends on each particular polypeptide.

Key words: Intermediate filament, Keratin, Cytoskeleton dynamics, Cell fusion

INTRODUCTION

Keratins, which form the intermediate filament (IF) cytoskeleton of epithelial cells, are a complex family of approximately 20 polypeptides that can be subdivided according to a number of criteria into two separate subfamilies, the acidic, or type I keratins, and the neutral-basic or type II keratins (for recent reviews see Fuchs and Weber, 1994; Quinlan et al., 1994). Contrary to other IF which are homopolymers, the keratins are heteropolymers containing equimolar amounts of type I and type II molecules (Fuchs et al., 1981; Moll et al., 1982; Schiller et al., 1982; Cooper et al., 1985). The first step in keratin assembly appears to be the formation of a heterodimer containing one molecule of each type aligned in parallel and in register (Coulombe and Fuchs, 1990; Hatzfeld and Weber, 1990; Steinert, 1990). These heterodimers tend to form

tetramers, which are thought to be the IF building blocks both in vitro and in vivo (Franke et al., 1983; Woods, 1983; Gruen and Woods, 1983; Quinlan et al., 1984, 1986; Parry et al., 1985; Eichner et al., 1986). In vivo, different types of epithelial cells are characterized by the strict combinations of specific keratins pairs that they synthesize, the so-called 'expression pairs' (Sun et al., 1984). This carefully-regulated keratin expression suggests that different keratins could play specific functions in cells. However, in vitro reconstitution experiments and electron microscopy analysis demonstrated that almost any equimolar combination of basic and acidic keratins leads to IF formation (Hatzfeld and Franke, 1985).

In contrast to other cytoskeletal proteins, which have been shown to be highly dynamic (Gawlitza et al., 1980; Glacy, 1983; Sammak et al., 1987; Schulze and Kirschner, 1986; Wadsworth and Sloboda, 1983), IF have long been considered

the most stable and consequently the least dynamic components of the cell cytoskeleton (Bloemendal and Pieper, 1989; Vikstrom et al., 1991). During certain cell processes, and especially during mitosis, however, IF proteins reversibly polymerize into structures different from the normal IF network (Franke et al., 1982; Lane et al., 1982). This indicates that these proteins undergo dynamic processes in which phosphorylation may play an important role (Chou et al., 1989, 1990; Chou and Omary, 1991; Klymkowsky et al., 1991; for a review see Skälli et al., 1993; Steinert, 1993). In the case of keratin IF, further evidence for these dynamic properties is derived from transfection of keratin genes or microinjection of mRNA coding for these proteins in cultured cells, in which the new proteins can be detected very early after transfection and are rapidly incorporated into the preexisting cytoskeleton (Albers and Fuchs, 1987, 1989; Franke et al., 1984; Paramio and Jorcano, 1994).

The majority of the dynamic studies on subcellular structures have been performed microinjecting the proteins under study after *in vitro* labelling. However, the relative insolubility of the IF proteins over a wide range of physiological conditions has made it difficult to use this methodology for the study of the IF dynamics until recently. Miller et al. (1991, 1993) and Vikstrom et al. (1989, 1991, 1992) have reported the microinjection of biotinylated or fluorescently-labelled IF subunits into cells in culture, and have demonstrated the high dynamic nature of these proteins. This technique could alter the dynamic process, however, due to the large amount of protein injected into the cells (Miller et al., 1993). In addition, no careful comparative study has thus far been made of the *in vivo* dynamic properties of the numerous members of the vast IF family. We have studied the dynamic properties of different keratins *in vivo* using an approach that does not alter the endogenous amount of these polypeptides. It also allows simultaneous observation of the temporal evolution of individual keratins and of the keratin cytoskeleton as a whole. Two different types of epithelial cells were fused by polyethyleneglycol (PEG) treatment and the formation of the heterokaryon cytoskeleton from the parental keratins was followed by double immunofluorescence using monospecific keratin antibodies. Our results indicate that the keratin IF network is a very dynamic structure and that different keratin polypeptides, even when they form an expression pair, can display different dynamic behaviours.

MATERIALS AND METHODS

Cell culture

PtK2 and MCA3D cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum (FCS, Gibco). BMGE+H cells were cultured in DMEM containing 20% FCS, 1 mg/ml insulin, 1 mg/ml prolactin and 1 mg/ml hydrocortisone (Sigma). The B.10-4 cell line was obtained by co-transfection of BMGE+H cells with a plasmid coding for bovine keratin K10 under the control of the bovine keratin K6 promoter and the plasmid pAG60 coding for neomycin resistance. This clone was selected from among the neomycin-resistant clones for its relatively high expression of the transfected keratin, without affecting cell morphology, doubling time or the appearance of the keratin cytoskeleton. All cells were cultured in plastic Petri dishes (Falcon, Beckton Dickinson) at 37°C in a 5% CO₂ atmosphere. The medium was changed every 3-4 days.

Cell fusion

Heterokaryons were generated as follows: the day before fusion,

appropriate cell numbers were plated onto glass coverslips and incubated for at least 16 hours. To promote fusion, cells were washed 3 times with serum-free medium, then 1 ml of PEG 1500 (Boehringer Mannheim) was added. The plates were incubated for 1 minute at room temperature, cells were washed 3-4 times using prewarmed serum-free medium, and complete medium was added. Cells were then incubated under normal conditions, and coverslips were collected at different times and processed for immunofluorescence (see below). Controls omitted the PEG treatment. Each type of fusion was repeated three to five times and was independently analyzed by two persons.

Immunofluorescence analysis

Cells cultured on glass coverslips were washed with phosphate-buffered saline (PBS) and fixed with methanol:acetone (2:1) at -20°C for 7 minutes, rehydrated with PBS and stained with either a 1/40 dilution of monoclonal antibody (mAb) K8.60 (Sigma) against K10 (Huszar et al., 1986), a 1/10 dilution of rat mAb TROMA1 against K8 (Kemler et al., 1981), a 1/5 dilution of mAb RCK107 against K14 (Raemakers et al., 1983), a 1/5 dilution of mAb LE61 against K18 (Lane, 1982), a 1/500 dilution of a rabbit antiserum against the K5 carboxy terminus (Roop et al., 1984), a 1/5 dilution of mAb AE14 against K5, a 1/500 dilution of a rabbit antiserum against the K14 carboxy terminus (kindly provided by Dr E. B. Lane), or a 1/10 dilution of mAb Dp1+2 (Progen) against desmoplakins 1 and 2. The specificity of these antibodies was confirmed in western blots using cytoskeleton-enriched fractions from the cells studied (not shown). Secondary fluorescent labelled antibodies raised in donkey and specific for multiple labelling purposes were purchased from Jackson Immunoresearch, and used at 1/50 (FITC labelled) or 1/400 dilution (Texas Red labelled). Mowiol (Hoechst) was used as aqueous mounting medium. In some cases, the fixation protocol was 3.7% paraformaldehyde for 10 minutes, followed by quenching in 10 mM NH₄Cl, permeabilization by immersion in cold acetone and subsequent washes in PBS, prior to incubation with the antibodies. Observations were performed on a Zeiss Axiophot Photomicroscope equipped with an epifluorescent illumination system and the appropriate filters to avoid cross-channel light contamination.

Protein synthesis inhibition

Cycloheximide (50 µM; Sigma) was used to inhibit protein synthesis, according to the procedure described elsewhere (Paramio and Jorcano, 1994). It was added to the culture at least 2 hours before PEG treatment and maintained thereafter.

Analysis of keratin polypeptides in the soluble fraction of BMGE+H cells

To determine the presence of keratins in the soluble pool, BMGE+H cells were cultured for 16 hours in labelling medium (methionine-deficient DMEM; Sigma) supplemented with hormones (see above), 20% dialysed FCS, 100 µCi [³⁵S]Met (Translabel, Amersham) per 10 cm dish and 5 µg/ml of non-radioactive methionine). At this time, cells were washed with non-radioactive complete medium containing cycloheximide, incubated for 2 hours in labelling medium with cycloheximide, and fusion was performed. Cells were then incubated for 3 hours in non-radioactive complete medium containing cycloheximide, washed three times with cold PBS and resuspended in 2 ml of low salt buffer (0.15 M NaCl, 0.1% Triton X-100, 2 mM EDTA, 1 mM DTT, 10 mM Tris-HCl pH 7.5, containing 5 mM PMSF, 1 mg/ml aprotinin and 1 mg/ml leupeptin). Cells were homogenized in this buffer and centrifuged at 300,000 *g* for 1 hour. The pellets were used to obtain a keratin-enriched fraction according to the method of Achtstätter et al. (1986). Urea (Fluka) was added to the supernatant fraction to a final concentration of 10 M and mixed with an excess (approximately 100 µg of protein) of unlabelled cytoskeletal proteins isolated from BMGE+H cells according to the method of Achtstätter et al. (1986) and resuspended in 10 M urea. This mixture was dialysed for 12 hours against 10 mM Tris-HCl, pH 6.0, and a further 24 hours

against 10 mM Tris-HCl, pH 7.0. The samples were cleared by centrifugation in a minifuge for 10 minutes at 12,000 rpm at 4°C, and pellets washed twice in low salt buffer. The final pellets were boiled in sample buffer and analyzed on a 9% SDS-PAGE. Fluorography was performed using EnHance (NEN) according to the manufacturer's recommendations.

Immunoprecipitation and chemical crosslinking

For immunoprecipitation experiments, the supernatant fraction obtained as above was precleared by incubation (30 minutes at room temperature) with Protein A-Sepharose (Pharmacia) and centrifugation. The supernatant was incubated with 1/100 dilution of the rabbit anti-K5 antiserum (1 hour, 4°C), and immunocomplexes were obtained by incubation with Protein A-Sepharose (1 hour, 4°C) and centrifugation (14,000 rpm in a microfuge). For the analysis of keratin phosphorylation, cells were treated as in the preceding paragraph, but omitting the [³⁵S]methionine labelling, and incubated for 2 hours after fusion in the presence of cycloheximide. Cells were then incubated for 1 hour with 500 µCi of [³²P]O₄Na₂ in phosphate-deficient medium (Sigma) containing cycloheximide. Thereafter, the soluble and filamentous fractions were obtained as above, and analyzed by immunoprecipitation (soluble fraction) using a mixture of rabbit polyclonal anti-K5 and RCK107 antibodies (see below) or by direct analysis in SDS-PAGE (filamentous fraction).

To analyze the molecular species present in the soluble fraction, chemical crosslinking was performed. Briefly, the [³⁵S]Met-labelled soluble fraction was incubated with 1% glutaraldehyde for 1 hour at 37°C, followed by quenching in 10 mM NH₄Cl. The crosslinked soluble fraction obtained was immunoprecipitated using the anti-K5 antiserum and analyzed in 9% SDS-PAGE.

RESULTS

We studied the dynamic behaviour of keratins, focusing on possible differences among them. We performed cytoplasmic fusions of two types of epithelial cultured cells and evaluated how the keratins reorganize to form the heterokaryon cytoskeleton. The availability of monospecific antibodies allowed comparison of the dynamic characteristics of the different keratins present in the fused cells. It has been shown that cell fusion is a suitable technique for the study of the reorganization of cytoplasmic structures (Zheng and Chang, 1991). The process consists of three main events. First, cytoplasmic bridges form after the membrane fusion promoted by the chemical treatment. Second, these bridges expand, allowing the cytoplasm of the fusing cells to merge. Finally, the nuclei of the fused cells move towards the center of the heterokaryon and the cytoplasmic structures are reorganized, forming a newly integrated system. We studied this process, focusing on the fate of the keratin IF from the parental cells. In general, these studies were performed scoring only two-cell fusions to maintain the contribution of each cell type constant and to avoid artifacts arising from an over-representation of one cell type with respect to the other.

Keratin dynamics in BMGE+H/PtK₂ hybrids

BMGE+H and PtK₂ were selected as parental cell lines, as they express very different keratins that can be individually tracked using existing monospecific antibodies. BMGE+H cells are derived from bovine mammary gland, and express K5, K6 and K14 as major keratins (Schmid et al., 1983). PtK₂ cells are derived from kidney epithelium and express K7, K8, K18 and K19, together with vimentin (Albers and Fuchs, 1989). We

fused these parental cell lines and followed the behaviour of their cytoskeletons by double immunofluorescence using different antibodies, comparing K14 versus K8 or K5 versus K18. Antibody specificity was demonstrated previously by western blotting using cytoskeleton-enriched fractions obtained from the cell lines used, and by immunostaining of mixed unfused cultures (not shown).

The initial events (15 minutes) after fusion are characterized by the apparent depolymerization of the K8 keratin filament cables, which are close to the fusion area (arrow in Fig. 1A). In this region, the filament bundles become shorter, thinner and less organized. In some cases, we observed the appearance of dots and punctuate staining. In contrast, K14, present in BMGE+H filaments, does not show such apparent depolymerization. In fact, these filaments appear unaltered by the fusion process at this time (Fig. 1A'). A short time later (30 minutes), the intercellular bridge has extended and the alteration of K8 filaments in this area is more evident (arrow in Fig. 1B). Weak, filamentous but poorly organized K8 staining can be seen in the cytoplasmic BMGE+H region adjacent to the fusion area. In contrast, the K14-containing filaments are still unaltered at this time (Fig. 1B').

One hour post-fusion, when the parental cells nuclei have migrated to the center of the heterokaryon, the TROMA1 antibody stains the entire heterokaryon diffusely, including the BMGE+H cytoplasm (Fig. 1C). In addition, original unrearranged K8-positive IF bundles are still present in those areas of the heterokaryon cytoplasm corresponding to the PtK₂ cell (arrowheads in Fig. 1C). In contrast, K14 from BMGE+H cells remains unaltered and confined to its cytoplasmic parental area (Fig. 1C'). It is important to note that in the BMGE+H part of the heterokaryon, K8 and K14 staining do not coincide. This indicates that, during its spreading, K8 does not co-integrate extensively into the pre-existing K14 cytoskeleton. We already observe K8 staining in the heterokaryon periphery at this early time, associated with the filamentous intercellular bridges characteristic of the BMGE+H cells (arrows in Fig. 1C), which have been described as associated with desmosomes (Schmid et al., 1983). In fact, double immunofluorescence staining of these hybrids 1 hour post-fusion using TROMA1 (Fig. 2A) and a mAb against desmoplakins 1+2 (Fig. 2A') demonstrate the colocalisation of both proteins (Fig. 2, arrowheads).

Three hours post-fusion, K8 appears distributed throughout the heterokaryon cytoplasm and well organized in filaments (Fig. 1D). At this time K14 is still almost exclusively restricted to filament bundles localized around the BMGE+H nucleus, probably in the pre-existing parental filament cables (Fig. 1D'). In some of these K14-containing filaments, it is already possible to detect K8 (arrowheads in Fig. 1D).

At 8 hours post-fusion (Fig. 1E,E'), K8 is already arranged in a normally organized IF cytoskeleton throughout the entire heterokaryon. However, although K14 has progressed towards heterokaryon colonization, there is only limited K8/K14 colocalisation and in most cases, only after 24 hours post-fusion was the complete colocalisation of K14 and K8 observed throughout the filaments of the entire heterokaryon cytoplasm (Fig. 1F,F').

In vivo, the natural partners of K8 and K14 are K18 and K5, respectively (Moll et al., 1982). To study whether the latter keratins behave similarly to K8 and K14, we repeated the above experiments, staining for K5 from BMGE+H and K18

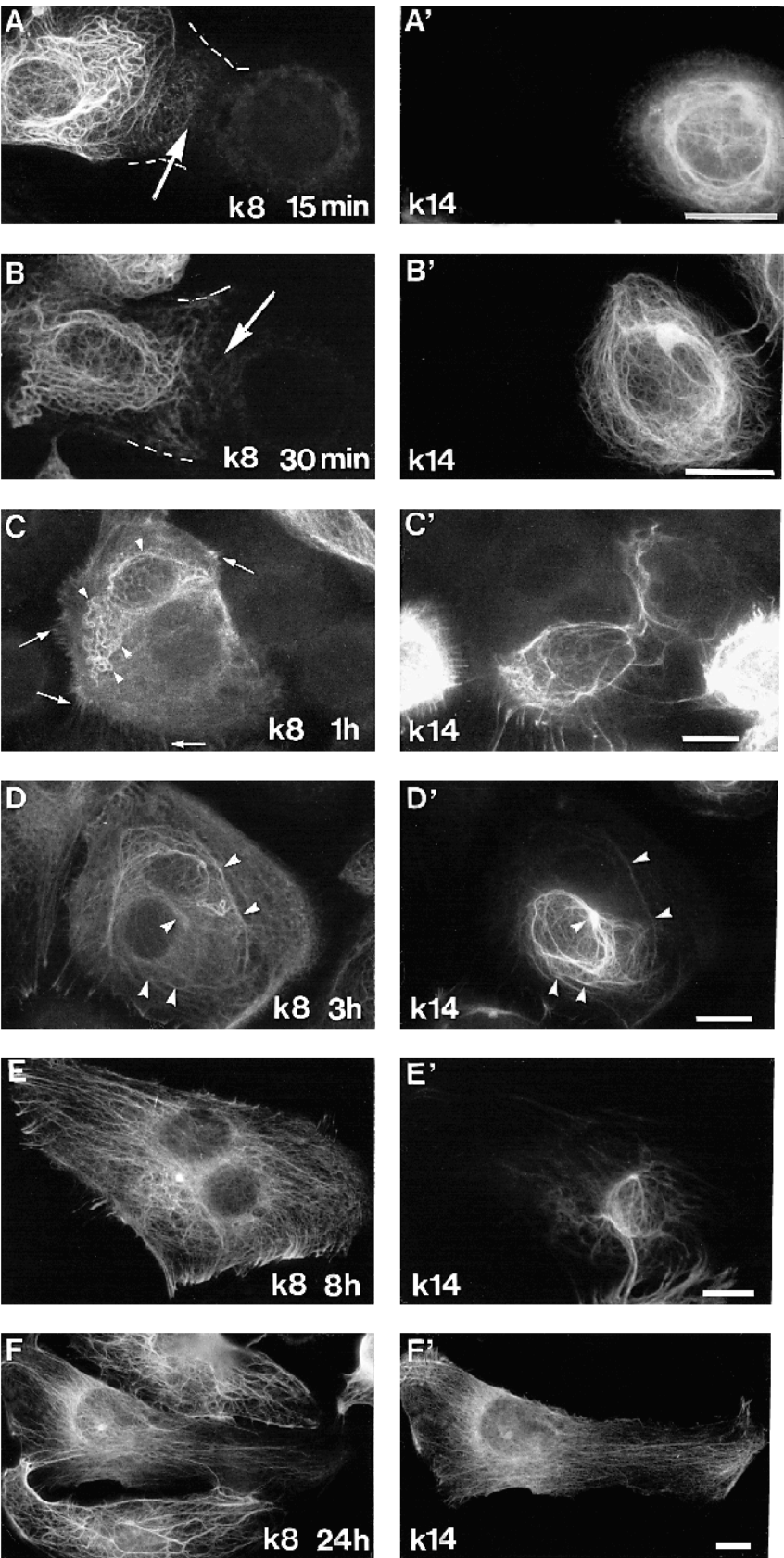
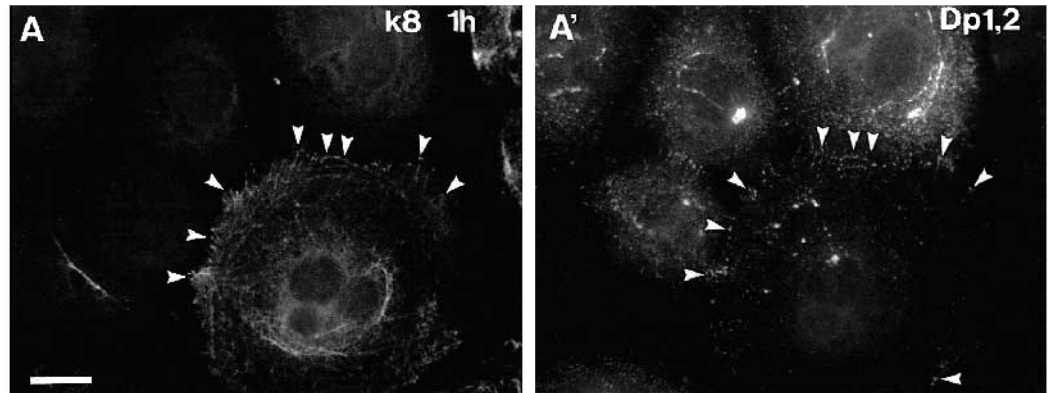


Fig. 1. Dynamics of K8 (A,B,C,D,E,F) versus K14 (A',B',C',D',E',F') in PtK₂/BMGE+H cell hybrids. Double immunofluorescence was performed at different times after fusion (A,A': 15 minutes; B,B': 30 minutes; C,C': 1 hour; D, D': 3 hours; E,E': 8 hours; F, F': 24 hours) using TROMA1 antibody against K8 and RCK107 against K14. Arrows in A and B denote the depolymerization of K8 in the fusion region (broken lines). Arrowheads in C denote presumed parental K8 positive filaments. Arrows in C denote the association of K8 positive filaments with the desmosomal junctions. Arrowheads in D and D' denote copolymerization of K8 and K14 in some but not all filaments. Bars, 10 μm

from PtK₂ cells. The initial events in these process were nearly identical for K18 as those described for K8. However K5,

contrary to K14, also displayed the depolymerization described for K8 at the fusion-adjacent areas (not shown).

Fig. 2. K8 associates with desmosomes early after fusion. BMGE+H/PtK₂ hybrids were stained 1 hour after fusion with TROMA1 (A) and Dp1 & 2 against desmoplakin 1+2 (A'). Note that K8 shows a clear tendency to colocalize with desmoplakins in the periphery of the heterokaryon (arrowheads). Bar, 10 μ m.



At 1 hour post-fusion, K18 staining is already found over broad areas of the heterokaryon (Fig. 3A'). In this short time, K18 also has a remarkable tendency to localize at the desmosomal junctions (arrows in Fig. 3A'). However, in spite of the apparent progress of the colonisation process by K18, some strong parental-type PtK₂ IF bundles are still apparent. On the other hand, K5 has clearly invaded the PtK₂ part of the heterokaryon (Fig. 3A), indicating a greater dynamic behaviour than K14, its natural partner (compare Fig. 3A with 1C'). As described for the case of K8/K14, at this time there is no great degree of K5/K18 co-staining, and when it exists, it occurs in thin, probably newly-formed filaments (arrowheads in Fig. 3A,A') and not in the parental filaments. At 3 hours (Fig. 3B,B'), both K5 and K18 are found throughout the heterokaryon and are organized into filaments in which the co-localisation of the two keratins is evident. However, there are some filaments in which there is no such co-localisation (arrowheads in Fig. 3B').

At 6 hours post-fusion (Fig. 3C,C') the process is at an advanced state, and keratin spreading and co-localisation are complete after 9 hours (data not shown). In comparison with the dynamic behaviour of K8 and K14, K18 is approximately as rapid as K8, however, K5 clearly appears more mobile than K14 (compare Fig. 3B and C with Fig. 1D' and E', respectively). Similar results were obtained using the mouse skin keratinocyte cell line MCA3D instead of BMGE+H, although the behaviour of K5 and K14 was slightly more dynamic in these cells (not shown; see also Fig. 6B,B').

Dynamics of keratin K10

So far, we have analyzed the behaviour of keratins characteristic of simple epithelia and basal cells of stratified epithelia. These cells are proliferation competent and one would suspect that their cytoskeletons might be more plastic than those of the suprabasal cells of stratified epithelia, such as epidermis. These latter cells are post-mitotic and the keratins that they synthesize (K1 and K10) form part of the cornified layer characteristic of this tissue. We have recently shown that, upon transfection in epithelial cells, K10 incorporates surprisingly quickly into the endogenous K8/K18 filaments in a process involving marked cytoskeletal rearrangements (Paramio and Jorcano, 1994). To study the behaviour of this keratin in heterokaryons, we fused PtK₂ and B10-4 cells. This cloned cell line was derived from BMGE+H cells permanently transfected with a plasmid containing the gene coding for bovine K10 (BK VI in the bovine notation) under the control of the bovine K6

promoter (BKIV in the bovine notation; for a description of this plasmid see Blessing et al., 1989). B10-4 shows no differences from the parental BMGE+H cell line with respect to its IF cytoskeleton appearance. The study of K5 and K14 dynamics using B10-4 as parental cells in fusions with PtK₂ cells revealed no significant differences compared with the results obtained using BMGE+H cells (data not shown).

The fusion was followed by double immunofluorescence using antibodies TROMA1 against K8 and K8.60 against K10. In this case, K10 seems to be much more dynamic than K14, since its spreading through the heterokaryon cytoplasm and its co-localisation with the simple epithelial keratin K8 occurs rapidly, with kinetics similar to that of K8 and K18. After 1 hour (Fig. 4A,A'), it is already possible to detect K10 in the PtK₂ region of the hybrid cytoplasm (arrows in Fig. 4A'). However, staining is diffuse and poorly-organized, and there is no apparent integration of K10 into the K8 filaments. At 2 hours post-fusion, K8 and K10 are dispersed throughout the entire heterokaryon and organized into thin filaments (Fig. 4B,B'). However, some thick parental K10 filaments are still associated with the B10-4 parental cell nuclei, indicating that they have not yet undergone the reorganization process. As described for the earlier BMGE+H/PtK₂ fusions, there is substantial colocalisation of K8 and K10 at this time (arrowheads in Fig. 4B,B'). This cointegration takes place in thin, possibly newly-formed filaments, however, whereas the thick parental BMGE+H filaments do not show major K8 integration. At 3 hours post-fusion (Fig. 4C,C'), the process is well-advanced for both K8 and K10, and K10 is co-localised in most of the K8-positive filaments. However, some parental K10 IF still form perinuclear bundles in which K8 is absent, or present in small amounts with respect to K10 (Fig. 4C'). At 6 hours (Fig. 4D,D') and 9 hours post-fusion (Fig. 4E,E'), the process of copolymerisation into the heterokaryon cytoskeleton is complete.

These results again suggest that the dynamic behaviour of keratins is dependent on each polypeptide, since K10 and K14 must initially form complexes in this cell line with the same type II partners (K5 and K6), but the dynamic behaviour of these two keratins is completely different.

Effect of protein synthesis on keratin dynamics

To study whether or not the formation of the heterokaryon cytoskeleton depends on the synthesis of new keratin polypeptides, or other accessory proteins, we performed a series of fusion experiments. In these, cells were treated with 50 μ M cycloheximide for 2 hours before promoting fusion, as under

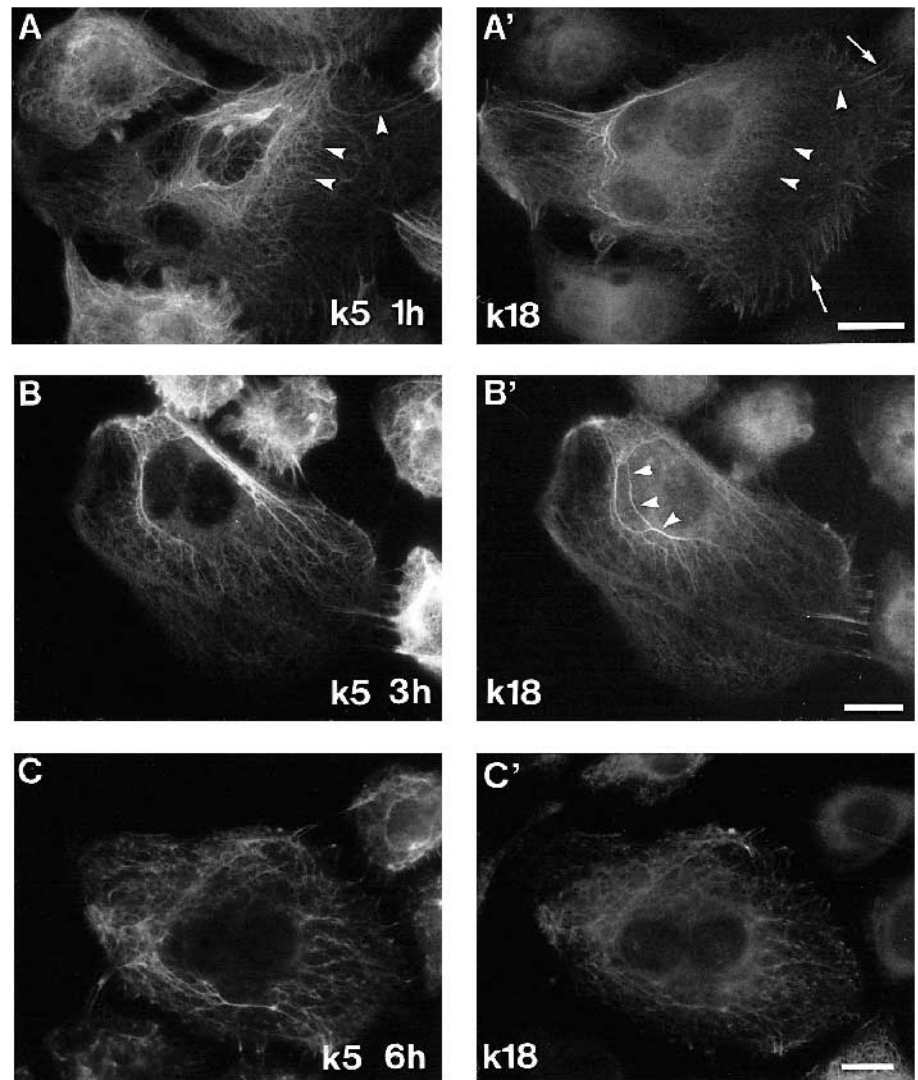


Fig. 3. Dynamics of K5 (A,B,C) versus K18 (A',B',C') in PtK₂/BMGE+H cell hybrids. Double immunofluorescence was performed at different times after fusion (A,A': 1 hour; B,B': 3 hours; C, C': 6 hours) using the LE61 antibody against K18 and a monospecific rabbit antibody against K5. Note limited K5/K18 colocalisation in these filaments (arrowheads in A,A') at 1 hour post-fusion. Extensive, although not complete colocalisation of these two keratins (arrowheads in 3B') is detected at longer times (3 hours, 6 hours). Arrows in A' denote K18 localisation at desmosomal junctions. Bars, 10 μ m.

these conditions protein synthesis is completely inhibited during the time that heterokaryons are studied (Paramio and Jorcano, 1994).

The results obtained for K8 and K10 after the fusion of B10-4 and PtK₂ cells are shown in Fig. 5. As can be observed, the kinetics of the process is similar to that observed in the absence of cycloheximide (compare Fig. 5 with Fig. 4). This indicates that the formation of the hybrid heterokaryon cytoskeleton does not depend on the synthesis of new proteins, but on the depolymerization of the preexisting filaments and their repolymerization or reassembly. Although the process of cytoskeleton rebuilding is similar in the heterokaryons in the absence or in the presence of protein synthesis, the kinetics, in particular of K10, appear to be slightly delayed in the presence of cycloheximide (compare Fig. 5B' with 4C', and Fig. 5C' with 4D').

Keratins K5 and K14, an expression pair, display different dynamics

Keratins K5 and K14 form an expression pair *in vivo*. Since K14 is the major acidic keratin in BMGE+H cells (Schmid et al., 1983; see also Fig. 7A), K5 and K14 must form complexes in these cells. Moreover, since K6 is minority

compared with K5 (Schmid et al., 1983), the K5-K14 should be the major complexes in these cells. However, the results shown in Figs 1 and 3, suggest that they behave with quite different dynamics. To study this problem more carefully, we performed cell fusions between BMGE+H and PtK₂ cells, and the resulting heterokaryons were double-stained using rabbit polyclonal anti-K5 and RCK107 mAb against K5 and K14, respectively (Figs 1 and 3). The results obtained (Fig. 6A,A') clearly confirm these observations. Thus, when K5 is spreading throughout the heterokaryon cytoplasm at 3 hours post-fusion, K14 is mostly restricted to the perinuclear area of the BMGE+H cells (Fig. 6A,A'). As a control, in unfused BMGE+H cells, or in PEG-treated BMGE+H, the antibodies against K5 and K14 recognize the same filaments (not shown; see also the cell at the left margin in Fig. 6A,A'). To further investigate this apparent difference between K5 and K14, we performed similar experiments using different cells (PtK₂ and MCA3D mouse keratinocytes) and the AE14 mAb against K5 and a rabbit polyclonal antibody against the carboxy terminus of K14. Staining of BMGE+H-PtK₂ hybrids with these antibodies gave results undistinguishable from those shown in Fig. 6A,A' (results not shown). In addition, to avoid potential

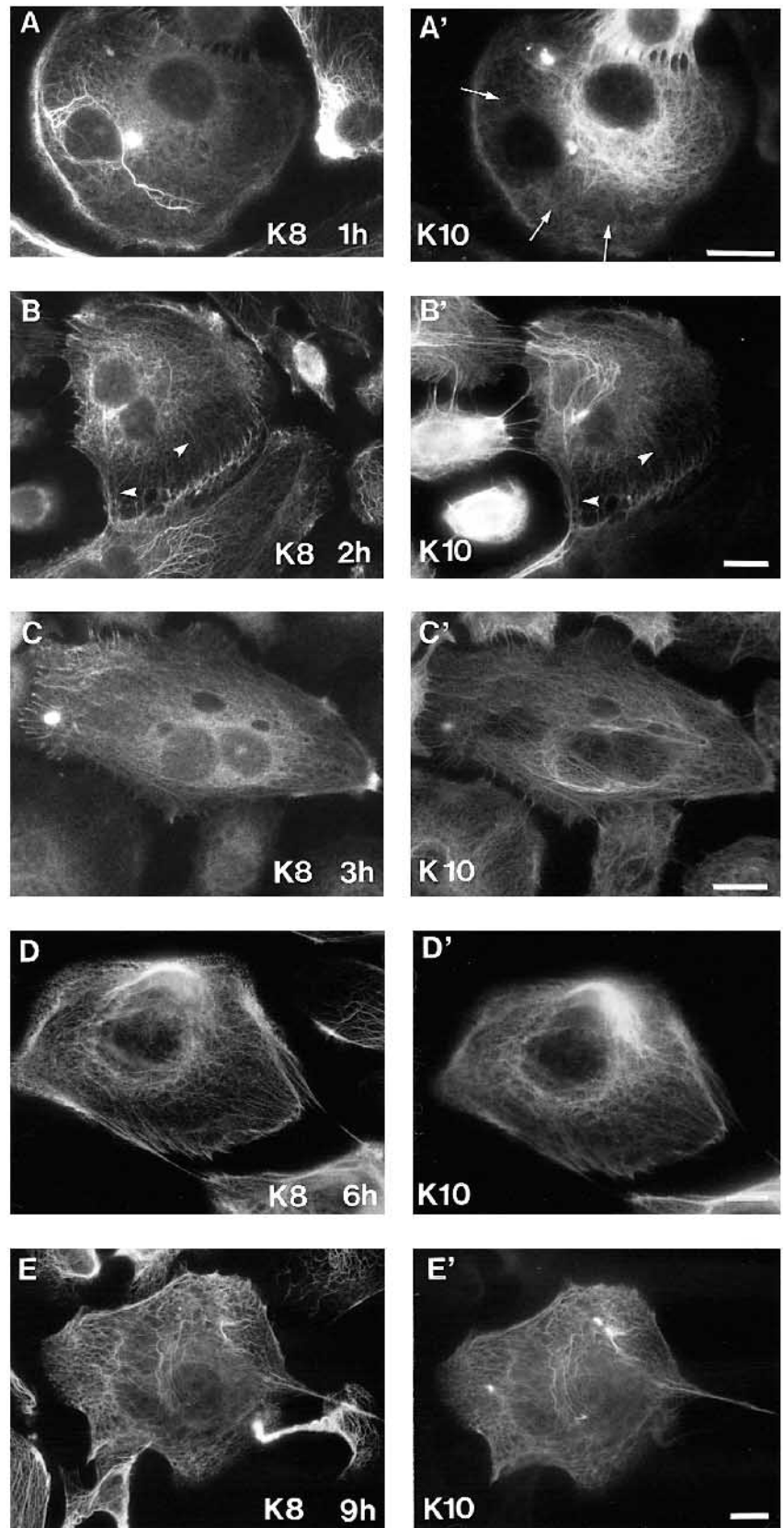


Fig. 4. Dynamics of K8 (A,B,C,D,E) versus K10 (A',B',C',D',E') in PtK₂/B10-4 cell hybrids. Double immunofluorescence was performed at different times after fusion (A,A': 1 hour; B,B': 2 hours; C,C': 3 hours; D,D': 6 hours; E,E': 9 hours) using TROMA1 antibody against K8 and K8.60 against K10. Arrows in A' denote the presence of K10 in the PtK₂ region of the hybrid cytoplasm. Arrowheads in B,B' denote the colocalisation of K8 and K10 in thin, presumably newly-formed filaments. After 6 hours most cell hybrids show nearly complete colonization by both keratins. Bars, 10 μ m.

artefacts due to the fixation method, MCA3D-PtK₂ hybrids were fixed using paraformaldehyde rather than methanol. The

results obtained (Fig. 6B,B') again demonstrated that K5 is more dynamic than K14. We also attempted to investigate the

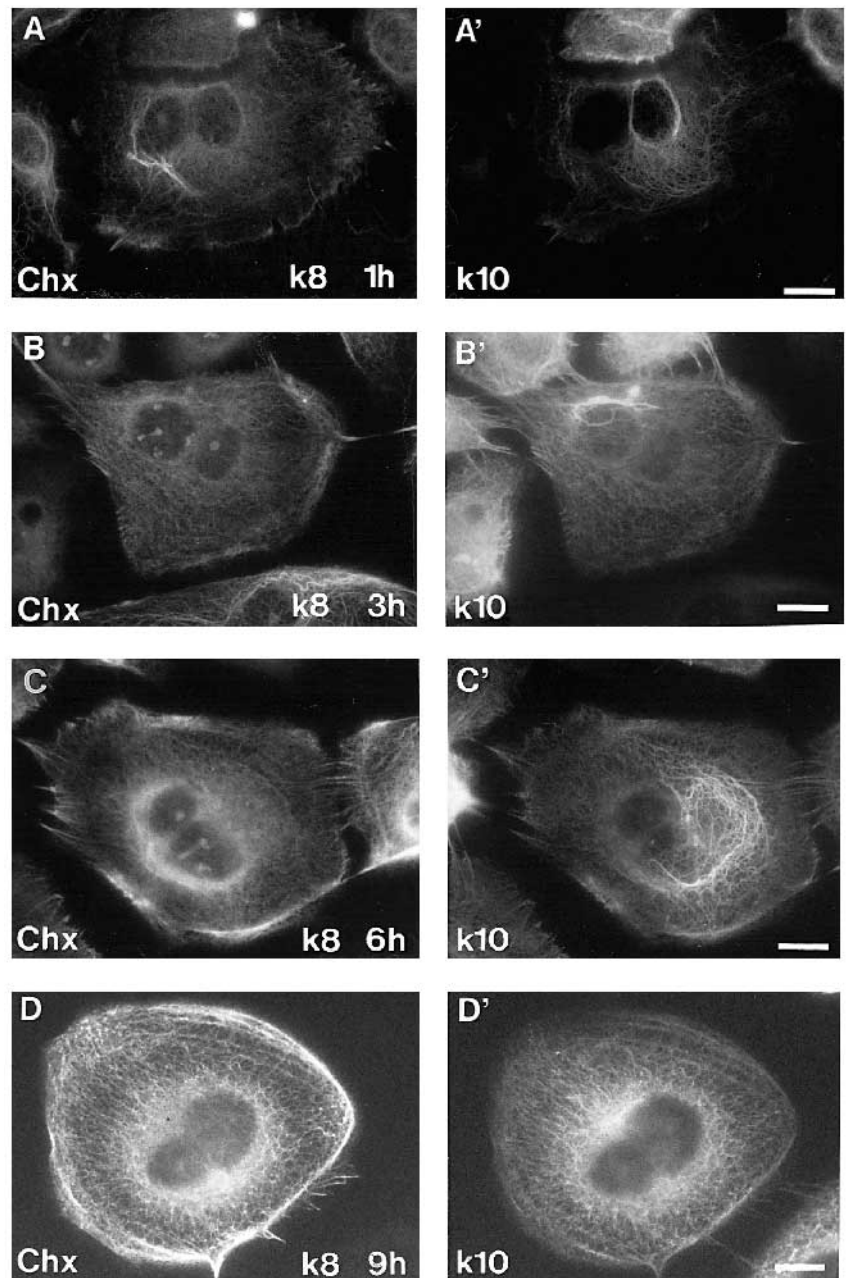


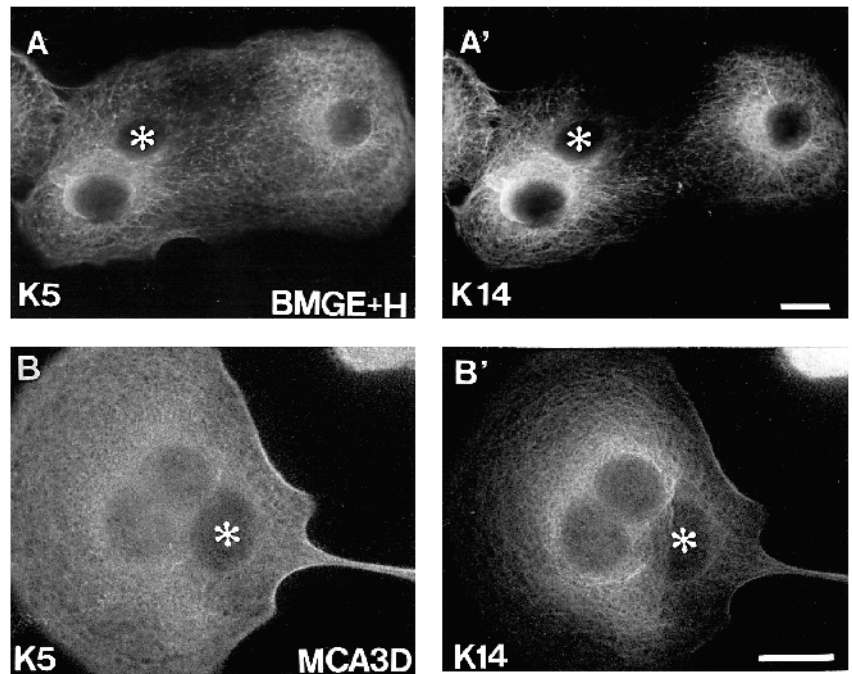
Fig. 5. Effect of protein synthesis inhibition on the dynamics of K8 (A,B,C,D) and K10 (A',B',C',D') in B10-4/PtK₂ hybrids. Cells were pretreated for 2 hours in the presence of 50 μ M cycloheximide, fused and, at different times post-fusion (A,A': 1 hour; B,B': 3 hours; C,C': 6 hours; D,D': 9 hours), analyzed by double immunofluorescence as in Fig. 4. K8 and K10 behaviour is similar in the absence of cycloheximide (Fig. 4). Bars, 10 μ m.

behaviour of K6 in these experiments, but the anti-K6 polyclonal antibody does not appear to stain BMGE+H cells. However, in MCA3D-PtK₂ hybrids, the behaviour of K6 is similar to that of K5 (results not shown). This result, together with the reduced amounts of K6 compared to K5 and K14 in BMGE+H cells (Schmid et al., 1983; see also Fig. 7A), suggests that the differences observed between K5 and K14 are not attributable to preferent pairing of K14 with K6 rather than K5, in agreement with Paladini et al. (1996) who showed that K14 pairs almost equally with K5 and K6. In another control, the BMGE+H keratin pattern did not change as a consequence of cell fusion as demonstrated by two-dimensional gel electrophoresis analysis of keratin-enriched cytoskeletal fractions of PEG-treated BMGE+H cells (results not shown).

Biochemical analysis of the soluble keratin pool

If we assume that, in the absence of protein synthesis, the amount of a given keratin in the soluble pool reflects and is a consequence of its dynamics, our results would predict that the amount of K5 and K14 in this soluble fraction must be different. To analyze this possibility, BMGE+H cells were cultured for 16 hours with [³⁵S]Met, fused by PEG treatment (in the absence of any other partner cells). They were incubated for 3 hours in non-radioactive medium in the absence of protein synthesis to allow integration of the newly-synthesized radioactive keratins into IF (see Materials and Methods). Subsequently, the insoluble and soluble fractions of these cells were isolated and the keratins present in the latter fraction were enriched by *in vitro* reconstitution using non-radioactive keratin isolated from BMGE+H cells. The radioactive keratins

Fig. 6. Different dynamics of K5 (A,B) and K14 (A',B') in BMGE+H/PtK₂ (A,A') and MCA3D/PtK₂ (B,B') cell hybrids observed by double immunofluorescence at 3 hours after fusion using a monospecific rabbit antibody against K5 and mAb RCK107 against K14 (A and A', respectively) or mAb AE14 against K5 and a rabbit polyclonal against K14 (B and B', respectively). Note in both type of fusions that the K5 staining (A,B) spreads throughout broad areas of the heterokaryon cytoplasm while the K14 staining (A',B') is mainly restricted to the perinuclear area of the parental BMGE+H or MCA3D cells. Asterisks denote the nucleus of the PtK₂ cells involved in the fusion. Bars, 10 μ m.



present in each fraction were analyzed by fluorography of SDS-PAGE (Fig. 7A). As can be observed, in the insoluble fraction corresponding to the keratins which are polymerized into filaments, the amounts of K5 and K14 are very similar while K6 is minority (Fig. 7A, lane 1), concurring with Schmid et al. (1983). However, in the soluble pool (Fig. 7A, lane 2) the amount of K5+K6 clearly exceeds the amount of K14. The bands which appear in the soluble fraction and do not correspond to keratins are also present in the total protein samples of this fraction (not shown). These results suggest the correlation between the different dynamic behaviour of K5 and K14 and the amounts of these polypeptides present in the soluble pool of BMGE+H.

In view of these results, we wanted to investigate the molecular forms of these soluble keratins. To this end, chemically crosslinked and non-crosslinked radioactive soluble fractions were immunoprecipitated (Fig. 7B). The immunoprecipitation of the non-crosslinked soluble fractions using an anti-K5 antibody gave two bands (Fig. 7B, lane 1) which correspond to K5 and K14, with a preponderance of the former. This result confirms the observation using *in vitro* reconstitution (Fig. 7A) suggesting the presence of greater amounts of K5 with respect to K14 in the soluble fraction. On the other hand, the coprecipitation of K14 with the anti-K5 antiserum indicates that both proteins are forming complexes in the soluble fraction, although the excess of K5 suggests the

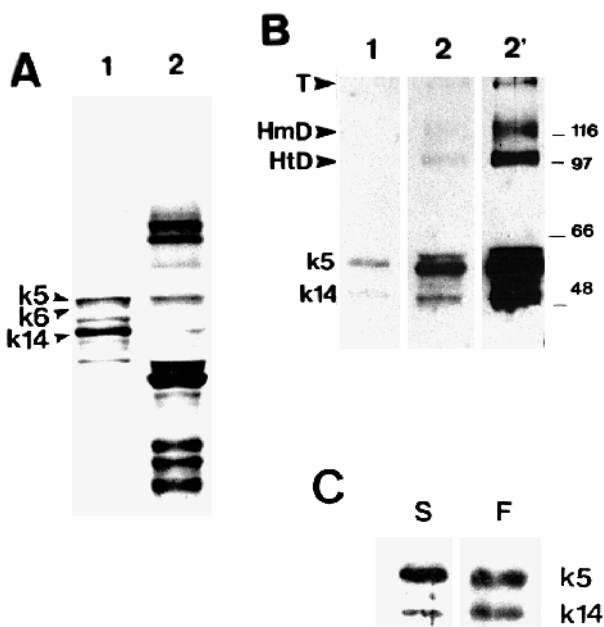


Fig. 7. Analysis of the keratin polypeptides present in the soluble and insoluble fractions from BMGE+H cells. (A) Fluorography of [³⁵S]Met labelled cytoskeletal proteins. Lane 1, polymerized, insoluble fraction; lane 2, soluble fraction after *in vitro* reconstitution using a non radioactive keratin fraction from BMGE+H. Arrowheads denote the position of K5, K6 and K14 as obtained by western blotting (not shown). (B) Immunoprecipitation of the [³⁵S]Met labelled soluble fraction using a monospecific rabbit polyclonal antibody against K5. Lane 1, non-crosslinked soluble fraction sample. Lane 2, chemically crosslinked soluble fraction. Note the presence of molecular species with mobilities compatible with K5/K14 heterodimers, K5 homodimers and tetramers (denoted by arrowheads and denoted by HtD, HmD and T, respectively). Lane 2' is a longer exposure of lane 2. (C) Keratin phosphorylation in the soluble (lane S) and insoluble (filamentous; lane F) ³²P-labelled fractions of BMGE+H cells. Note the similar labelling of K5 and K14 in the filamentous fraction. Note also that the differences in labelling observed in the soluble fractions are compensated by the differences in the amounts of K5 and K14 present in this fraction (compare C, lane S with A, lane 2 and B, lane 1). Insoluble, polymerized keratins were directly analyzed in SDS-PAGE. Soluble keratins were immunoprecipitated with a mixture of polyclonal anti-K5 and mAb RCK107 and analyzed by SDS-PAGE. The figure shows the autoradiograph of the gel.

presence of other molecular forms constituted predominantly by K5. To study these forms, the immunoprecipitation was repeated using soluble fractions which were previously chemically crosslinked by glutaraldehyde treatment. The results (Fig. 7B, lanes 2 and 2') demonstrate the presence of multimers (denoted by arrowheads in Fig. 7B) in addition to monomeric keratins. The apparent molecular mass of these multimers is compatible with K5/K14 heterodimers, K5 homodimers and tetrameric forms (labelled as HtD, HmD and T in Fig. 7B). However, we cannot discard the possibility that these high molecular mass species can be due to the interaction of K5 with proteins other than keratins, although their relative abundance and strong interaction with K5, suggest that they are in fact keratin complexes.

Phosphorylation has been suggested to promote changes in IF dynamics (Chou et al., 1989, 1990; Chou and Omary, 1991; Klymkowsky et al., 1991; for reviews see Skälli et al., 1992; Steinert, 1993). We therefore analysed whether the differences observed between K5 and K14 dynamics can be attributed to variations in the phosphorylation state of these proteins in the filamentous or soluble forms. To this end, in the absence of protein synthesis, 2 hours after fusion BMGE+H cells were labelled with ^{32}P for one additional hour and used to isolate the soluble and insoluble fractions. Gel electrophoresis followed by autoradiographic analysis of K5 and K14 in the filamentous fraction indicates that both proteins were labelled to a similar extent (Fig. 7C, lane F). Immunoprecipitation of the ^{32}P -labelled soluble fraction, using a mixture of anti-K5 and anti-K14 antibodies (Fig. 7C, lane S) showed predominant K5 labelling. However, densitometric analysis taking into consideration the greater amount of K5 than K14 in this fraction (Fig. 7A, lane 2; Fig. 7B lane 1) indicated that the degree of phosphorylation of these two keratins was similar. Therefore, differential keratin phosphorylation is probably not responsible for the different amounts of K5 and K14 observed in the soluble fraction. This does not eliminate, however, the possibility that phosphorylation may indeed play an important role in the generation of a soluble pool of keratins, modifying the overall solubility of these proteins within the cells, although Chou et al. (1993) demonstrated that, in simple epithelial cells, K8/K18 phosphorylation does not play any obvious role in generating the soluble keratin pool.

DISCUSSION

The cell cytoskeleton is a highly dynamic structure, and this dynamic behaviour is fundamental for its function in the cell. Probably due to their *in vitro* biochemical properties, IF have been considered as the most stable components of cells (Bloemendal and Pieper, 1989; Vikstrom et al., 1991). However, experiments of mRNA microinjection (Kreis et al., 1983; Franke et al., 1984), DNA transfection (Albers and Fuchs, 1987, 1989; Chin and Liem, 1989; Wong and Cleveland, 1990; Ngai et al., 1990; Sarria et al., 1990; Raats et al., 1990; Paramio and Jorcano, 1994), and, more recently, microinjection of IF-labelled proteins (Vikstrom et al., 1989, 1991, 1992; Miller et al., 1991, 1993) have shown that IF can also undergo dynamic processes *in vivo*, such as disassembly-reassembly or subunit exchange events (for reviews see Skälli et al., 1993; Steinert, 1993). In particular, a soluble pool of IF protein complexes,

which includes recently synthesised polypeptides, seems to be in dynamic equilibrium with IF-polymerized proteins (e.g. see Vikstrom et al., 1991; Miller et al., 1993). Besides this local IF dynamic exchange, little is known concerning the global IF cytoskeleton stability. In this study we present new aspects of the IF dynamic behaviour using the fusion of different types of epithelial cells in culture.

Shortly after cell fusion, the cytoskeleton of the heterokaryon is composed of the IF proteins of the parental cells, which form two different and separate networks. These proteins tend to combine and reorganize into a new, hybrid network. Therefore, cell fusion in combination with double immunofluorescence using monospecific antibodies can be used to study how a keratin from a cell spreads throughout and colonizes the cytoplasm of the other cell in the heterokaryon, and to observe how it combines with other keratins during this process. Using this approach, it is also possible to study how the pre-existing parental cytoskeletons evolve towards the formation of the heterokaryon cytoskeleton. In addition, this approach avoids potential artifacts arising from the use of modified keratins, or from changing the amount of keratin present in the cells, as in the case of microinjection or transient transfection experiments (see Miller et al., 1993). Therefore, in terms of changes in the total keratin amounts present in the cells, our results could be assumed to represent a steady state situation. In addition, proper selection of the parental cells to be fused has allowed the study of the behaviour of different keratins.

Our results demonstrate that keratins are highly dynamic proteins. Collectively, our data are in agreement with those of Miller et al. (1991, 1993), who showed that biotinylated type I keratins microinjected into epithelial cells incorporate readily into the pre-existing cytoskeleton. This incorporation is thought to take place due to the dynamic exchange of keratins between IF and the soluble pool (for a careful discussion, see Skälli et al., 1993; Steinert, 1993). However, we stress that, during the formation of the heterokaryon cytoskeleton, the main process we observe is not this dynamic exchange between pre-existing IF and the soluble pool, but a complete reorganization of the parental cell cytoskeletons. Our experiments, in particular those performed in the absence of protein synthesis, show that the heterokaryon cytoskeleton is formed via the depolymerization and co-polymerization of the parental cell cytoskeletons into a new hybrid network and that the keratin cytoskeleton is so dynamic that this process takes place within 3–6 hours. This indicates that dynamic exchange is not the only process taking place in IF, since the polypeptides that exit from IF into the soluble pool seem to have a stronger tendency to polymerize into new filaments than to reintegrate into pre-existing IF. This interpretation concurs with our observation that, after fusion, the first signs of co-polymerization of BMGE+H and PtK₂ keratins seem to be in short and thin, probably newly formed hybrid filaments, whereas the integration into pre-existing filaments is limited and is observed later. The result of these complex dynamics is the continuous and rapid reshaping of the keratin cytoskeleton manifest in these cell fusions, but which probably also takes place in normal cells. These dynamics are similar to that observed upon transient transfection of K10 into epithelial cells, which leads to the transient dismantling of the endogenous keratin cytoskeleton and its very rapid reconstruction (Paramio and

Jorcano, 1994). The observed time scale is in agreement with that required for incorporation of other microinjected or transfected IF proteins to incorporate into the endogenous filaments (Vikstrom et al., 1989; Wong and Cleveland, 1990).

We found that different keratins have diverse dynamics. Keratins K8, K10, K18 and K5 behave similarly, but they are much more dynamic than K14. Keratin assembly does not seem to require accessory proteins or nucleotide hydrolysis, depending only on the nature of keratin subunit interactions. Therefore, the hydrophobic and ionic interactions among these subunits should in principle be similar *in vivo* and *in vitro*. In this regard, the behaviour observed during the cell fusion experiments can be related to *in vitro* studies of keratin complex melting (Franke et al., 1983). These studies also showed that complexes containing K8, K18 or K10 are much less stable than complexes containing K14. However, they do not explain the different behaviour we found between K5 and K14. In fact, other types of *in vitro* experiments suggest that K5/K14 filaments are more stable than those composed by other keratins (Coulombe and Fuchs, 1990).

The diverse behaviour of K5 and K14 is also difficult to reconcile with the idea that keratins have a strong tendency to associate into type I/type II heterodimers and heterotetramers, and that these complexes are the only subunits polymerizing into IF and participating in the dynamic exchange processes (e.g. Miller et al., 1993; see also for recent reviews Steinert, 1993; Fuchs and Weber, 1994). Besides being natural partners in basal cells of stratified epithelia, K5 and K14 must necessarily form complexes in BMGE+H cells, since in these cells K14 is the only major type I keratin and the amounts of keratin K6 are small compared to those of K5 (Schmid et al., 1983; see also Fig. 7A, lane 1). The simplest explanation of our results would be that single polypeptides or homodimers or homopolymers can participate in the dynamic processes. The finding of greater amounts of type II than type I keratin in the soluble pool fraction of fused BMGE+H cells in the absence of protein synthesis (Fig. 7A), when both polypeptide types are present in similar amounts in the polymerized filament fraction, supports this hypothesis. It supports the greater mobility of K5 with respect to K14 detected by indirect immunofluorescence experiments. This hypothesis would also help to explain transfection (e.g. Albers and Fuchs, 1987, 1989; Chin and Liem, 1989; Paramio and Jorcano, 1994) and microinjection experiments (Miller et al., 1991, 1993) in which excess amounts of single polypeptides are expressed *de novo* in the cells and are readily incorporated into the IF network, even in the absence of protein synthesis (Miller et al., 1991, 1993; Paramio and Jorcano, 1994). Moreover, the immunoprecipitation and *in vitro* reconstitution experiments (Fig. 7A and B, lane 1) also indicate the existence of greater amounts of K5 than K14 in the soluble pool, and the chemical crosslinking experiments have revealed the presence in the soluble pool of high molecular mass species that could correspond to not only K5/K14 heterodimers but also K5 homodimers and probably larger homo- and heteropolymers (Fig. 7B, lanes 2 and 2'). The existence of keratin homodimers and homopolymers (Steinert, 1990, 1991) has been detected *in vitro* and these complexes have been proposed to participate in the dynamic exchange process (Steinert, 1991; Miller et al., 1993; for a contrary opinion see Fuchs and Weber, 1994).

We have studied whether keratin phosphorylation plays a role in generating these different amounts of K5 and K14 keratins in the soluble pool. Our results, however, indicate that this post-translational modification does not seem to be relevant in the differential dynamics displayed by K5 and K14, since the two keratins appear equally phosphorylated in both soluble and filamentous pools (Fig. 7C). The apparent differences in ³²P content detected in the soluble pool (Fig. 7C, lane S) can be directly attributed to the different amounts of each protein in this fraction (Fig. 7A and B, lanes 1). This absence of an obvious role for phosphorylation in the generation of this difference in the soluble pool is in agreement with the results of Chou et al. (1993) and suggest an alternative mechanism for the observed differences in keratin solubility.

Initial events occurring after fusion involve the disassembly of those filament bundles reaching the fusion area, but apparently not in other areas of the fused cell cytoplasm. This fact could probably be associated with the disappearance of the cell membrane in this region, and the lack of attachment of the filaments. In this regard, the tendency of the PtK₂ keratins to reassociate quickly at the intercellular keratin bridges, associated with desmosomes in the heterokaryons (Fig. 2; see also Figs 1C and 4B) would suggest that these junctions are sites at which a highly dynamic exchange of keratins takes place. An intimate association between keratin IF and desmosomes (Green and Jones, 1990; Schwarz et al., 1990; Stappenbeck and Green, 1992), and a preferential association of desmoplakin with type II keratins have been reported (Kouklis et al., 1994). The reassembly process was otherwise homogeneous throughout the entire heterokaryon cytoplasm, indicating that keratin IF assembly under these conditions does not require a juxtanuclear organizing center, in agreement with microinjection of keratin mRNA (Kreis et al., 1983; Franke et al., 1984) or microinjection of keratin polypeptides (Miller et al., 1991, 1993).

Collectively, our results confirm the dynamism of keratin IF polypeptides. However, the specific dynamics appears to be dependent upon the different polypeptides involved and related to the amount of each polypeptide in the soluble pool.

Our thanks to M. Navarro, A. Ramírez, R. Kemler, B. Lane, F. Raemakers and D. Roop for their generous gifts of materials and helpful comments. We also thank M. Aldea and E. Cerezo for their expert technical support, and I. McLean and C. Mark for a critical revision of the manuscript. This work was partially funded by grants PM 92-0203 and PB90-0390 from D.G.I.C.Y.T. (Spain).

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(Received 7 January 1997 - Accepted 5 March 1997)