Phosphorylation of Keratin and Vimentin Polypeptides in Normal and Transformed Mitotic Human Epithelial Amnion Cells: Behavior of Keratin and Vimentin Filaments during Mitosis

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ABSTRACT Analysis by means of two-dimensional gel electrophoresis (IEF) of [^{32}P]orthophosphate-labeled proteins from mitotic and interphase transformed amnion cells (AMA) has shown that keratins IEF 31 (M_r = 50,000; Hela protein catalogue number), 36 (M_r = 48,500), 44 (M_r = 44,000), 46 (M_r = 43,500), as well as vimentin (IEF 26; M_r = 54,000) are phosphorylated above their interphase level during mitosis. Similar studies of normal human amnion epithelial cells (AF type) confirmed the above observations except in the case of keratin IEF 44 whose relative proportion was too low to be analyzed.

Immunofluorescent staining of methanol/acetone-treated mitotic transformed amnion cells with a mouse polyclonal antibody elicited against human keratin IEF 31 showed a dotted staining (with a fibrillar background) in all of the cells in late anaphase/early telophase (characteristic "domino" pattern) and in a sizeable proportion of the cells in other stages of mitosis. Normal mitotic amnion cells on the other hand showed a fine fibrillar staining of keratins at all stages of mitosis. Similar immunofluorescent staining of normal and transformed mitotic cells with vimentin antibodies revealed a fibrillar distribution of vimentin in both cell types.

Taken together the results indicate that the transformed amnion cells may contain a factor(s) that modulates the organization of keratin filaments during mitosis. This putative factor(s), however, is most likely not a protein kinase as transformed amnion cells and amnion keratins are modified to similar extents. It is suggested that in general the preferential phosphorylation of intermediate-sized filament proteins during mitosis may play a role in modulating the various proposed associations of these filaments with organelles and other cellular structures.

Epithelial cells contain intermediate-sized filaments (10 nm) composed of proteins that are related to the epidermal keratins (for a recent review see reference 1). In addition, many cultured epithelial cells also express vimentin (2, 3). In mitotic HeLa cells these proteins are phosphorylated above their interphase level (4–6) and we have suggested that these modifications may be instrumental in assuring the process of cell division (4–6). A transient disintegration of keratin filaments throughout mitosis has been recently reported in several cultured epithelial cells (7–9), but this phenomenon is not general (9, 10).

As yet there is no information as to whether there is any correlation between keratin aggregation and phosphorylation during mitosis (9). Towards this aim we present in this report a comparative gel electrophoresis and immunofluorescence study of the behavior of intermediate filament proteins during mitosis in normal and transformed human epithelial amnion cells. The results indicate that transformed amnion cells (AMA) may contain a factor(s) that affects the dynamics of keratin organization during mitosis. This putative factor(s), however, is most likely not a protein kinase as AMA and amnion keratins are phosphorylated to a similar extent during

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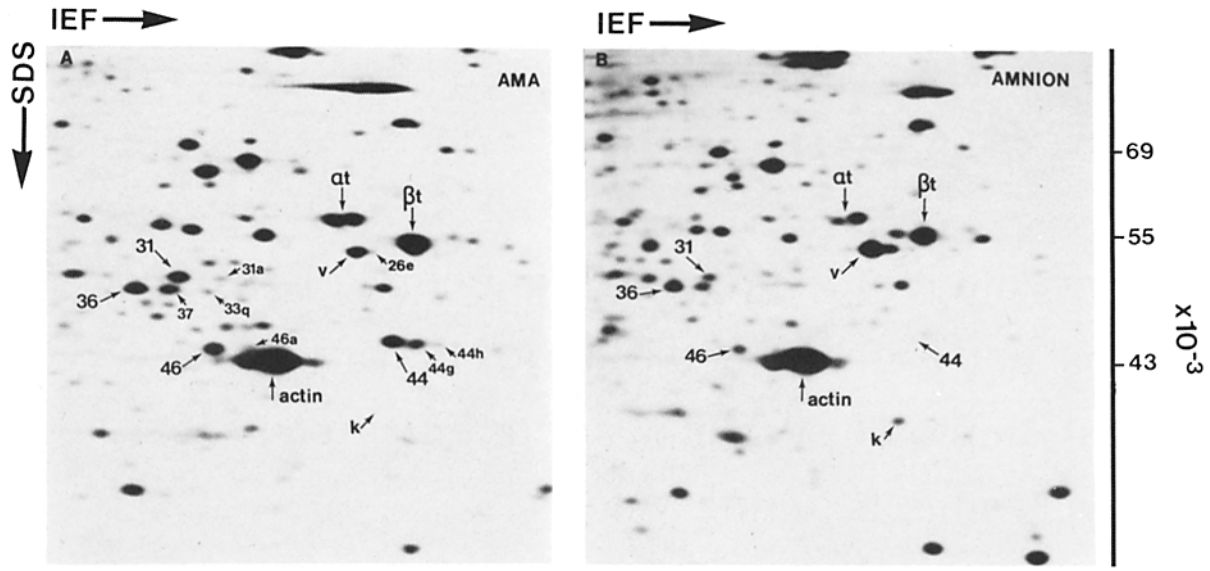

FIGURE 1 Two-dimensional gel electrophoresis (*IEF*) of [^{35}S]methionine-labeled proteins from asynchronous AMA cells (P103; *A*) and human epithelial amnion cells (*B*; AF type) labeled for 16 h. IEF *31, 36, 44, 46,* and *k* correspond to keratins. *v*, vimentin; *αt*, *α*-tubulin; *βt*, *β*-tubulin. Some modified variants of the keratins and of vimentin are indicated in *A* (see also Table II and Fig. 2, *A* and *B*).

mitosis. In general, the preferential phosphorylation of intermediate-sized filament proteins during mitosis is suggested to modulate filament organelle interactions.

MATERIALS AND METHODS

Cultured cells free of mycoplasm were grown routinely as monolayer cultures in Dulbecco's modified Eagle's minimal essential medium containing 10% fetal calf serum and antibiotics (100 u/ml penicillin, 50 μg/ml streptomycin). The normal human amnion epithelial cells (from amnioncentesis, AF type [11, 12] passage 2) were kindly provided by the Department of Human Genetics, Århus University, Denmark.

The preparation and characterization of the mouse polyclonal antibody against human keratin IEF 31 has been described in detail elsewhere (6). The vimentin antibody was a gift from Dr. S. Blose (Cold Spring Harbor Laboratory).

The procedures for preparing mitotic cells by mechanical detachment (4, 5, 13), labeling with [^{35}S]methionine (14, 15) and [^{32}P]orthophosphate (4), two-dimensional gel electrophoresis (4), and indirect immunofluorescence of methanol/acetone-fixed cells (16) have been previously described.

RESULTS

Phosphorylation of Keratins and Vimentin during Mitosis in Normal and Transformed Human Epithelial Amnion Cells

Fig. 1 *a* shows the appropriate region of a two-dimensional gel fluorograph (IEF)[1] of [^{35}S]methionine-labeled polypeptides from asynchronous human AMA labeled for 20 h (6, 17). The positions of the four keratins present in these cells (IEF's 31, M_r = 50,000; 36, M_r = 48,500; 44, M_r = 44,000, and 46, M_r = 43,500; HeLa protein catalogue number, 18–20, Table I) (6) as well as of vimentin (*v*), *α*- and *β*-tubulin (*αt*, *βt*), and total actin are indicated as reference. At least three of the AMA keratins (IEF 31, 36, and 46) as well as vimentin are present in normal cultured epithelial amnion cells (AF type; Fig. 1 *b*) (6) but these cells synthesized lower amounts of keratins IEF 31 and 46 and higher amounts of vimentin (see Fig. 1, *a* and *b*; Table I) (6, 17). The keratin IEF 44 was either present in only very small amounts or was absent in amnion cells. Cultured epithelial amnion cells contain in addition a low molecular weight keratin (M_r = 40,000; *k* in Fig. 1 *b*, Table I) that was not detected in AMA cells (Fig. 1 *a*). This protein can be immunoprecipitated with a broad specificity keratin antibody but it has not been thoroughly characterized.

In mitotic AMA cells the keratins as well as vimentin were phosphorylated above their interphase level (Fig. 2, *a* and *b*; 21). The relevant fraction of IEF gels of [^{32}P]orthophosphate-labeled proteins (15-min labeling) from mitotic and interphase AMA cells depleted of mitotic figures are shown in Fig. 2, *a* and *b*, respectively. For reference the positions of the non-phosphorylated keratins and of vimentin are indicated in Fig. 2 *a* (mitotic AMA cells) and are listed in Table II. The phosphorylated spots are indicated with their corresponding number in the HeLa protein catalogue (18–20). The authenticity of the modified proteins has been verified by immunoprecipitation using a broad specificity keratin antibody (21). The following modification products of IEF 31 (*31c*), 36 (*33q*, *35b*, *33p*), 44 (*44h*, *44h1*), 46 (*46b*), and vimentin (*26e1*, *26e2*) were preferentially detected during mitosis (Table II) although variable amounts of some of these polypeptides were also observed in interphase cells depleted of mitotic cells (Fig. 2 *b*). Since most of the mitotic cells were in prometaphase and metaphase (~65% of the cell population; not shown) it is likely that these modifications may take place in or very near these phases of the cell cycle. Phosphoproteins IEF *31a*, *37*, *44g*, *46a*, and *26e* correspond to the first modification products of the keratins and of vimentin (Fig. 2 *a*; Table II) and are the main phosphorylated form of these proteins in interphase (Fig. 2 *b*) (see also references 4, 5, and 21). A summary of the levels of modification of the various intermediate-sized filaments proteins in interphase as well as in mitosis is given in Table I. The data have been calculated from the analysis of [^{35}S]methionine-labeled proteins. Clearly, the level of modification of keratins IEF 36 and 44 in interphase is significantly higher than that of the two other keratins and of vimentin (see Table I). In all cases, however, an

[1] *Abbreviations used in this paper:* IEF, isoelectric focusing.

TABLE I

Intermediate-sized Filament Proteins of Normal and Transformed Human Amnion Cells

Intermediate-sized filament proteins	Mol wt	AMA/Amnion*	AMA		Amnion	
			Interphase	Mitosis	Interphase	Mitosis
	Kdalton		%‡	%	%‡	%
Keratin						
IEF 31	50	2.50	18	38	17	ND§
IEF 36	48.5	1.36	34	57	27	50
IEF 44	44	—¶	27	54	ND	ND
IEF 46	43.5	2.68	14	23	15	30
IEF K‖	40	—¶			ND	ND
Vimentin						
IEF 26	54	0.21	13	33	9	29

*Ratio based on the quantitation of [^{35}S]methione-labeled proteins. AF type amnion (11, 12).
‡% modification based on the quantitation of [^{35}S]methione-labeled proteins.
§In three preparations, the separation of IEF 31 from 31a was not optimal to allow quantitation.
‖This protein is not present in AMA cells.
¶ Barely detected or absent.

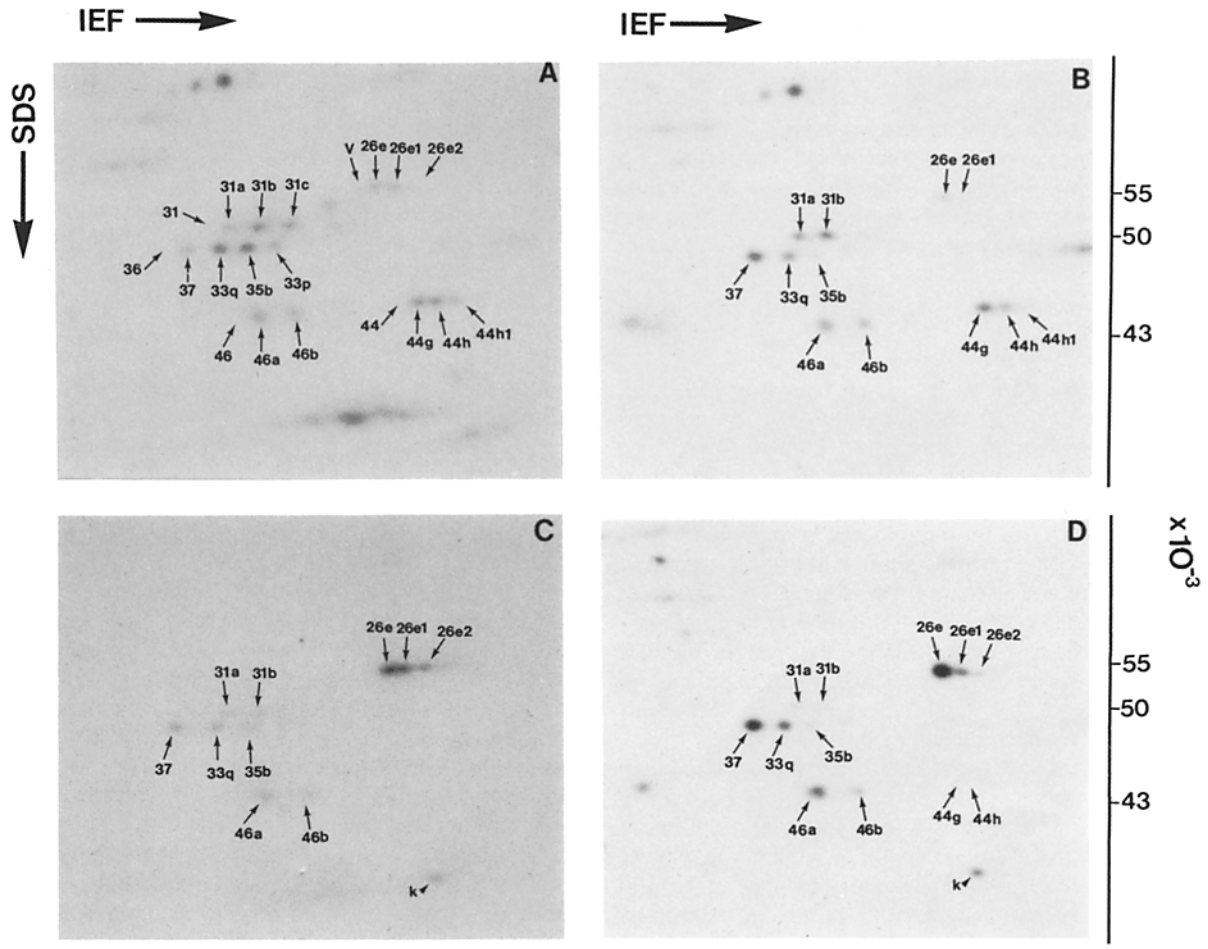

FIGURE 2 Two-dimensional gel electrophoresis (*IEF*) of [^{32}P]orthophosphate-labeled proteins (15-min labeling). (*A*) Mitotic AMA cells (P120); (*B*) asynchronous AMA cells depleted of mitotic cells; (C) normal mitotic amnion cells; and (*D*) asynchronous amnion cells depleted of mitotic cells. In *A* the position of the nonphosphorylated keratins and of vimentin are indicated as a reference. About 80% of the cells obtained by shake-off were in mitosis as determined by phase contrast microscopy.

increased modification during mitosis has been observed (~50%; Table I).

Similar gel analyses of [^{32}P]orthophosphate-labeled proteins from normal mitotic amnion cells and of interphase amnion cells depleted of mitotic figures are shown in Fig. 2, *c* and *d*. Since the number of normal mitotic amnion cells that can be obtained by mechanical detachment is very small, the gel in Fig. 2 *c* contains much fewer counts than those shown in other panels of Fig. 2. In spite of this limitation it is possible to see that keratins IEF *31*, *36*, *46*, and vimentin were phosphorylated preferentially during mitosis. The above analysis further showed that the 40-kdalton keratin present in normal

TABLE II

Main Phosphorylated Products of Keratins and Vimentin in Transformed Human Epithelial Amnion Cells

Intermediate-sized filament protein	Phosphorylated spots (IEF)*	Predominant spots‡
Keratins		
IEF 31	31a§, 31b§, 31c§	31c
IEF 36	37, 33q, 35b, 33p	33q, 35b, 33p
IEF 44	44g, 44h, $44h_1$§	44h, $44h_1$
IEF 46	46a§, 46b§	46b
Vimentin		
Vimentin‖	26e, $26e_1$§, $26e_2$§	$26e_1$, $26e_2$

*The authenticity of the modified variants has been assessed by immunoprecipitation (21).
‡Spots were observed in mitosis as compared with interphase cells.
§These numbers are new additions to the HeLa protein catalogue (18–20).
‖Vimentin corresponds to IEF 26 in the HeLa protein catalogue (18–20).

amnion cells (*k* in Fig. 1 *b*) is also phosphorylated (short arrows in Fig. 2, *c* and *d*). A summary of the levels of modification of the various intermediate-sized filament proteins is given in Table I. Again, we found that the level of phosphorylation in interphase was different for the various proteins (higher for IEF 36 and 44), but in all cases there was a substantial increase in mitosis (~50%; Table I). This increase was similar to that observed in AMA cells. Only in the case of keratin IEF 31 were we not able to quantitate the extent of modification.

Behavior of Keratin and Vimentin Filaments during Mitosis

Mouse polyclonal antibodies raised against human keratin IEF 31 were used to determine the pattern of keratin distribution during mitosis in normal and human AMA. The antibody reacted mainly with protein IEF 31, although it shows a small but significant cross-reactivity with keratins IEF's 36, 44, and 46 (6). Immunofluorescence microscopy of AMA at various stages during mitosis showed a granular, dotted staining in a significant proportion of the cells at all stages of mitosis but not in early Gl (Fig. 3, *b*, *f*, *j*, *n*, and *r*; prophase is not shown). In most cases, however, a fine filamentous network was also detected but this was difficult to picture as it lay in a different focal plane as the round fluorescent dots. We have encountered preparations, however, in which the dotted pattern was not observed (see also reference 9), indicating as suggested by Lane et al. (9) that keratin reorganization is taking place close to its threshold limiting condition. Based on the observation of 20 preparations (showing the dotted pattern), the following percentage of mitotic phases showed keratin aggregation: prophase, 20% (not shown); metaphase, 40% (Fig. 3 *b*); anaphase, 80% (Fig. 3 *f*); late anaphase/early telophase, 100% (Fig. 3 *j*), and late telophase, 46% (Fig. 3 *n*) (see also reference 9). Nearly all cells in early Gl showed a fibrillar staining (Fig. 3 *r*) and in some spreading cells it was possible to see short, unconnected stained filaments similar to those described in Ptk2 by Aubin et al. (10) (not shown). Interestingly, only late anaphase/early telophase cells consistently showed a dotted pattern in all cases. The staining pattern was most striking as it resembled a domino (Fig. 3 *j*); the fluorescent dots were usually aligned in the central part between the two sets of chromosomes. The nature of the interaction involved in maintaining this structure is at present unknown. The domino pattern rapidly disappeared in late telophase as the dots first disorganized and later appeared to concentrate in the central part as well as in the opposite end of the cells (not shown). The "domino" pattern has not been described before but Lane et al. (9) have shown that keratin aggregation reaches a peak in anaphase.

The above results contrasted with those obtained with vimentin antibodies which revealed a fibrillar distribution of vimentin in all stages of mitosis (Fig. 3, *c*, *g*, *k*, and *o*, prophase not shown) (10, 22).

Similar studies of normal amnion cells using the keratin IEF 31 antibody failed to reveal the dotted pattern at any stage during mitosis, instead a fine fibrillar distribution of keratins was observed (Fig. 3, *d*, *h*, *l*, and *p*; prophase is not shown). Essentially the same results were obtained with mouse polyclonal antibodies raised against human epidermal keratin k2 (23). In this case the dots showed a much stronger staining. Treatment of normal amnion cells with demecolcine for 3 h (10 μg/ml) did not result in the appearance of the dotted pattern as judged by immunofluorescence using the two keratin antibodies (not shown). Incubation of normal mitotic amnion cells with vimentin antibodies also revealed a fibrillar staining throughout mitosis (results not shown).

DISCUSSION

Previous studies by immunofluorescence and electron microscopy have shown a breakdown of keratin filaments during mitosis in various immortal cell lines (7–9) exhibiting various degrees of transformation. This phenomenon is not general, however, as many cell lines show intact keratin filaments throughout mitosis (9, 10).

Our study of a pair of normal (limited lifespan) and transformed human epithelial amnion cells indicated that the transformed cells contain a factor(s) that modulate keratin organization during mitosis. Since normal and transformed amnion cells show mainly quantitative differences in polypeptide synthesis (17, 24), it is likely that this putative factor is synthesized at low levels by the amnion cells and that its synthesis (or activity) is enhanced as a result of transformation. This factor(s), however, is most likely not a protein kinase as both AMA and normal amnion keratins are phosphorylated to similar extents during mitosis. This result is in line with the observation that vimentin filaments remained intact during mitosis in both normal and transformed amnion cells despite the higher level of phosphorylation of vimentin during mitosis. The possibility must be considered, however, that keratin breakdown may be due to the phosphorylation of only certain keratins. In the cell pair we have used, only keratin IEF 44 was not present (or present in very low amounts) in amnion cells and therefore may be responsible for the dotted pattern observed in mitotic AMA cells. We consider this possibility unlikely, however, as human MCF-7 cells (breast adenocarcinoma) that do not express IEF 44 (1) show keratin aggregation during mitosis (9). From these results it would seem that the preferential phosphorylation of intermediate-filament proteins observed during mitosis may serve a more subtle function (5, see also reference 24 for further references). For example, phosphorylation may modulate some of the proposed filament associations with organelles or other cell structures (16, 21, 25–35). Recently, we have presented evidence indicating that in AMA cells the intermediate-sized filament proteins are coordinately modi-

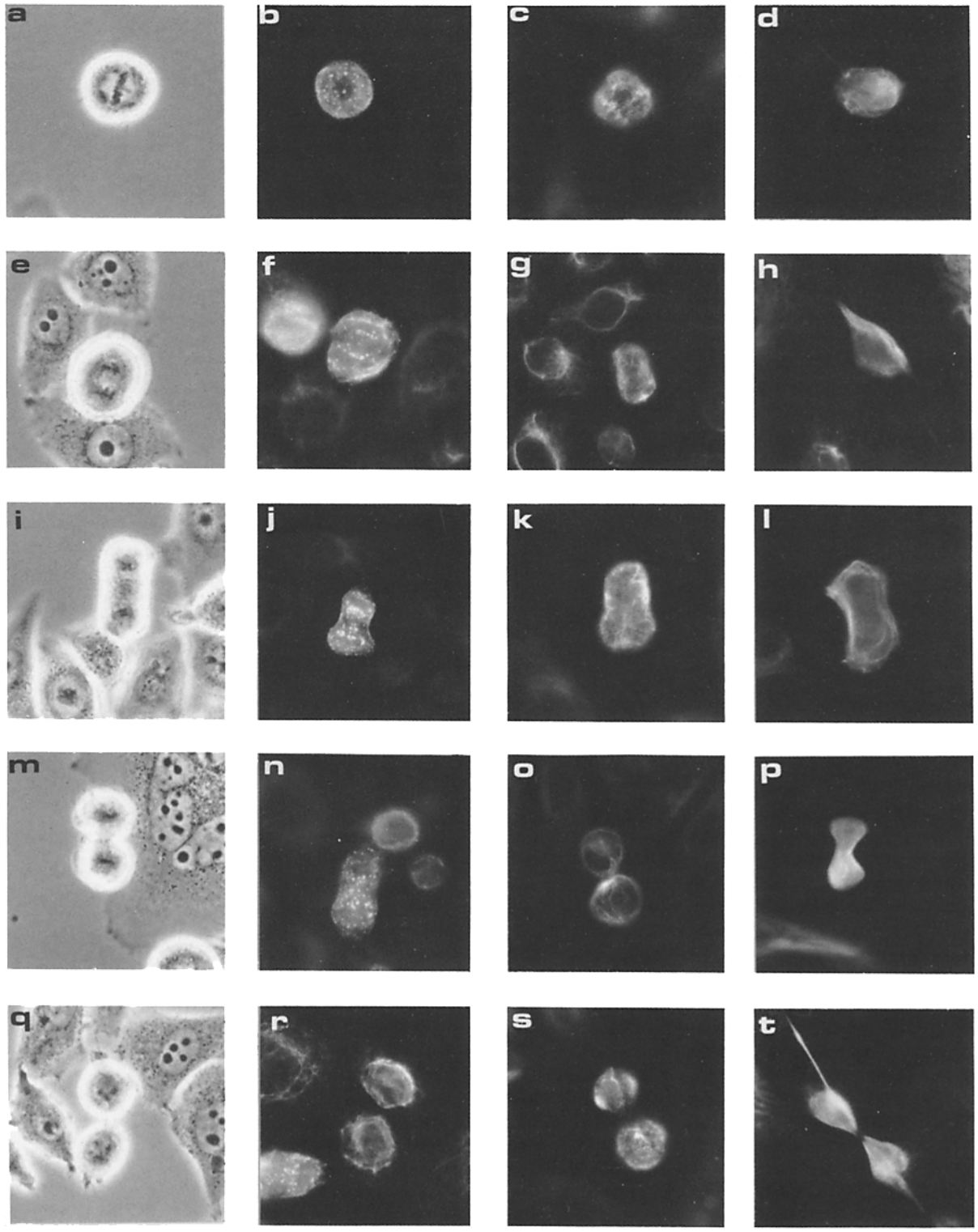

FIGURE 3 Immunofluorescent staining of normal and transformed mitotic amnion cells using keratin and vimentin antibodies. Sparse-growing cultured cells were fixed with methanol/acetone and reacted with the antibodies as previously described (14). Phase contrast of mitotic AMA cells (P125): (*a*) metaphase, (*e*) anaphase, (*i*) late anaphase/early telophase, (*m*) late telophase, and (*q*) early Gl. Immunofluorescence: (*b–d*) metaphase, (*f–h*) anaphase, (*j–i*) late anaphase/early telophase, (*n–p*) late telophase, and (*r–t*) early Gl. (*b, f, j, n*) Mitotic AMA cells reacted with the keratin IEF 31 antibody. (*c, g, k, o*) as above but reacted with the vimentin antibody; (*d, h, l, p*) Normal mitotic amnion cells reacted with the keratin IEF 31 antibody. × 150.

fied during mitosis and that the half-life of the phosphate is ~13 min for the keratins and 11 min for vimentin (21). These data are compatible with a regulatory role for phosphorylation during mitosis. There is increased evidence, however, from microinjection of intermediate-sized filament antibodies (31, 36–38) indicating that a collapse of these filaments has no obvious effect on the distribution of organelles. We should emphasize, however, that organelles (mitochondria, for ex-

ample) may be linked to more than one filamentous system (see 16 and 35 for references) and that these interactions may not be equally susceptible to various treatments.

It is unlikely that keratin degradation in a fashion similar to that described for vimentin (4, 5, 39, 40) may account for the drastic reorganization of keratins observed during mitosis in AMA cells. This is mainly deduced from the analysis of [^{35}S]methionine- and silver-stained gels that showed that the total amount of a given keratin (unmodified plus modified products) in mitosis is similar to that of their interphase level. Interestingly, however, one of the mitotic specific proteins described in HeLa cells (m9; [4, 5]) has recently been shown to have a mobility similar to that of a degradation product of either keratin IEF 31 or 36 as determined by immunoprecipitation using a broad specificity keratin antibody (unpublished observations). Further studies will be necessary to determine whether specific degradation of keratins takes place during mitosis.

It is intriguing why some keratin filaments need to be broken down (aggregated) during mitosis in transformed amnion cells. Obviously, this reorganization is not essential for normal cell division and must reflect some anomaly developed during transformation. Indeed, cultures of AMA cells showed an unusually high mitotic index that would not be expected from the duration of mitosis. At present we do not know at what stage during the transformation process filament fragmentation begins, and further studies of early passaged transformed cells will be needed to answer this question. So far, properties such as immortality, tumorigenicity, anchorage independence, ploidy, and duration of the mitotic cycle do not seem to correlate with keratin filament aggregation (9, and this article).

Clearly, the rearrangement of keratin filaments observed during mitosis in some transformed cells must play a role in the process of cell division; but clarification of this role will require a more refined analysis of mitosis.

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