J. Cell Sci. **62**, 129–147 (1983)
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POLYACRYLAMIDE GEL ELECTROPHORETIC SCREENING OF MAMMALIAN CELLS CULTURED *IN VITRO* FOR THE PRESENCE OF THE INTERMEDIATE FILAMENT PROTEIN VIMENTIN

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SUMMARY

A total of 63 different cell lines originating from a variety of mammalian species were cultured *in vitro* and analysed for the presence of vimentin, employing polyacrylamide gradient slab gel electrophoresis in urea/acetic acid as buffer system. Irrespective of the cell culture conditions, and the growth potential and morphology of the cells, vimentin was expressed in all cell lines examined, with two exceptions: MPC-11 mouse myeloma and MOPC-31C mouse plasmacytoma cells. Immunoblotting with the monoclonal antibody α-IFA, which is directed against an antigenic determinant shared by all classes of intermediate filaments, did not detect any other of the known intermediate filament proteins in MPC-11 and MOPC-31C cells.

Vimentin synthesized by various cell lines was characterized by four different criteria: (1) its extractability with Triton X-100 under various ionic conditions; (2) its behaviour in $(NH_4)_2SO_4$ fractionation of cellular extracts; (3) its electrophoretic mobility in polyacrylamide gel electrophoresis in urea/acetic acid; and (4) the co-isolation of polypeptides of higher electrophoretic mobility, which, by comparison with degradation products of vimentin obtained with the Ca^{2+}-activated proteinase specific for intermediate filament proteins *in vitro*, were identified as products of Ca^{2+}-dependent proteolysis of vimentin. Although the degradation products occurred in different ratios in extracts of different cell lines, they constituted the same characteristic set of proteins whenever degradation of vimentin was observed. The formation of proteolytic breakdown products could be partially to totally suppressed when the cells were harvested, washed and processed in the presence of EGTA and proteinase inhibitors.

The experimental data show that: (1) vimentin, as well as the Ca^{2+}-activated proteinase specific for intermediate filament proteins, is highly conserved during the evolution of mammalian species; (2) the proteolytic breakdown products of vimentin, which give rise to a characteristic 'staircase' in two-dimensional gel electrophoresis, are probably artefacts of isolation; (3) the expression of vimentin is neither a prerequisite for nor necessarily indicative of rapid cell proliferation *in vitro*; and (4) the techniques described can be used for the routine identification of vimentin in cells and tissues in case vimentin-specific antibodies are not available.

INTRODUCTION

Indirect immunofluorescence microscopy of vertebrate tissues has shown that *in situ* certain cell types are characterized by only one type of intermediate filament system (for reviews on intermediate filaments, see Anderton, 1981; Goldman *et al.*

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1979; Franke *et al.* 1982; Lazarides, 1980, 1982*a*; Osborn *et al.* 1982). However, during development more than one intermediate filament system can contribute to the cytoskeletal makeup of the same cell (Bignami, Raju & Dahl, 1982; Dahl, Strocchi & Bignami, 1982; Lazarides, 1982*b*; Schmid *et al.* 1982; Schnitzer, Franke & Schachner, 1981; Shaw, Osborn & Weber, 1981; Tapscott, Bennett & Holtzer, 1981*a*; Tapscott *et al.* 1981*b*; Yen & Fields, 1981). In all cases reported, vimentin was the subunit protein that was expressed in addition to tissue-specific filament proteins. In astrocytes of rat brain and spinal cord also, the muscle cell-specific filament protein desmin could be localized as well as glial fibrillary acidic protein (Dahl & Bignami, 1982). Moreover, when differentiated vertebrate cells were removed from their natural environment and taken into cell culture, they all expressed vimentin as a second intermediate filament protein (Cremer *et al.* 1981; Darmon, Buc-Caron, Paulin & Jacob, 1982; Franke *et al.* 1979, 1981; Jacobs, Choo & Thomas, 1982; Osborn, Franke & Weber, 1980; Quinlan & Franke, 1982; Sharp, Osborn & Weber, 1982; Virtanen *et al.* 1981*a*,*b*). From this observation that all proliferating cultured cells contain vimentin-type filaments in addition to tissue-specific filaments, it was concluded that vimentin expression is connected with the adaptation of cells to *in vitro* culture conditions (Franke *et al.* 1979; Paulin *et al.* 1982; Virtanen *et al.* 1981*a*). There was, thus far, only one exception to this rule: MH_1C_1 hepatoma cells, which, although they proliferate rapidly, do not synthesize vimentin (Franke *et al.* 1981).

In the course of our studies on several biochemical aspects of intermediate filaments, particularly on the structure–function relationship of vimentin, we have propagated 63 different mammalian cell lines and analysed them for the presence of vimentin. In this paper we describe techniques for the extraction of vimentin from these cells and its polyacrylamide gel electrophoretic identification. Although these methods allow only a semiquantitative estimation of the vimentin content of individual cell lines, they are nevertheless useful in the screening of mammalian cells for the presence of vimentin in case vimentin-specific antibodies are not available.

MATERIALS AND METHODS

Materials

Horse serum and all media used for cell culture were obtained from Flow Laboratories (Irvine, Scotland). Foetal calf serum was purchased from Boehringer Mannheim (Mannheim, FRG) and Seromed (München, FRG), penicillin G, streptomycin, mycostatin, phenylmethylsulphonyl fluoride (PMSF) and Triton X-100 from Serva (Heidelberg, FRG). L-1-tosylamido-2-phenylethylchloromethyl ketone (TPCK) was supplied by Sigma (St Louis, Mo., U.S.A.). All other chemicals were of reagent grade and obtained from Merck AG (Darmstadt, FRG). The monoclonal antibody α-IFA was a gift from Dr A. Dowding and Dr B. Anderton (London, England).

Cells

The following cell lines were obtained from The American Type Culture Collection (Rockville, Md, U.S.A.): MMT 060562, mouse mammary tumour, CCL51; Neuro-2a, mouse neuroblastoma, CCL131; Balb/3T3 clone A31, mouse embryo, CCL163; SV-T2 mouse embryo, SV40 virus-transformed Balb/3T3, CCL163.1; TCMK-1, mouse kidney, SV40 virus-transformed, CCL139;

3T6-Swiss albino, mouse embryo, CCL96; K-Balb (K-234), mouse embryo, Kirsten murine sarcoma virus-transformed Balb/3T3, CCl163.3; 3T3-Swiss albino, mouse embryo, CCL92; LC-540, rat Leydig cell testicular tumour, CCL43; Dede, Chinese hamster lung, CCL39; 104Cl, guinea pig embryo, transformed, CRL1405; IMR-33, gerbil fibroma, CCL146; TblLu (NBL-12), bat lung, CCL88; Sfl Ep (NBL-11), cotton tail rabbit epidermis, CCL68; PtK1 (NBL-3), marsupial kidney, CCL35; PtK2 (NBL-5), marsupial kidney, CCL56; MvlLu (NBL-7), mink lung, CCL64; AK-D, cat lung, CCL150; CRFK, cat kidney, CCL94; D-17, canine primary osteogenic sarcoma, CCL183; MDCK (NBL-2), canine kidney, CCL34; $DoCl_1$ (S^+L^-), canine kidney, Moloney murine sarcoma virus-infected, CCL34.1; ChlEs (NBL-8), goat oesophagus, CCL73; PK(15), porcine kidney, CCL33; EBTr (NBL-4), bovine embryonic trachea, CCL44; MDBK (NBL-1), bovine kidney, BVD infected, CCL22; E.Derm (NBL-6), equine dermis, CCL57; LLC-MK_2 original, Rhesus monkey kidney, CCL7; BS-C-1, African green monkey kidney, CCL26; CV-1, African green monkey kidney, CCL70; Vero, African green monkey kidney, CCL81; WI-38, human lung, diploid, CCL75; WI-38 VA13 subline 2RA, human lung, SV40 virus-transformed, CCL75.1; WI-26 VA4, human lung, SV40 virus-transformed, CCL95.1; Detroit 532, human skin, CCL54; Detroit 550, human skin, CCL109; MPC-11, mouse myeloma, CCL167; MOPC-31C, mouse plasmacytoma, CCL130.

Dr G. Darai (Universität Heidelberg, FRG) provided us with the following cell lines: LET, lamb embryo testis; SK, porcine kidney; TEF, tupaia embryo fibroblasts; TEK-646-1, tupaia embryo kidney; HF-TAV, adenovirus-transformed marmoset skin fibroblasts; OMK2/22, owl monkey kidney.

Dr M. Schweiger and Dr M. Hirsch-Kaufmann (Universität Innsbruck, Austria) made the following cell lines available to us: SV3T3, mouse embryo, SV40 virus-transformed; RoII, human amnion; KoII, human skin.

From Dr J. R. Sheppard (University of Minnesota, Minneapolis, Minn., U.S.A.) we obtained the following cell lines: C6-2, rat glial tumour; Novikoff hepatoma, rat liver tumour; HTC-1, rat liver tumour.

Ehrlich ascites tumour (EAT) cells adapted to cell culture conditions were obtained from Dr A. Shatkin (The Roche Institute, Nutley, N.J., U.S.A.), HeLa S3 cells (cervic carcinoma) from Dr D. Gallwitz (Universität Marburg, FRG), BHK-21/C13/$A_3$281 cells (baby hamster kidney) from Dr S. H. Revell (Institute for Cancer Research, London, England) and L929 cells (mouse connective tissue) and RK13 cells (rabbit kidney) from Dr G. Rutter (Pette Institut, Hamburg, FRG). MESF (mouse embryo skin fibroblasts), RESF (rat embryo skin fibroblasts), HEF (hamster embryo fibroblasts), GPEF (guinea pig embryo fibroblasts), CESF (cat embryo skin fibroblasts) as well as cell lines derived from cat embryo kidney, cat embryo liver and cat embryo thymus were obtained from whole embryos or foetal organs by standard techniques.

Cell culture

The cells obtained from The American Type Culture Collection were propagated in media whose compositions are specified in the Catalogue of Strains II, 2nd edn (1979). The remaining cell lines were propagated in the following media: In MEM: SV3T3 (5% foetal calf serum (FCS)), MESF (10% FCS), RESF (10% FCS), HEF (10% FCS), GPEF (10% FCS), RK13 (10% FCS), CESF (10% FCS), TEF (10% FCS), TEK-646-1 (10% FCS plus non-essential amino acids), RoII (20% FCS), KoII (20% FCS); in DMEM: C6-2 (10% FCS), HTC-1 (10% FCS), BHK-21/C13/$A_3$281 (10% FCS); in RPMI 1640: LET (10% FCS), SK (10% FCS), HF-TAV (10% FCS), OMK2/22 (15% FCS). All cells were grown in monolayer culture in standard-sized roller bottles in the presence of 100 mg/ml streptomycin, 63·5 mg/l penicillin G and 5·7 mg/l mycostatin and 5% CO_2 in the atmosphere. L929, HeLa S3, EAT and Novikoff hepatoma cells were propagated in suspension culture using MEM supplemented with antibiotics and 5% FCS. The MEM used for suspension culture did not contain Ca^{2+}. MPC-11 cells were grown in suspension culture in Dulbecco's modified Eagle's medium supplemented with 20% heat-inactivated horse serum and antibiotics, MOPC-31C cells were also grown in suspension culture in Leibovitz's medium L-15 supplemented with 20% FCS and antibiotics.

When the monolayer-grown cells had reached confluency, the media were decanted and the roller bottles cooled on ice/water. The cells were scraped off the substratum with the aid of a rubber policeman in cold 10 mM-Tris-acetate (pH 7·6), 0·15 M-NaCl (Tris-saline) in the absence or the

presence of 1 mM-EGTA (ethylene glycol bis (β-aminoethylether)-*N*,*N*′-tetra-acetic acid), 1 mM-PMSF, 1 mM-TPCK and 2% DMSO (dimethyl sulphoxide) as specified in Results. They were pelleted by centrifugation at 1500 g_{av} for 5 min, washed with harvesting buffer and immediately frozen in liquid N_2. The cells were stored at −80 °C until use. Suspension-grown cells were pelleted by centrifugation at 1500 g_{av} for 5 min and processed as specified above. All following operations were carried out, if not specified otherwise, at 0 °C.

Extraction of cells

In the first series of extractions, on average 2 g portions of frozen cells were thawed at 4 °C in 30 mM-Tris-acetate (pH 7·6), 3 mM-EDTA, 6 mM-2-mercaptoethanol, 0·5% (w/v) Triton X-100 in the absence or in the presence of 1 mM-PMSF and 1 mM-TPCK, as specified in Results. The cells

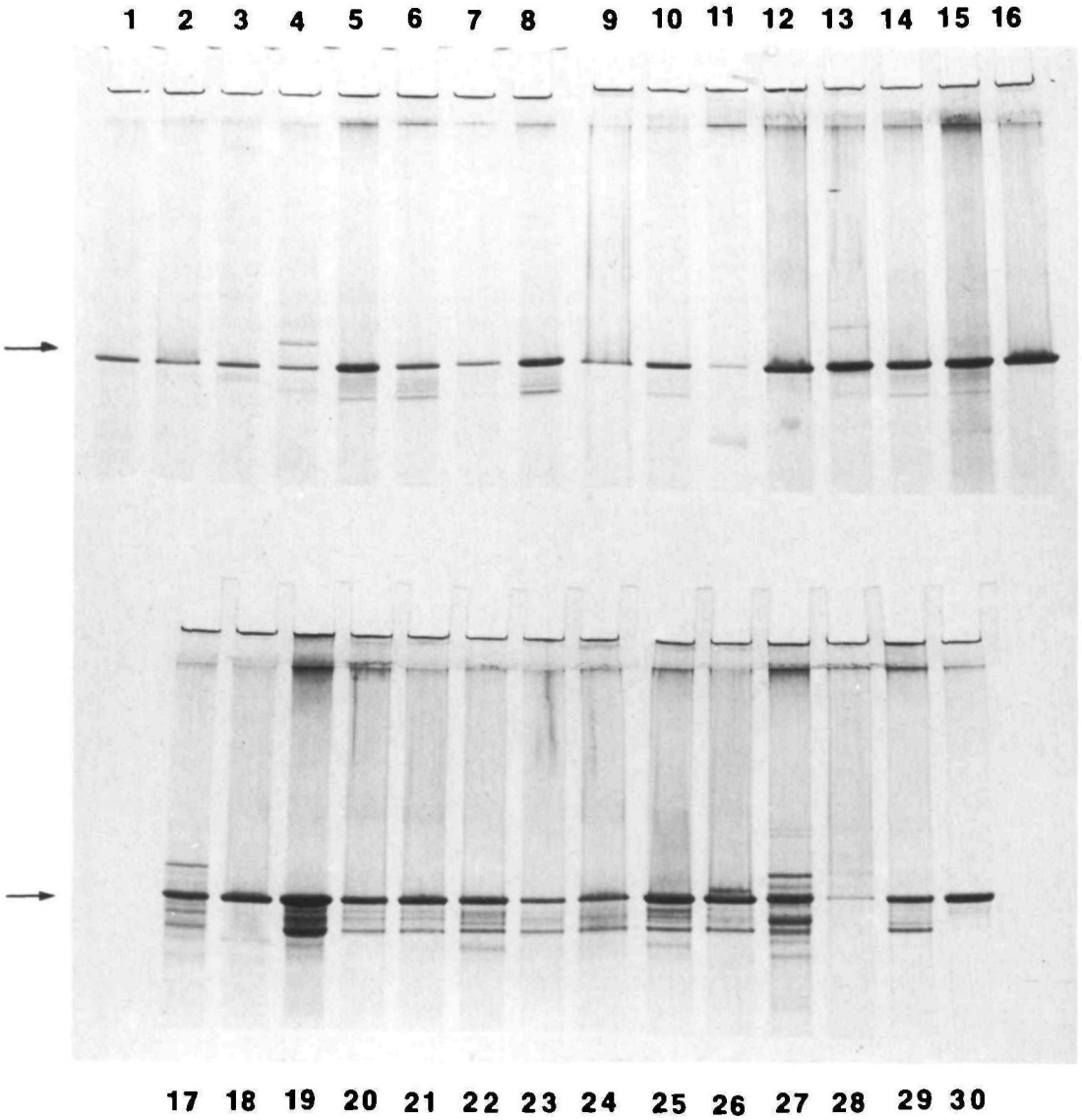

Fig. 1

were extracted with one 15 ml and two 10 ml portions of extraction buffer by 10 strokes each time in tightly fitting all-glass Dounce homogenizers. After each extraction, the nuclear material was pelleted by centrifugation at 35 000 g_{av} for 5 min. The combined supernatants were recentrifuged at 35 000 g_{av} for 5 min and the pellets were combined with the corresponding major pellets of nuclear material.

The volume of the supernatants was adjusted to 39 ml and vimentin was precipitated by dissolving 5·2 g solid $(NH_4)_2SO_4$ (23 % saturation). After shaking for 1 h, the precipitates were collected by centrifugation at 35 000 g_{av} for 30 min. Whereas the vimentin-free supernatants were discarded, the pellets were dissolved in 10 mM-Tris-acetate (pH 7·6), 3 mM-EDTA, 6 mM-2-mercaptoethanol by sonication. The volume (in ml) was equal to the mass of cells (in g) used for extraction; 0·2 ml of each solution was mixed with 0·5 ml 10 % trichloroacetic acid. The precipitates were collected by centrifugation at 2000 g_{av} for 5 min, washed with 1 ml acetone and dissolved in 0·2 ml 6 M-urea, 0·6 % acetic acid, 10 % 2-mercaptoethanol by sonication. A sample (20 μl) of each solution was used for polyacrylamide gradient slab gel electrophoresis in urea/acetic acid as buffer system. For the results, see Figs 1 and 2.

The nuclear pellets were suspended in distilled water in tightly fitting all-glass Dounce homogenizers. The final volume was 5·5 ml; 0·6 ml 2·5 M-HCl was added to each suspension and the mixtures were shaken for 2 h. Insoluble material was removed by centrifugation at 35 000 g_{av} for 5 min and solubilized protein was precipitated from the supernatants by the addition of 60 ml acetone. The precipitates were collected by centrifugation at 2000 g_{av} for 5 min and dissolved in 6 M-urea, 0·6 % acetic acid, 10 % 2-mercaptoethanol by brief sonication. The final volume (in ml) of each solution was again equal to the mass of cells (in g) used for extraction. A sample (20 μl) of each solution was subjected to polyacrylamide gradient slab gel electrophoretic analysis in urea/acetic acid as buffer system. For the results, see Figs 1 and 2.

Another set of 2-g portions of frozen cells were extracted with one 15 ml and two 10 ml portions of 10 mM-Tris-acetate (pH 7·6), 1 mM-EGTA, 4 mM-magnesium acetate, 6 mM-2-mercaptoethanol, 1 mM-PMSF, 1 mM-TPCK, 0·5 % (w/v) Triton X-100 by 10 strokes each time in tightly fitting all-glass Dounce homogenizers. Interval centrifugation was at 2000 g_{av} for 5 min. While the vimentin-free supernatants were aspirated and discarded, the Triton-resistant residual cell structures were extracted with 0·25 M-HCl exactly as described above. The solubilized and acetone-precipitated

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Fig. 1. Polyacrylamide gel electrophoretic analysis of vimentin extracted from various mammalian cell lines in the absence of proteinase inhibitors. *In vitro* cultured cells were washed with Tris-saline and extracted with Triton X-100 in a low ionic strength buffer free of divalent cations. Vimentin was precipitated from the extracts at 23 % $(NH_4)_2SO_4$ saturation and analysed by polyacrylamide gradient slab gel electrophoresis in urea/acetic acid as buffer system. Equivalent (with respect to the mass of wet cell pellet used for extraction) amounts of protein were applied to the gel; only the amount of HF-TAV protein (slot 26) was reduced by a factor of 3. The arrows indicate the position of vimentin. The satellite bands of higher electrophoretic mobility represent proteolytic breakdown products of vimentin. Only the upper third of the gels is shown. Slots 1, L929, mouse connective tissue cells; 2, MMT, mouse mammary tumour cells; 3, Neuro-2a, mouse neuroblastoma cells; 4, SV3T3, SV40 virus-transformed mouse embryo cells; 5, SV2T2, SV40 virus-transformed mouse embryo cells; 6, Balb/3T3, mouse embryo cells; 7, TCMK-1, SV40 virus-transformed mouse kidney cells; 8, HEF, hamster embryo cells; 9, BHK-21/C13/$A_3$281, baby hamster kidney cells; 10, Dede, hamster lung cells; 11, PtK1, potoroo kidney cells; 12, TB1Lu, bat lung cells; 13, Mv1Lu, mink lung cells; 14, CESF, cat embryo skin fibroblasts; 15, AK-D, cat lung cells; 16, D-17, dog primary osteogenic sarcoma cells; 17, MDCK, dog kidney cells; 18, $DoCl_1$, moloney murine sarcoma virus-infected dog kidney cells; 19, Ch1Es, goat oesophagus cells; 20, LET, lamb embryo testis cells; 21, EBTr, bovine embryo trachea cells; 22, E.Derm, horse dermis cells; 23, PK15, pig kidney cells; 24, SK, pig kidney cells, 25, TEF, tupaia embryo fibroblasts; 26, HF-TAV, adenovirus-transformed marmoset skin fibroblasts; 27, LLC-MK_2, Rhesus monkey kidney cells; 28, HeLa S3, human epitheloid carcinoma cells; 29, WI-38VA13, SV40 virus-transformed human lung cells; 30, EAT, mouse Ehrlich ascites tumour cells.

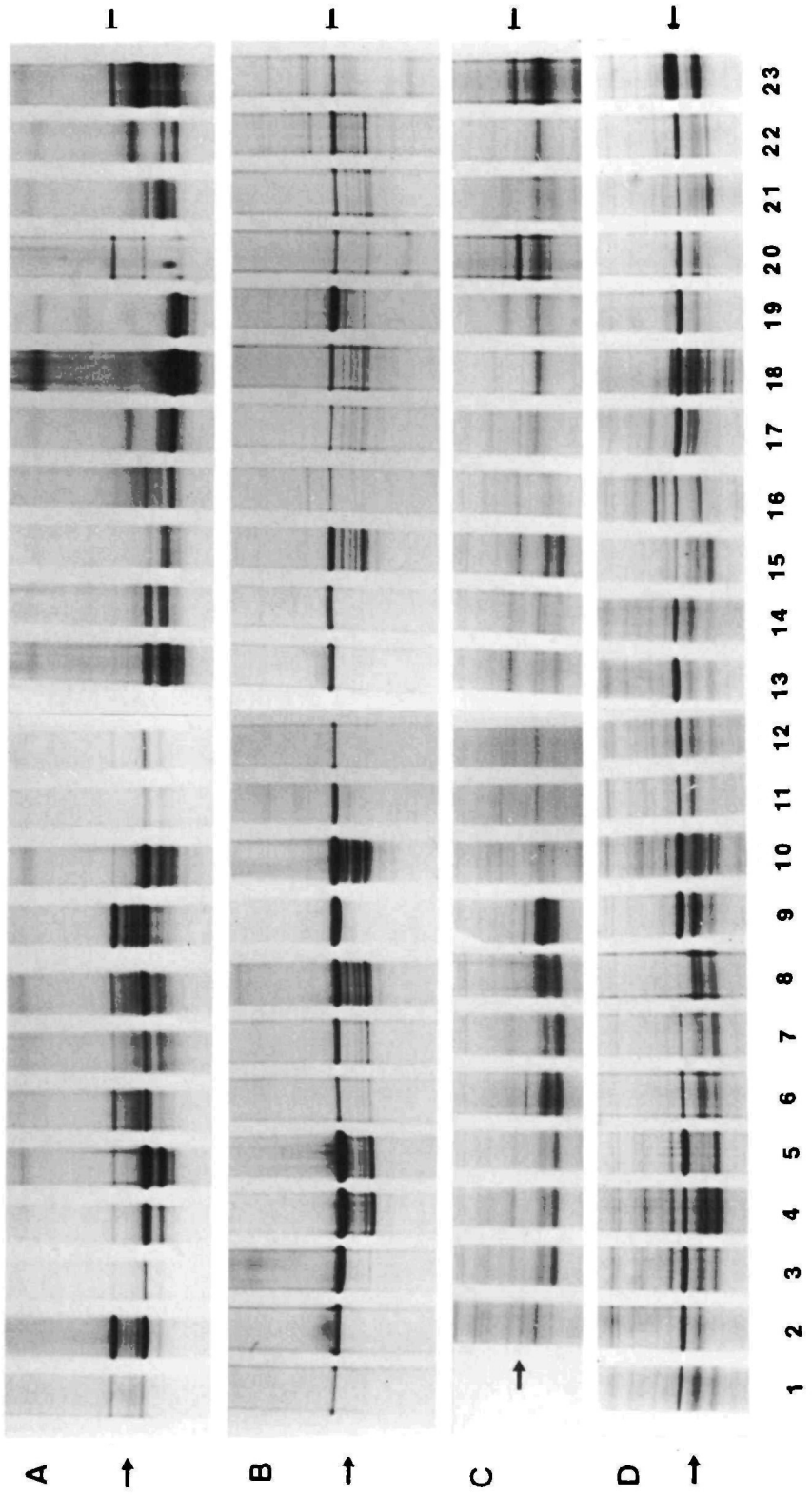

Fig. 2

protein fractions were dissolved in 6 M-urea, 0·6 % acetic acid, 10 % 2-mercaptoethanol by sonication. The final volume (in ml) was equal to the mass of cells (in g) used for extraction. A sample (20 μl) of each protein solution was analysed by polyacrylamide gradient slab gel electrophoresis in urea/acetic acid as buffer system. For the results, see Fig. 3.

The amount of cells and, correspondingly, the buffer volumes used for extraction can be reduced by a factor of 10 in the case of the first extraction series and by a factor of 20 in the case of the second extraction series (Triton-resistant residual cell structures). When only small amounts of cells are available, cell homogenization is carried out in Eppendorf tubes by vortexing instead of using Dounce homogenizers.

The complexity of the protein composition of the $(NH_4)_2SO_4$ precipitates can be considerably reduced when, instead of direct extraction of cells with Triton X-100 in the presence of EDTA, first Triton-resistant residual cell structures are prepared in the presence of 4 mM-Mg^{2+} and these are then extracted with a low ionic strength buffer in the absence of Mg^{2+} (Nelson & Traub, 1982*a*; Traub & Nelson, 1982). This procedure removes, besides lipids, substantial amounts of cytosolic proteins, RNA, ribosomes and membrane proteins, which otherwise might co-precipitate with vimentin during $(NH_4)_2SO_4$ fractionation; whereas vimentin is quantitatively retained in the cell residues. Triton-resistant residual cell structures are prepared as described above and extracted with two 10-ml and one 15-ml portion of 10 mM-Tris-acetate (pH 7·6), 1 mM-EGTA, 6 mM-2-mercaptoethanol by 10, 15 and 20 strokes, respectively, in tightly fitting all-glass Dounce homogenizers. Interval centrifugation is at 35 000 g_{av} for 5 min. The supernatants are combined, adjusted to 1 mM-Mg^{2+} or 50 mM-KCl and recentrifuged at 35 000 g_{av} for 5 min. The pellets are combined with the corresponding major fractions of chromatin. The supernatants are subjected to $(NH_4)_2SO_4$ fractionation and the nuclear pellets extracted with 0·25 M-HCl exactly as described above for the first extraction series.

As an alternative to the $(NH_4)_2SO_4$ precipitation method, vimentin can also be quantitatively precipitated with 2–3 mM-spermidine (Traub & Nelson, 1982). Since nucleic acids and nucleoproteins are also precipitated, the precipitate has to be extracted with 0·25 M-HCl before polyacrylamide gel electrophoresis. This method has the advantage of the quantitative removal of nucleic acids, which interfere with the gel electrophoretic analysis of vimentin in urea/acetic acid as buffer system.

Polyacrylamide gradient slab gel electrophoresis and immunoblotting

The protein moieties of the $(NH_4)_2SO_4$ precipitates, nuclear fractions and Triton-resistant residual cell structures, all dissolved in 6 M-urea, 0·6 % acetic acid, 10 % 2-mercaptoethanol, were

Fig. 2. Polyacrylamide gel electrophoretic analysis of vimentin extracted from various mammalian cell lines in the absence (A) and the presence (B) of proteinase inhibitors. *In vitro* cultured cells were harvested, washed and processed as outlined in the legend to Fig. 1 in the absence of proteinase inhibitors (A) and the presence of 1 mM-EGTA, 1 mM-PMSF, 1 mM-TPCK(B). The protein compositions of the corresponding nuclear fractions from cells processed in the absence of proteinase inhibitors (C) and the presence of 1 mM-EGTA, 1 mM-PMSF, 1 mM-TPCK (D) are also shown. The nuclear proteins were solubilized with 0·25 M-HCl and precipitated with acetone. Equivalent amounts of all protein fractions were analysed by polyacrylamide gradient slab gel electrophoresis in urea/acetic acid as buffer system. The arrows indicate the position of vimentin. Slots 1, 3T6, mouse embryo cells; 2, WI-26VA4, SV40 virus-transformed human lung cells; 3, WI-38, human lung cells; 4, Detroit 532, human skin cells; 5, Detroit 550, human skin cells; 6, IMR-33, gerbil fibroma cells; 7, MESF, mouse embryo skin fibroblasts; 8, RESF, rat embryo skin fibroblasts; 9, GPEF, guinea pig embryo fibroblasts; 10, OMK2/22, owl monkey kidney cells; 11, RoII, human amnion cells; 12, KoII, human skin cells; 13, K-Balb, Kirsten murine sarcoma virus-transformed mouse embryo cells; 14, 3T3, mouse embryo cells; 15, LC-540, rat Leydig testicular cells; 16, BS-C-1, African green monkey kidney cells; 17, RK13, rabbit kidney cells; 18, MDBK, bovine kidney cells; 19, Sf1Ep, rabbit epidermis cells; 20, CRFK, cat kidney cells; 21, 104Cl, guinea pig embryo cells; 22, CV-1, African green monkey kidney cells; 23, PtK2, potoroo kidney cells.

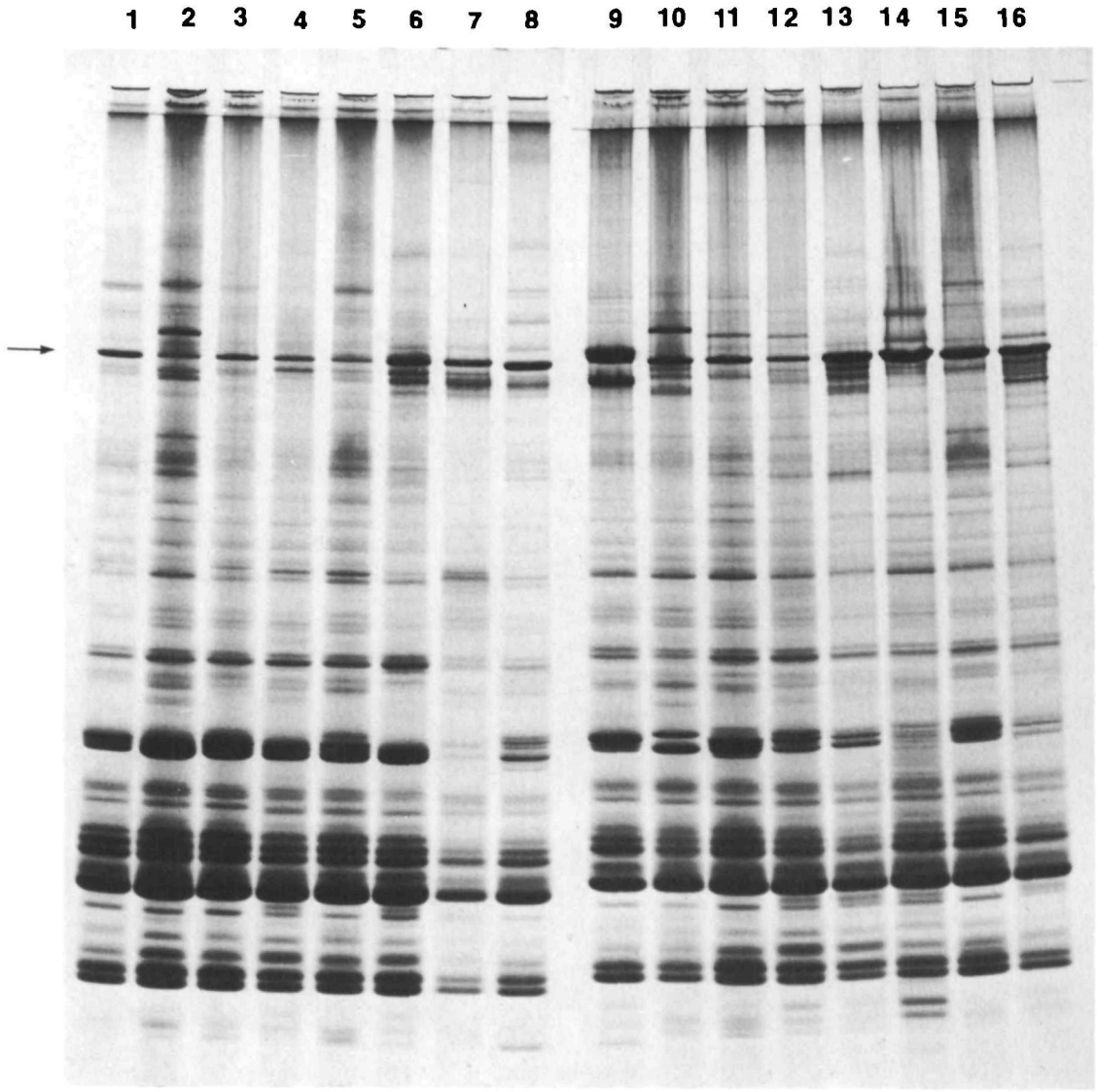

Fig. 3. Polyacrylamide gel electrophoretic analysis of the protein composition of Triton-resistant residual cell structures derived from various mammalian cell lines. Cells were extracted with Triton X-100 in a low ionic strength buffer containing 4 mM-Mg^{2+}, 1 mM-EGTA, 1 mM-PMSF and 1 mM-TPCK. The proteins of the cell residues were solubilized with 0·25 M-HCl, precipitated with acetone and analysed by polyacrylamide gradient slab gel electrophoresis in urea/acetic acid as buffer system. The position of vimentin is indicated by an arrow. Slots 1, L929, mouse connective tissue cells; 2, SV3T3, SV40 virus-transformed mouse embryo cells; 3, TCMK-1, SV40 virus-transformed mouse kidney cells; 4, C6-2, rat glial tumour cells; 5, Novikoff hepatoma, rat liver tumour cells; 6, HTC-1, rat liver tumour cells; 7, IMR-33, gerbil fibroma cells; 8, TB1Lu, bat lung cells; 9, PtK1, potoroo kidney cells; 10, MDCK, dog kidney cells; 11, TEK-646-1, tupaia embryo kidney cells; 12, Vero, African green monkey kidney cells; 13, OMK2/22, owl monkey kidney cells; 14, HF-TAV, adenovirus-transformed marmoset skin fibroblasts; 15, WI-26VA4, SV40 virus-transformed human lung cells; 16, Detroit 550, human skin cells.

analysed by polyacrylamide gradient slab gel electrophoresis in 6 M-urea, 6 % acetic acid as buffer system, as described previously (Egberts, Hackett & Traub, 1976). For immunoblotting, the monoclonal antibody α-IFA was used, which cross-reacts with an antigenic determinant shared by all intermediate filament subunit proteins (Pruss *et al.* 1981). The electrophoretic transfer of proteins from urea/acetic acid gels to nitrocellulose filters and the subsequent immunoblotting using the peroxidase–Hanker/Yates method has been described in detail previously (Nelson & Traub, 1982*b*).

RESULTS

As described recently, vimentin is almost quantitatively solubilized when Ehrlich ascites tumour cells grown in suspension culture are extracted with Triton X-100 in a low ionic strength buffer free of di- and polyvalent cations (Traub & Nelson, 1982). It can be quantitatively precipitated from the postnuclear supernatant of this extract at 23 % $(NH_4)_2SO_4$ saturation. When analysed by polyacrylamide gradient slab gel electrophoresis in urea/acetic acid as buffer system, the $(NH_4)_2SO_4$ precipitate seems to consist almost exclusively of vimentin. The filament protein appears as a sharp, major band occasionally accompanied by some minor bands of higher electrophoretic mobility representing proteolytic degradation products of vimentin (Fig. 1, slot 30). However, when analysed by polyacrylamide gradient slab gel electrophoresis in the presence of sodium dodecyl sulphate, the electrophoresis profile turns out to be rather complex (data not shown); if the amount of vimentin in the $(NH_4)_2SO_4$precipitate is low, then the filament protein can only be identified with difficulty.

We have employed this procedure of extraction and gel electrophoretic analysis in urea/acetic acid for the screening of a large variety of mammalian cell lines for their potentials to synthesize vimentin. Whereas in Fig. 1 those cell lines are summarized that have released most of their vimentin in its intact form into the postnuclear supernatant, in Fig. 2A those cases are presented in which vimentin has been proteolytically degraded to a great extent. The nuclear pellets, which had also been analysed for their residual vimentin contents, were virtually free of vimentin (Fig. 2C, not shown for cell lines listed in Fig. 1).

Judging from the gel electrophoretic band patterns of the breakdown products of vimentin, which were identical to those obtained *in vitro* with purified vimentin and the intermediate filament-specific, Ca^{2+}-activated proteinase (Nelson & Traub, 1981, 1982*c*) (data not shown), the degradation of vimentin seemed to be due to the activation of the proteinase during harvesting and processing of the cells. In order to avoid Ca^{2+}-dependent proteolysis of vimentin, a second batch of the cells listed in Fig. 2A were harvested and washed with Tris-saline in the presence of the Ca^{2+}-chelating agent EGTA. The proteinase inhibitors phenylmethylsulphonyl fluoride (PMSF) and L-1-tosylamido-2-phenylmethylchloromethyl ketone (TPCK) were included in the wash buffer to prevent non-specific proteolytic degradation of vimentin. Also, the extraction of the cells with Triton X-100 was performed in the presence of PMSF and TPCK. As shown in Fig. 2B, the precautions taken indeed had a positive effect on the yield of intact vimentin. In all cases examined, the extent of proteolytic degradation of vimentin was drastically reduced in comparison with the situation illustrated in Fig. 2A; vimentin appeared as the major protein constituent of the $(NH_4)_2SO_4$

precipitates. However, if one compares in Fig. 2A and B the total staining density of vimentin and its degradation products extracted from corresponding cell batches, in some cases (for instance, in those of samples 4, 5, 6, 11, 12, 13, 14) the yields of vimentin and its derivatives seemed to be higher when the cells had been washed and processed in the absence of any inhibitors. This contradiction is only apparent since, as shown in Fig. 2D, the corresponding nuclear pellets remaining after extraction of the cells in the presence of inhibitors still contained substantial amounts of mostly intact vimentin. Under proteinase-inhibitory conditions, the solubilization of vimentin with Triton X-100 in low ionic strength buffer was obviously less efficient.

As was also shown previously, vimentin is quantitatively retained in detergent-resistant residual cell structures when EAT cells are extracted with Triton X-100 in a low ionic strength buffer containing 4 mM-Mg^{2+} (Traub & Nelson, 1982). The protein moiety of the residual cell structures, including vimentin and histones as the major components, can be solubilized with 0·25 M-HCl, precipitated with acetone and finally dissolved in urea/acetic acid buffer for polyacrylamide gel electrophoretic analysis. In order to avoid the splitting of the vimentin complement of cells into a soluble and a nuclear fraction as indicated in Fig. 2B and D, we have also prepared, under proteinase-inhibitory conditions, Triton-resistant residual cell structures from a variety of mammalian cells that had been harvested in the presence of EGTA, PMSF and TPCK. As depicted in Fig. 3, in all cases examined, vimentin was, besides the histones, the major protein constituent. In most cases it was accompanied by small amounts of its high molecular weight proteolytic degradation products.

To demonstrate that the major polypeptides that, in Figs 1 and 2, migrated faster than vimentin were indeed derived from vimentin by proteolysis, we have characterized them further by immunoblotting. We have used the monoclonal antibody α-IFA, which defines an antigenic determinant shared by all classes of intermediate filaments (Pruss *et al.*1981). Fig. 4A, B clearly illustrates that the satellite bands of vimentin strongly reacted with the antibody and, moreover, that proteolysis of vimentin during the handling and processing of the various cells created a constant set of identical degradation products in which, however, corresponding polypeptides were represented in varying amounts in the different cases examined.

Altogether, we have analysed 63 different mammalian cell lines for the presence of vimentin and its degradation products. Those that proved to be vimentin-positive are presented in Figs 1–4 and are also listed in Table 1. Among the 63 cell lines we detected only two, MPC-11 mouse myeloma and MOPC-31C mouse plasmacytoma cells, that, no matter how they had been propagated, handled and processed, never contained vimentin or its high molecular weight proteolytic degradation products. To exclude the possibility that vimentin was synthesized only in trace amounts that escaped detection in the urea/acetic acid gel electrophoresis system, we also analysed the total cellular protein content of both cell preparations in the sodium dodecyl sulphate gel electrophoresis system, in combination with the sensitive immunoblotting assay. Cells harvested in the presence of EGTA, PMSF and TPCK were extracted, under proteinase-inhibitory conditions, with Triton X-100 in a low ionic strength buffer free of divalent cations. The nuclei and the postnuclear supernatants were

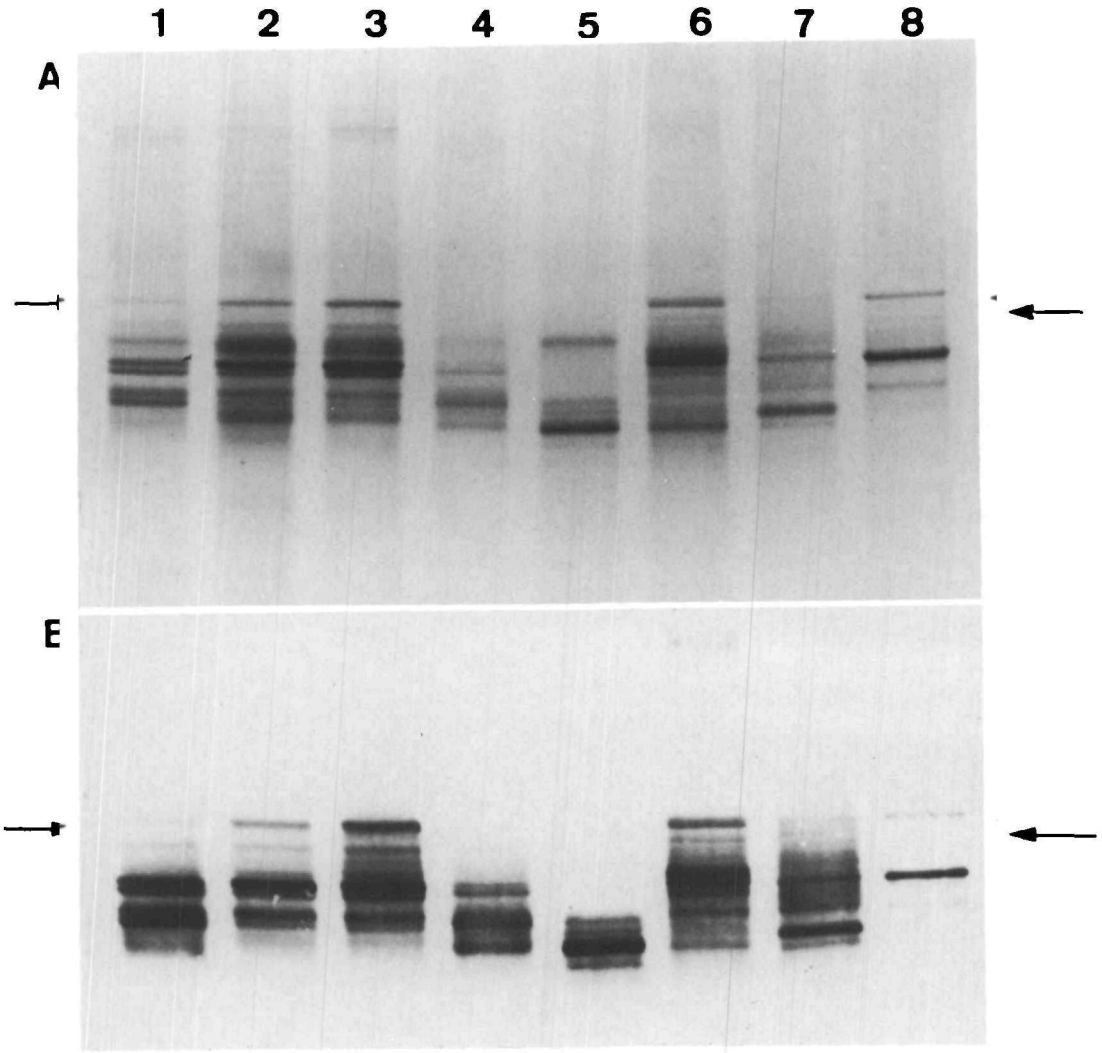

Fig. 4. Cross-reactivity of the monoclonal antibody, α-IFA, with vimentin and its proteolytic degradation products extracted from various mammalian cell lines in the absence of proteinase inhibitors. Vimentin was extracted from various mammalian cell lines, which had been harvested in the absence of proteinase inhibitors, with Triton X-100 in a low ionic strength buffer free of divalent cations and precipitated at 23 % $(NH_4)_2SO_4$ saturation. After polyacrylamide gradient slab gel electrophoretic separation of the protein constituents in urea/acetic acid as buffer system, these were transferred onto nitrocellulose paper and reacted with the monoclonal antibody α-IFA, which defines a common antigenic determinant shared by all intermediate filament proteins (Pruss *et al.* 1981). The antibody-labelled proteins were visualized by incubation with a horseradish peroxidase-conjugated immunoglobulin G preparation and Hanker/Yates solution plus H_2O_2. A. Coomassie Brilliant Blue-stained gel; B. immunoblot. The position of vimentin is indicated by arrows. Slots 1, 3T3, mouse embryo cells; 2, MESF, mouse embryo skin fibroblasts; 3, RESF, rat embryo skin fibroblasts; 4, 104Cl, guinea pig embryo cells; 5, RK13, rabbit kidney cells; 6, PtK2, potoroo kidney cells; 7, BS-C-1, African green monkey kidney cells; 8, WI-38, human lung cells.

Table 1. *Compilation of vimentin-synthesizing, cultured cells orginating from different mammalian species*

Cell line	Species	Origin	Morphology[b]	Fig./Slot[c]			
				1	2	3	4
L 929	Mouse	Connective tissue	F	1		1	
MMT	Mouse	Mammary tumour	E	2			
Neuro-2a	Mouse	Neuroblastoma	N	3			
Balb/3T3	Mouse	Embryo	F	6			
SV3T3	Mouse	Embryo (SV40)[a]	F	4		2	
SV2T2	Mouse	Embryo (SV40)[a]	F	5			
TMCK-1	Mouse	Kidney (SV40)[a]	E	7		3	
EAT	Mouse	Ehrlich ascites tumour	S	30			
3T6	Mouse	Embryo	F		1		
MESF	Mouse	Embryo skin	F		7		2
K-Balb	Mouse	Embryo (KMSN)[a]	F		13		
3T3	Mouse	Embryo	F		14		1
RESF	Rat	Embryo skin	F		8		3
LC-540	Rat	Leydig cell testicular tumour	F		15		
C6-2	Rat	Glial tumour	F			4	
Novikoff hepatoma	Rat	Liver tumour	S			5	
HTC-1	Rat	Liver tumour	E			6	
BHK-21/C13/A$_3$281	Hamster	Kidney	F	9			
Dede	Hamster	Lung	F	10			
HEF	Hamster	Embryo	F	8			
GPEF	Guinea pig	Embryo	F		9		
104 Cl	Guinea pig	Embryo	F		21		4
IMR-33	Gerbil	Fibroma	F		6	7	
TB1 Lu	Bat	Lung	E	12		8	
RK13	Rabbit	Kidney	E		17		5
Sf1 Ep	Rabbit	Epidermis	E		19		
PtK1	Potoroo	Kidney	E	11		9	
PtK2	Potoroo	Kidney	E		23		6
Mv1 Lu	Mink	Lung	E	13			
CESF	Cat	Embryo skin	F	14			
		Embryo kidney	F				
		Embryo liver	E				
		Embryo thymus	F				
AK-D	Cat	Lung	E	15			
CRFK	Cat	Kidney	E		20		
D-17	Dog	Primary osteogenic sarcoma	E	16			
MDCK	Dog	Kidney	E	17		10	
DoCl	Dog	Kidney (MMSV)[a]	E	18			
ChlEs	Goat	Oesophagus	F	19			
LET	Sheep	Embryo testis	F	20			
PK15	Pig	Kidney	E	23			
SK	Pig	Kidney	E	24			
EBTr	Bovine	Embryo trachea	F	21			
MDBK	Bovine	Kidney	E		18		
E. Derm	Horse	Dermis	F	22			
TEF	Tupaia	Embryo	F	25			
TEK-646-1	Tupaia	Embryo kidney	E			11	

Table 1. *continued*

Cell line	Species	Origin	Morphology[b]	Fig./Slot[c]			
				1	2	3	4
HF-TAV	Monkey	Marmoset skin (AV)[a]	F	26		14	
LLC-MK$_2$	Monkey	Rhesus m.kidney	E	27			
OMK 2/22	Monkey	Owl m.kidney	E		10	13	
BS-C-1	Monkey	African green m.kidney	E		16		7
CV-1	Monkey	African green m.kidney	F		22		
Vero	Monkey	African green m.kidney	F			12	
HeLa S3	Man	Epitheloid carcinoma	E,S	28			
WI-38	Man	Lung	F		3		8
WI-38 VA13	Man	Lung (SV40)[a]	E	29			
WI-26 VA4	Man	Lung (SV40)[a]	E		2	15	
Detroit 532	Man	Skin	F		4		
Detroit 550	Man	Skin	F		5	16	
RoII	Man	Amnion	F		11		
KoII	Man	Skin	F		12		

[a] SV40, SV40 virus-transformed; MSV, Kirsten murine sarcoma virus-transformed; MMSV, Moloney murine sarcoma virus-infected; AV, adenovirus-transformed.

[b] E, epithelial-like; F, fibroblast-like; N, neurone-like; S, suspension culture.

[c] In the last four columns the numbers of the slots of Figs 1–4 are listed in which the vimentin complements of the individual cell lines have been analysed by polyacrylamide gradient slab gel electrophoresis in urea acetic acid.

subjected directly to polyacrylamide gradient slab gel electrophoretic analysis in the presence of sodium dodecyl sulphate and the separated proteins were transferred onto nitrocellulose paper. However, as shown in Fig. 5, neither vimentin nor its proteolytic degradation products were detected by the monoclonal antibody α-IFA.

DISCUSSION

Irrespective of the cell culture conditions (suspension or monolayer culture) and the growth potential (logarithmic or stationary growth phase, transformed or non-transformed cells) and morphology (fibroblast- or epithelial-like) of a large variety of mammalian cell lines, with two exceptions, they all synthesized vimentin in relatively large quantities. Although this study did not emphasize quantitative aspects, our experimental results, nevertheless, give some evidence as to the relative amounts of vimentin present in different cell lines. Thus, adenovirus-transformed marmoset skin fibroblasts (Fig. 3, slot 14) were found to contain the highest amount of vimentin. But also hamster embryo fibroblasts (Fig. 1, slot 15), dog osteogenic sarcoma cells (Fig. 1, slot 16), goat oesophagus cells (Fig. 1, slot 19), tupaia baby fibroblasts (Fig. 2, slot 25), human skin fibroblasts (Fig. 2, slots 4, 5) and Ehrlich ascites tumour cells (Fig. 1, slot 30), to mention only a few cell lines, proved to be very rich in vimentin. Principally, all these cell lines can be used for the large-scale isolation of vimentin. Since Ehrlich ascites tumour cells, however, have a generation time of only 12 h and

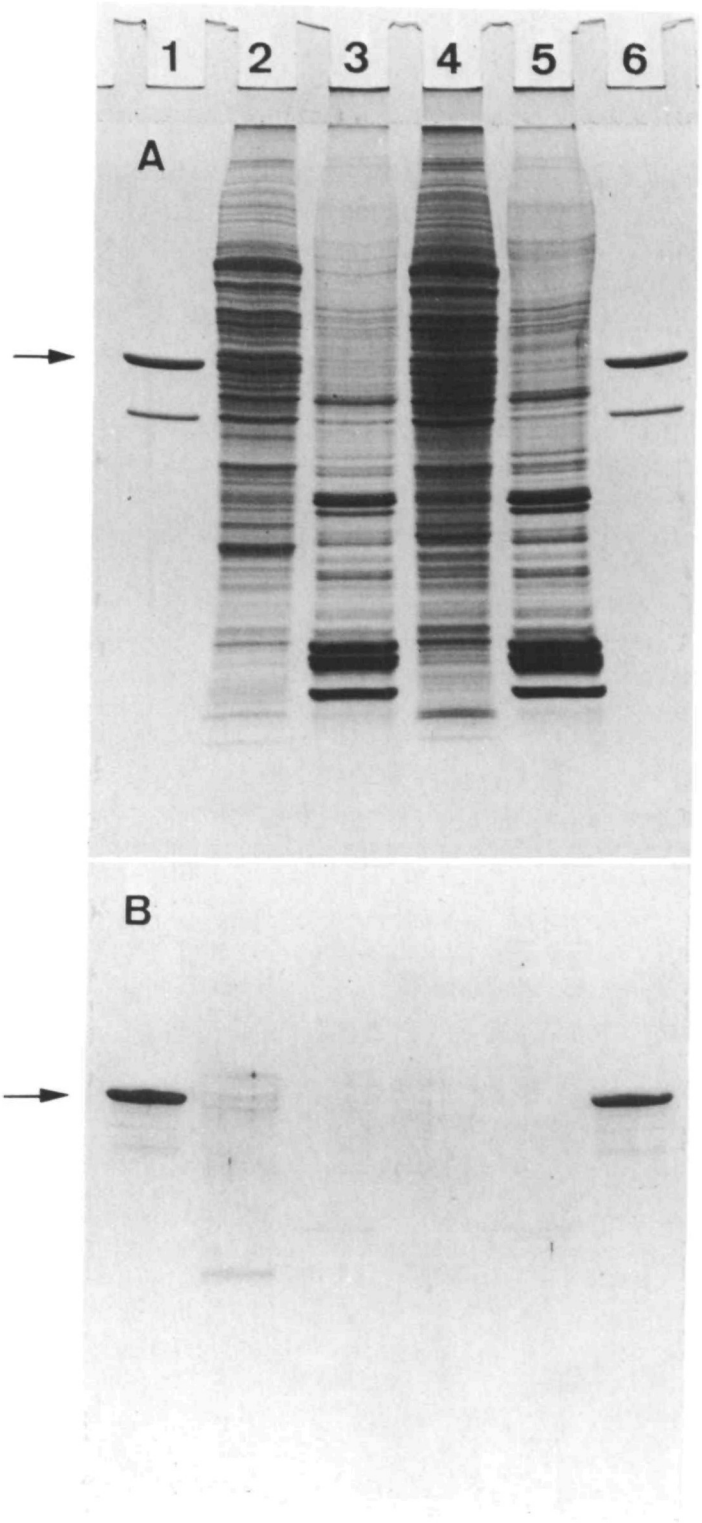

Fig. 5

can be conveniently grown in suspension culture with heat-inactivated normal calf serum to high cell densities, they are most suitable for the preparation of large amounts of pure vimentin (Nelson & Traub, 1982*b*; Nelson, Vorgias & Traub, 1982).

One might be confronted with the problem of having to identify vimentin as a cellular constituent, and a vimentin-specific antibody is not available. In such cases, the technique described here can be used as a relatively fast, alternative identification method. Vimentin is easily characterized: (1) by its retention in detergent-resistant residual cell structures when cells propagated *in vitro* are extracted with Triton X-100 at low ionic strength and in the presence of 4 mM-Mg^{2+}; (2) by its extractability from detergent-resistant residual cell structures, and also from whole cells, at low salt concentration and in the absence of Mg^{2+}; (3) by its precipitability from such extracts at 23% $(NH_4)_2SO_4$ saturation; and (4) by its electrophoretic mobility in polyacrylamide gradient slab gel electrophoresis in the urea/acetic acid buffer system (see also Traub & Nelson, 1982).

Among the various cell lines examined, we found many that were apparently vimentin-negative or seemed to contain only low amounts of vimentin (Fig. 2A). However, we detected substantial amounts of degradation products of vimentin in these cell lines. That the polypeptides represented by the satellite bands of higher electrophoretic mobility are indeed derived from vimentin was ascertained by: (1) their reactivity with the monoclonal antibody α-IFA (Pruss *et al.* 1981) in immunoblotting (Fig. 4); (2) their identity with polypeptides generated by the Ca^{2+}-activated proteinase from purified vimentin *in vitro* (data not shown); and (3) their characteristic arrangement in a 'staircase' in two-dimensional polyacrylamide gel electrophoretic analysis (data not shown here, but for the presentation of such a staircase see, for instance, Nelson, Vorgias & Traub; 1982; Ochs, McConkey & Guard, 1981). Our observations show that vimentin is extremely sensitive to Ca^{2+}-dependent proteolysis and that the degradation can be largely avoided by harvesting and processing of the cells in the presence of Ca^{2+}-chelating agents and proteinase inhibitors (Fig. 2c). These precautions should be taken whenever vertebrate cells are analysed for the presence of intermediate filament proteins or used for the preparation of cytoskeletal frameworks and the isolation of intermediate filament subunit proteins.

The fact that cell lines originating from a large variety of mammalian species are capable of synthesizing the same vimentin convincingly shows that there has been

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Fig. 5. Analysis of nuclear and cytoplasmic fractions from MOPC-31C mouse plasmacytoma and MPC-11 mouse myeloma cells for the presence of vimentin by immunoblotting. Suspension-grown MOPC-31C and MPC-11 cells were harvested and washed in the presence of EGTA, PMSF and TPCK, and extracted in the presence of the same proteinase inhibitors, with Triton X-100 in a low ionic strength buffer free of divalent cations. The nuclear fractions as well as the postnuclear supernatants were subjected directly to polyacrylamide gradient slab gel electrophoresis in the presence of sodium dodecylsulphate. While one gel was stained with Coomassie Brilliant Blue (A), the proteins separated on a second gel were analysed further by immunoblotting using the monoclonal antibody α-IFA (B). Slots 1 and 6, vimentin plus actin; 2, MOPC-31C postnuclear supernatant; 3, MOPC-31C nuclear fraction; 4, MPC-11 postnuclear supernatant; 5, MPC-11 nuclear fraction. The position of vimentin is indicated by arrows.

a high degree of conservation of this protein during evolution. Our results of polyacrylamide gel electrophoretic analysis of the distribution of vimentin among different species are in good agreement with those of previous investigations employing indirect immunofluorescence microscopy and also biochemical analysis (for references, see the Introduction). In this study, vimentin was characterized not only by its constant physicochemical behaviour during extraction, $(NH_4)_2SO_4$ precipitation and gel electrophoresis, but also by its potential to give rise to a characteristic and constant set of polypeptides in response to degradation by the specific, Ca^{2+}-activated proteinase. Irrespective of the evolutionary distance between the various species, Figs 1–4 show constant patterns of satellite bands of vimentin, although the degradation products are quantitatively differentially represented. The reason for this variability is unknown; it might be due to the presence of different activators or inhibitors of the Ca^{2+}-activated proteinase in different cell lines or to slight differences in the handling of the cells during their harvesting and extraction. Since the supplementation of the wash and extraction buffers with EGTA and proteinase inhibitors led to a considerable, in some cases even total, suppression of the formation of breakdown products, these polypeptides must be regarded as artefacts of isolation. From this we conclude that *in vivo* the major part of vimentin is somehow protected from proteolytic degradation and that, if the Ca^{2+}-activated proteinase found to be specific for intermediate filament proteins has a genuine function in the processing of vimentin, only a small number of vimentin molecules are degraded, probably at very distinct locations within the cell. However, not only vimentin but also the Ca^{2+}-activated proteinase is conserved during evolution, as published recently (Nelson & Traub, 1982*a*; Traub & Nelson, 1981). This high degree of evolutionary stability points to an important cellular function for the vimentin–proteinase system that is still a matter of conjecture.

Among the 63 different cell lines examined, we found only two that did not express vimentin: MPC-11 mouse myeloma and MOPC-31C mouse plasmacytoma cells. Even after cautious harvesting of the cells in the presence of EGTA and proteinase inhibitors, their extraction under proteinase-inhibitory conditions and the employment of the sensitive immunoblotting assay (α-IFA), the nuclear and cytoplasmic fractions of both cell lines turned out to be totally devoid of vimentin and other intermediate filament proteins (Fig. 5). This result is in agreement with our previous failure to detect vimentin-specific mRNA in the poly(A)$^+$ mRNA populations of logarithmically growing MPC-11 and MOPC-31C cells (McTavish, Nelson & Traub, 1983). Correspondingly, the activity of the Ca^{2+}-activated proteinase specific for intermediate filament proteins from these cells was found to be, at the most, 20% of that in Ehrlich ascites tumour cells (our unpublished results). The complete absence of vimentin from MPC-11 and MOPC-31C cells shows that vimentin expression is neither a prerequisite for nor necessarily indicative of rapid cell proliferation as suggested previously (Franke *et al.* 1979; Paulin *et al.* 1982; Virtanen *et al.* 1981*a*), but was then questioned when rapidly proliferating MH_1C_1 hepatoma cells were found not to synthesize vimentin (Franke *et al.* 1981). It cannot be excluded, however, that cell lines that do not accumulate vimentin in amounts detectable by conventional

analytical methods still produce small amounts of the filament protein that are sufficient to maintain rapid cell growth. It would be interesting to find out whether cell lines that do not synthesize vimentin still contain an intact vimentin gene and if so, how the production of vimentin can be induced. This would allow a deeper insight into the mechanism(s) governing the regulation of the synthesis of intermediate filament subunit proteins *in vitro* and *in situ*.

We are grateful to Mrs Margot Bialdiga for her invaluable help in growing all the cells used in this investigation and to Miss Brigitte Geisel, Miss Annemarie Scherbarth and Mrs Gabi Shoeman for skilful technical assistance, especially in the performance of polyacrylamide gel electrophoresis. We also thank Dr G. Darai (University of Heidelberg, FRG), Dr M. Schweiger and Dr M. Hirsch-Kaufmann (University of Innsbruck, Austria), Dr J. R. Sheppard (University of Minnesota, Minneapolis, Minn., U.S.A.), Dr A. Shatkin (The Roche Institute, Nutley, N.J., U.S.A.), Dr D. Gallwitz (University of Marburg, FRG), Dr S. H. Revell (Institute for Cancer Research, London, U.K.) and Dr G. Rutter (Pette Institut, Hamburg, FRG) for the provision of various cell lines and Dr A. Dowding and Dr B. Anderton (St George's Hospital Medical School, London, U.K.) for the generous gift of the monoclonal antibody α-IFA.

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(Received 24 January 1983 – Accepted 18 February 1983)