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Tissue-specific expression of a vimentin - desmin hybrid gene in transgenic mice

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We have introduced a hybrid gene, pVVim2, composed of the 5′ region of the hamster vimentin gene encoding the head and rod domain of vimentin and the 3′ region of the hamster desmin gene encoding the tail domain of desmin, into the germ line of mice by pronuclear injection. RNA and protein analysis of mice transgenic for this construct showed that the pVVim2 gene was expressed at high levels in a developmental and tissue-specific manner. This indicates that the vimentin-derived segment of the fusion gene contains all the regulatory elements required for vimentin-specific expression. Immunohistochemical staining of fibroblast cultures derived from the transgenic mice with antibodies specific for vimentin and desmin demonstrated that the pVVim2 protein is assembled into filaments that co-localize with the endogenous vimentin filaments. The expression of pVVim2 protein in mesenchymal cells does not interfere with the function of vimentin in these cells.

Introduction

The intermediate filaments (IFs) have been characterized as a unique set of cytoskeletal structures, composed of cell type-specific proteins. These proteins can be divided into five distinct classes, which are expressed in a developmentally regulated and tissue-specific fashion (Franke *et al.*, 1982; Osborn and Weber, 1982). For example, desmin is only expressed in muscle cells, while vimentin is almost exclusively found in cells of mesenchymal origin (Lazarides, 1982). In addition, vimentin is found in many cells cultured *in vitro* (Franke *et al.*, 1979; Virtanen *et al.*, 1981; Traub, 1985). The intermediate filament proteins are strongly related to the nuclear lamins and have been assigned to the same multigene family (McKeon *et al.*, 1986; Fisher *et al.*, 1986). The IF subunits have a similar structural organization: a central conserved α-helical domain (rod) of ~320 amino acid residues is flanked by a non-helical amino-terminal head domain and a carboxy-terminal tail domain of variable length (Geisler *et al.*, 1982; Steinert *et al.*, 1985). *In vitro*, the head and rod domains play a pivotal role in the polymerization of IF subunits (Kaufmann *et al.*, 1985; Traub and Vorgias, 1983).

The close relationship between the IFs is also reflected in their gene structure. The exon-intron organization of vimentin, desmin and the glial fibrillary acidic protein is similar and coding sequences show up to 65% homology (in case of vimentin and desmin, Quax *et al.*, 1983, 1985; Roop and Steinert, 1986). For most IF genes little is known about the regulatory sequences controlling their developmental and tissue-specific expression. For hamster desmin we have shown that the region between −89 and +25 bp relative to the cap site is sufficient for cell type-specific transient expression. The flanking 5′ region of the vimentin gene contains several regulatory elements. These elements are instrumental both in the downregulation of vimentin gene expression during myogenesis (Piper *et al.*, 1987, in press) and in the transcriptional modulation of vimentin gene expression during myogenesis (Pieper *et al.*, 1987, in press) Baserga, 1987).

Determination of the cell type-specific function of vimentin and the characterization of the regulatory pathways controlling vimentin gene expression is partly hampered by the fact that expression of vimentin is induced in most cells by *in vitro* culture. Therefore, we have introduced a vimentin-desmin hybrid gene construct into the germ line of mice to study the tissue-specific regulation of vimentin gene expression *in vivo* and to obtain insight in tissue-specific IF functions. In this construct the last three exons of the hamster vimentin gene covering the complete tail domain were replaced by the last three exons of the hamster desmin gene. This allows recognition of the gene product in the presence of endogenous vimentin, while retaining the structural features characteristic for intermediate filaments. The expression of this fusion gene was analyzed in transgenic mice and in cell lines derived from these mice.

Results

The pVVim2 construct

For the construction of the vimentin-desmin hybrid gene, further referred to as pVVim2 gene, the hamster vimentin and hamster desmin genes served as starting material. Figure 1 shows a physical map of both genes. Introns in both genes map at identical positions (Quax *et al.*, 1983, 1985). In the pVVim2 construct a 9.2 kbp *Bam*HI-*Bgl*II fragment comprising the promoter region and the first six exons encoding head and rod domain of the vimentin gene were fused via the *Eco*RI site in the pUC19 polylinker to a 3.4 *Eco*RI-*Stu*I fragment harboring the last three exons of the desmin gene encoding the tail domain. In this construct 3.1 kbp of 5′ flanking DNA is present. No other constructs were examined for tissue-specific expression in transgenic mice in this study. The pVVim2 gene construct is depicted in Figure 1a, and

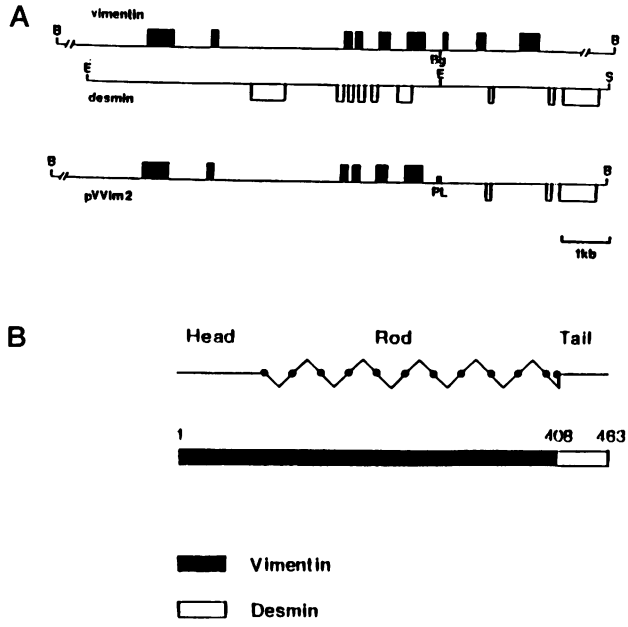

Fig. 1. (**A**) Physical map of the hamster vimentin gene, the hamster desmin gene and the hybrid gene pVVim2. Bars represent exon sequences, *Bam*HI, *Bgl*II, *Eco*RI, and *Stu*I restriction sites are indicated as B, Bg, E and S, respectively; PL represents the polylinker of the pUC19 vector. (**B**) Schematic representation of the hybrid protein encoded by the pVVim2 gene.

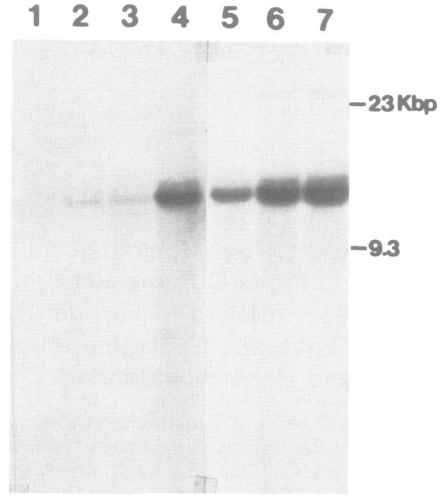

Fig. 2. Southern blot analysis of tail DNA from transgenic mice. 8 μg of DNA was digested with *Stu*I, run on 0.6% agarose gel, transferred to nitrocellulose and hybridized with the desmin-specific probe AA85; *Stu*I cleaves once in the pVVim2 fragment and in a head-to-tail tandem array of pVVim2 copies it generates a single hybridizing fragment of unit size; in all transgenic mouse lines multiple copies of pVVim2 were integrated. **Lane 1**, DNA from a control mouse; **lane 2**, DNA from founder No. 32; **lane 3**, DNA from offspring of founder No. 32; **lane 4**, DNA from founder No. 34; **lane 5**, DNA from founder No. 44; **lanes 6** and **7**, DNA from offspring of founder No. 44.

the composition of the expected hybrid protein in Figure 1b. Amino acids 1–408 comprise the head and rod domain of vimentin and amino acids 408–463 the tail domain of desmin. Cotransfection of the pVVim2 plasmid with the pSV2-neo plasmid into tissue culture cells (HeLa and hamster lens) results in stable transformants expressing the pVVim2 gene, as assayed with a polyclonal desmin antiserum (Van den Heuvel *et al.*, 1987). The data show that the hybrid protein encoded by the pVVim2 construct is able to assemble into a cytoskeletal network with normal IF appearance.

Transgenic mice

For the generation of transgenic mice the pVVim2 hybrid gene was excised from the vector and injected into the most accessible pronucleus of fertilized mouse eggs. Three transgenic mice were obtained (Nos. 32, 34 and 44). Southern blot analysis of tail DNA showed that the three transgenic founders had incorporated multiple copies of the gene in a head-to-tail arrangement (Figure 2). None of the transgenic mice showed any abnormalities. Two founders (Nos. 32 and 44) transmitted the pVVim2 gene to their offspring. Southern blot analysis indicated that founder No. 44 was mosaic for the pVVim2 gene, as its offspring showed a much stronger hybridization signal. This was also confirmed by immunofluorescence studies on tissues sections (see below).

Tissue-specific expression

The expression of the pVVim2 protein was analyzed by the indirect immunofluorescence technique on tissue sections using polyclonal rabbit antisera directed against desmin (poly-Des), vimentin (poly-Vim) and cytokeratin (poly-Ker) (Ramaekers *et al.*, 1983). The immunohistochemical analysis was performed on blood cells, sections from tail-skin, ear-shell, liver, spleen and testis. For blood cell analysis, blood was collected from the tail vein and smeared onto cover slips. Blood cells from transgenic mice displayed intensive staining after incubation with poly-Des antiserum, whereas cells from control mice were non-reactive (Figure 3a–c). Staining of macrophages was most pronounced. The staining patterns often show a filamentous organization. This suggests that the pVVim2 molecules are able to assemble into intermediate filaments. Sections of tail-skin and ear-shell from transgenic line 44 were used to analyze the tissue-specific expression of the pVVim2 gene. A strong staining with the poly-Des antiserum was observed in all tissues that normally express vimentin e.g. fibroblasts and cartilage (Figure 3d–f). No reaction was seen, however, in epithelial tissues (epidermis and hair root sheath) that stained with the polyclonal keratin antiserum (data not shown). In frozen sections from transgenic spleen nearly all cells stain with poly-Des, whereas in spleen from control mice only smooth muscle tissue of blood vessels are positive (data not shown). In testis of transgenic mice Leydig cells and Sertoli cells express the hybrid protein (Figure 3h). In liver of pVVim2 transgenic mice, Kupffer cells which express vimentin stain strongly with the poly-Des serum (Figure 3k). A comparison of liver sections from founder No. 44 with liver sections from its positive offspring confirmed the mosaic character of transgene integration in founder No. 44; only Kupffer cells in distinct liver segments react with poly-Des antiserum in the founder (Figure 3m), whereas in positive offspring all Kupffer cells stain with this antibody.

Identification of the pVVim2 mRNA and protein in vitro

Ear-shell fibroblasts from transgenic founder No. 32 were immortalized by SV40, and the cells were cloned and used for RNA and protein analysis. It appears that the hybrid pVVim2 protein is present in a filamentous network with normal IF appearance (Figure 4). Double immunofluor-

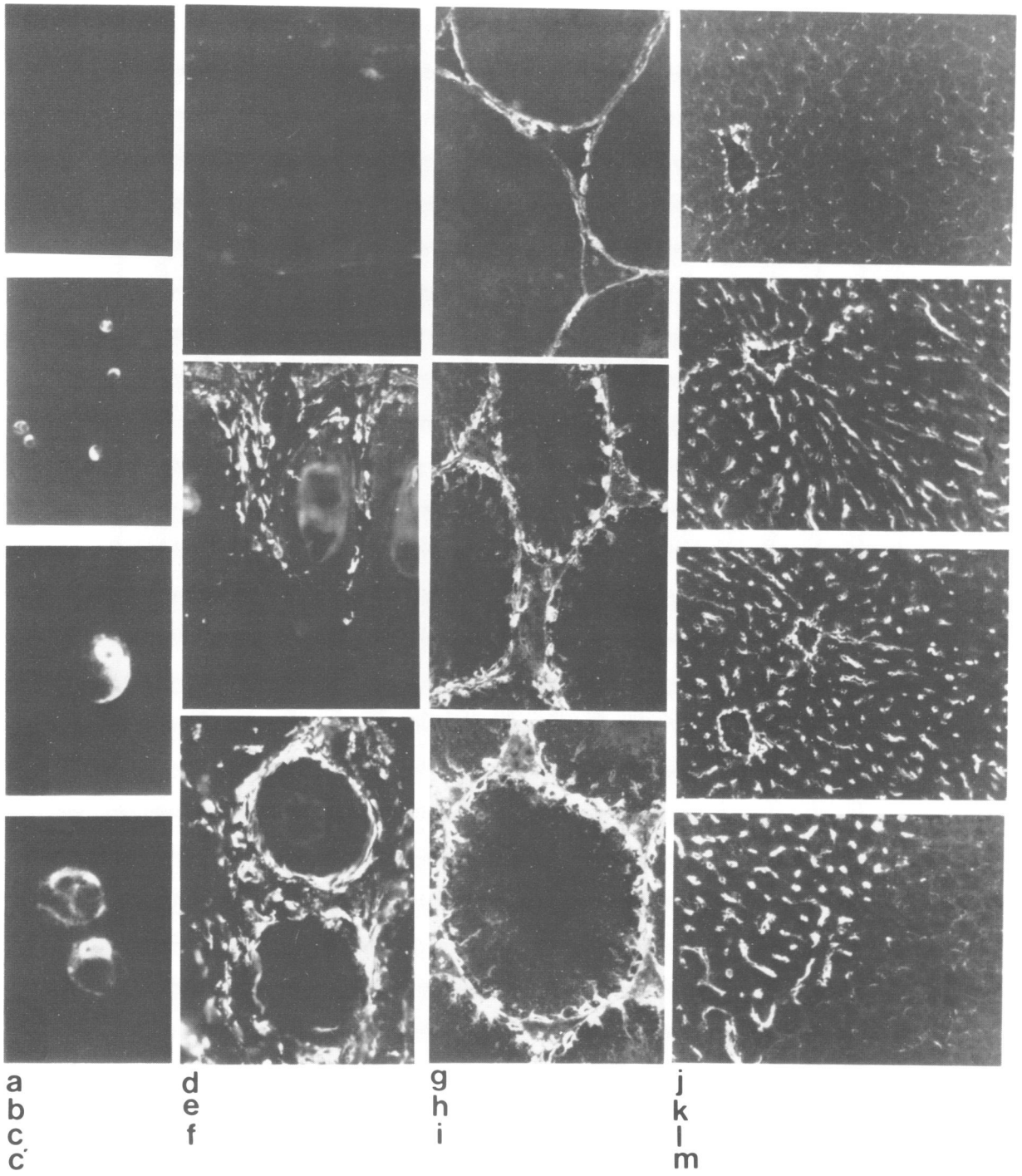

Fig. 3. Indirect immunofluorescence studies. Immunofluorescence was performed on blood smears (**a–c**), frozen sections of tail skin (**d–f**), testis (**g–i**) and liver (**j–m**) from control mice (**a,d,g,j**), positive offspring of founder No. 44 (**b,c,e,f,h,i,k,l**) and of founder No. 44 showing mosaic expression (**m**). Sections depicted in panels **a–h,j,k**, and **m** are reacted with the poly-Des antiserum; **i** and **l** are stained with the poly-Vim antiserum. Note that incubation of the smears and tissues from control mice with a polyclonal desmin antiserum does not show any staining of blood cells (**a**) or epidermis and dermis (**d**). Staining is exclusively seen in cells known to express desmin, i.e. smooth muscle cells of blood vessel walls (**j**) or myoid cells surrounding the seminiferous epithelium of the testis (**g**). In mice harboring the pVVim2 transgene, cells normally expressing only vimentin, i.e. macrophages (**b,c**), fibroblasts (**e,f**), Leydig cells and Sertoli cells in testis (**h**), and Kupffer cells in liver (**k,m**), now also express the pVVim2 protein, as visualized with poly-Des. For comparison the frozen sections were also incubated with poly-Vim (**i,l**). Mosaic expression can be seen in the liver section depicted in frame **m**. Magnifications; **a,b**, 60×; **c**, 350×; **d,e,f,g,h,i,j,k,l**, 150×; **m** 200×.

escence studies of the same cells with desmin and vimentin antibodies show that the hybrid protein is co-localized with the endogenous vimentin filaments (Figure 4c,d).

RNA was isolated from these cell lines and analyzed by Northern blot (Figure 5). Clearly, only in fibroblastic cell lines derived from a pVVim2 transgenic mouse was an mRNA of the appropriate size (2.2 kb) observed with the desmin probe X54, whereas fibroblasts from control mice

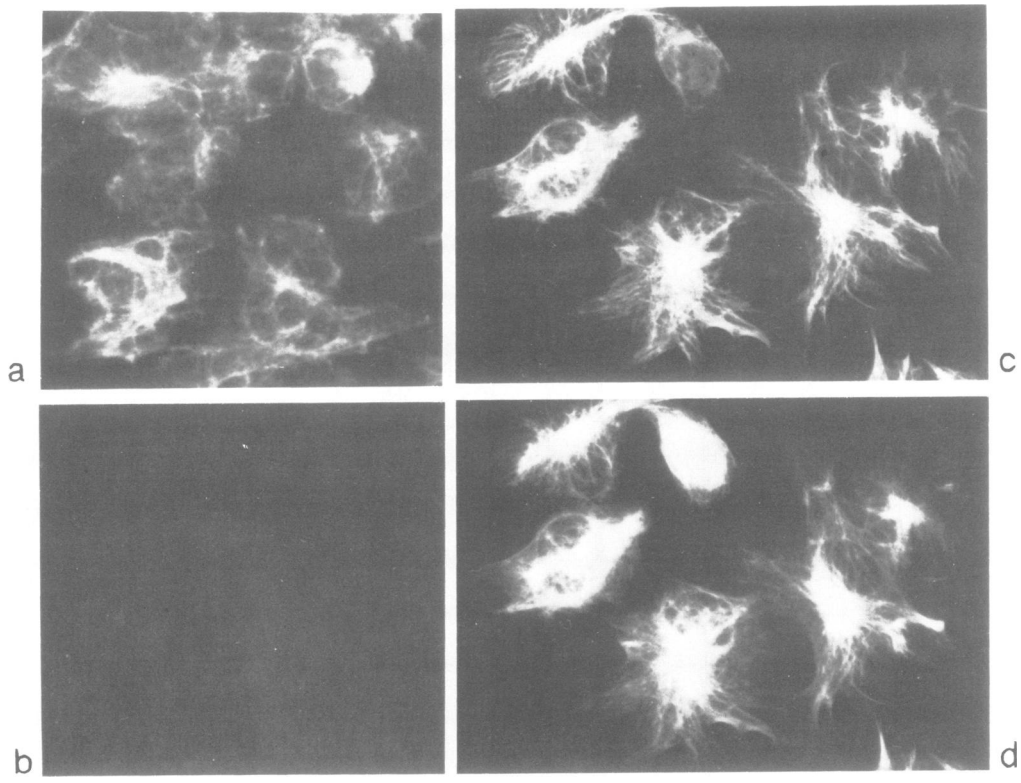

Fig. 4. Double-label immunofluorescence staining of transformed fibroblasts from transgenic mouse No. 32. Co-localization of pVVim2 protein and vimentin filaments is shown by staining with the polyclonal vimentin antibody poly-Vim (**c**) and the monoclonal desmin antibody RD301 (**d**). Fibroblasts from control mice react with poly-Vim (**a**) and not with RD301 (**b**). **a**–**d**, ×280.

were nonreactive. Hybridization with the vimentin-specific probe E49 showed the presence of the 2.0 kb long mouse vimentin mRNA in cell lines from both control and transgenic mice.

The hybrid gene is expressed at a level similar to that of the endogenous vimentin. Comparison of mRNA levels in fibroblasts from transgenic and control mice (Figure 5, compare lanes 1 and 2 to lane 3) clearly shows that the expression of the endogenous vimentin mRNA is not influenced by pVVim2 expression.

Identification of the pVVim2 protein

The immunohistochemical data were further substantiated by biochemical identification of the pVVim2 protein. Protein analysis by Western blotting was performed on the SV40-transformed fibroblastic pVVim2 positive cell clones which were also used for RNA analysis. In addition the *in vivo* synthesized pVVim2 protein was characterized. We chose lens cell tissue which normally expresses vimentin (Ramaekers *et al.*, 1980).

Western blots of SDS-polyacrylamide gels were reacted with a monoclonal antibody to desmin (RD301) and with a monoclonal antibody specific for vimentin (RV202). The epitope recognized by RD301 is located in the tail domain of desmin encoded by the last three exons of the hamster desmin gene (Van den Heuvel *et al.*, 1987). Lysates from BHK cells and a crude desmin preparation from mouse muscle were used as positive controls. SV40-transformed fibroblasts and lenses from control mice were used as negative controls. The results (Figure 6) clearly show the

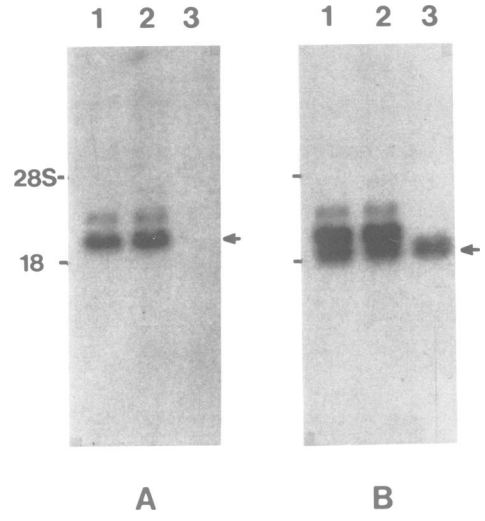

Fig. 5. Northern blot analysis of RNA from fibroblastic cell lines derived from pVVim2 transgenic mice. 15 μg total RNA was electrophoresed on a 1% agarose gel, transferred to nitrocellulose and hybridized with desmin-specific probe X54 (**panel A**), and with the vimentin specific probe E49 (**panel B**). **Lanes 1** and **2**, RNA isolated from two independent poly-Des positive cell lines derived from founder No. 32; **lane 3**, RNA isolated from a poly-Des negative control cell line.

presence of the pVVim2 protein with an apparent mol. wt of 57 kd in the transgenic fibroblastic cell lines and lens cells. In skeletal muscle tissue no pVVim2 or vimentin protein was

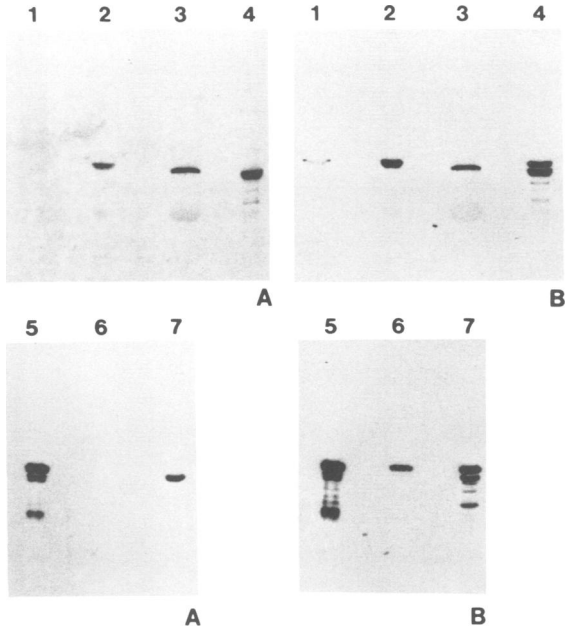

Fig. 6. Biochemical characterization of intermediate filament proteins from tissues of transgenic and control mice. Western blots prepared from tissue proteins were incubated with the monoclonal desmin antibody RD301 (**panel A**) and subsequently with RV202 (**panel B**). **Lane 1**, transformed fibroblasts from control mouse; **lane 2**, transformed fibroblasts from transgenic mouse No. 32; **lane 3**, muscle, only positive for desmin; **lane 4**, BHK cells, positive control for desmin and vimentin; **lane 5**, eye lens from transgenic mouse No. 32; **lane 6**, lens from control mouse; **lane 7**, BHK cells; additional protein bands below desmin represent characteristic IF breakdown products.

detected (Figure 6, lane 3). Since we used different antibodies to detect the hybrid protein it seems unlikely that this negative reaction is caused by masking of epitopes due to conformational change during myogenesis. This was confirmed by immunohistochemical analyses of muscle tissue (data not shown). Both striated and smooth muscle cells from different organs were stained with mono- and polyclonal antibodies against vimentin and desmin. In transgenic mice the desmin staining pattern was similar to that in control mice. No staining with the vimentin antibodies was observed in striated muscle cells indicating that both pVVim2 and vimentin are not expressed in these cells. Smooth muscle cells which do not express vimentin in control mice also do not express pVVim2 or vimentin in transgenic mice.

The pVVim2 expression level

In order to determine the expression level of pVVim2 cytoskeletal fractions of eye lenses from control and transgenic mice (founders No. 32 and No. 44) were analyzed by one-dimensional SDS–PAGE (Figure 7a). In lenses of offspring from founder No. 44 high levels of IF expression were detected clearly exceeding vimentin expression in lenses from control mice. Lenses of offspring from founder No. 32 also showed elevated, although much less, IF expression levels. Two-dimensional PAGE analysis of total lens extract from control and transgenic offspring from founder No. 44 confirmed these findings (Figure 7b). Immunoblotting of two-dimensional gels positively identified the hybrid protein and vimentin which were not separated on these gels. Identical results were obtained with cytoskeletal preparations (data not shown).

Discussion

In this paper we describe the construction and transgenic expression of a hybrid gene between the genes of the cytoskeletal proteins vimentin and desmin. A DNA fragment containing 3.1 kb of 5′ flanking DNA and the 5′ part of the vimentin structural gene up to the 6th exon, encoding the head and rod of the vimentin, was fused to the three last exons of the desmin gene encoding the tail portion of desmin. This chimeric gene, pVVim2, was introduced into the germ line of mice and its expression was analyzed at the mRNA and protein level using specific DNA probes and immunoreagents. The data show that a properly sized mRNA is transcribed from the pVVim2 gene. The pVVim2 mRNA is translated into a protein of the expected molecular weight.

Immunohistochemical analysis of tissue sections indicates that the expression pattern of the transgenic pVVim2 gene is indistinguishable from that of the endogenous vimentin. The expression levels differ in the two transgenic mouse strains investigated. Offspring mice from founder No. 32 expressed the hybrid gene at levels similar to the endogenous vimentin gene, whereas offspring from founder No. 44 expressed pVVim2 to a significantly higher level. RNA analysis shows that endogenous vimentin expression is not measurably influenced by transgenic pVVim2 expression. All the regulatory sequences required for the tissue-specific expression of vimentin are included in the 9.2 kb *Bam*HI–*Bgl*II fragment, starting 3.1 kb upstream from the transcriptional initiation site. The immunofluorescent staining pattern with poly-Des antiserum, especially of transformed fibroblasts (Figure 4) demonstrates that the pVVim2 protein under these circumstances can assemble into filamentous structures. Double-label immunofluorescence studies which combine monoclonal antibodies RD301 and RV203 with the poly-Vim and poly-Des antisera, respectively, show that the vimentin containing filaments match completely with the pVVim2 containing structures, strongly suggesting that pVVim2 protein copolymerizes with vimentin and becomes a part of the vimentin skeleton. This is in agreement with cross-linking studies in which copolymerization has been shown between vimentin and desmin (Quinlan and Franke, 1982; Steinert *et al.*, 1981). Since the pVVim2 protein contains the vimentin α-helical domain and the desmin carboxy terminus, which is supposed to protrude from the skeletal backbone (Geisler and Weber, 1986), copolymerization between pVVim2 and vimentin is not surprising.

Intermediate filament proteins are expressed in a developmentally regulated and tissue-specific fashion, and this pattern of expression has been conserved among vertebrate species. However, some differences which may relate to a slight divergence in the developmental pattern of a distinct cell lineage, have been noted (Ngai *et al.*, 1987). This supports the view that intermediate filaments fulfill an important role in development. Furthermore, it suggests that alterations in highly conserved protein domains, which are supposed to be of crucial importance for proper functioning of IFs, are probably not tolerated. The amino acid sequence of the carboxy-terminal domain of vimentin is much more conserved among species than the sequence of the amino

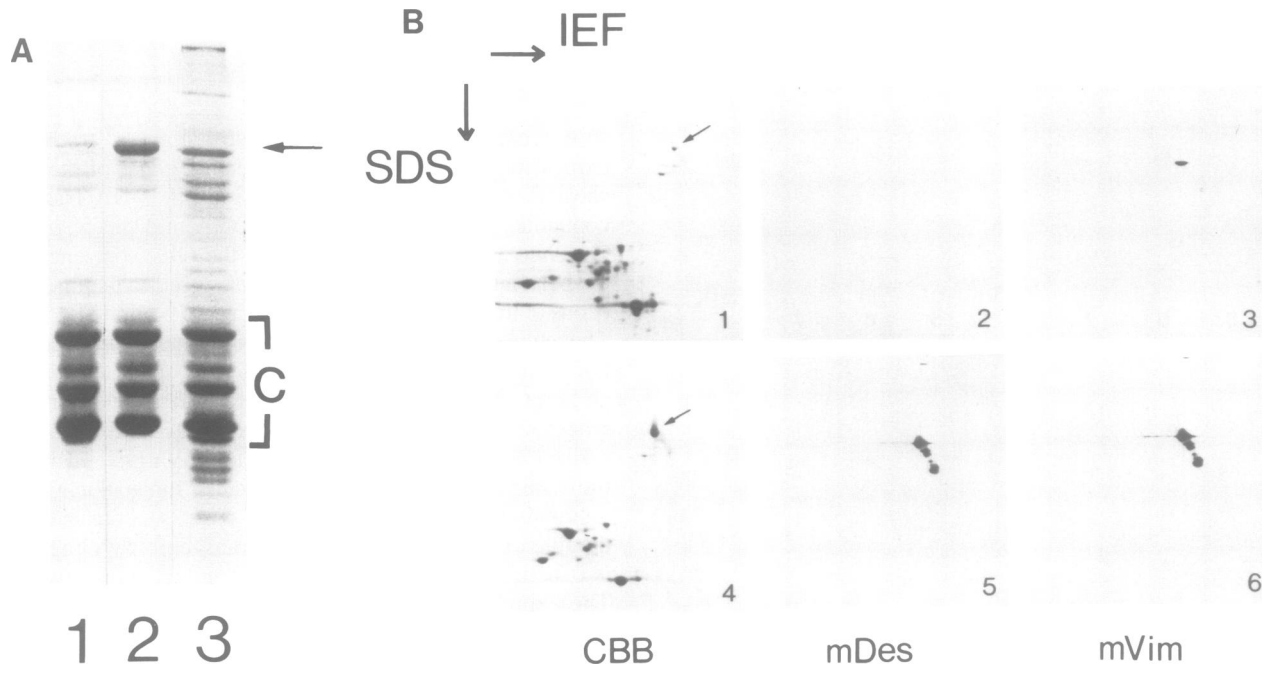

Fig. 7. Biochemical characterization and analysis of expression levels of IF proteins from eye lenses of transgenic and control mice. (**A**) Coomassie Brilliant Blue (CBB) staining of one dimensional SDS gels of cytoskeletal preparations from control and transgenic mice. **Lane 1**, lens extract from control mouse; **lane 2**, lens extract from transgenic mouse, derived from founder No. 44; **lane 3**, lens extract from transgenic mouse derived from founder No. 32. Arrow indicates pVVim2 protein and vimentin position. C, crystallin polypeptide chains serve as standards for the amount of protein applied onto the gel. (**B**) Total lens extracts of control (**1–3**) and transgenic mice (**4–6**) analyzed by two dimensional gel electrophoresis and stained with CBB or immunoblotted and stained with the monoclonal desmin antibody RD301 (**2,5**) and subsequently with the monoclonal vimentin antibody RV202 (**3,6**). Both mice were derived from founder No. 44. Arrows indicate IF protein position. Additional spots on the blots represent characteristic IF breakdown products.

terminal domain and approximates the conservation of the rod domain (Zehner *et al.*, 1987). This is also observed for desmin (Quax *et al.*, 1984). However, the carboxy-terminal regions of different classes of IF subunits have significantly diverged. This is suggestive for the importance of the carboxy terminal region in IF-specific function. Recently, it has been shown that the carboxy-terminal domain of both vimentin and desmin can associate with lamin B at the nuclear envelope (Georgatos and Blobel, 1987; Georgatos *et al.*, 1987). Our results show that the presence of significant levels of pVVim2 protein in all vimentin-expressing cells does not interfere with normal mouse development. Preliminary results using transgenic mice in which desmin is expressed in a vimentin-specific fashion also indicate that the expression of desmin in mesenchymal cells does not interfere with normal development. This suggests that, if vimentin fulfills an essential function in development, this function is not hampered by the co-expression of pVVim2 or desmin.

Materials and methods

Plasmid construction

Isolation and characterization of the hamster vimentin and desmin genes have been described previously (Quax *et al.*, 1983, 1984, 1985; Quax-Jeuken *et al.*, 1983). Intervening sequences map at similar positions in the gene. The last three exons of the vimentin gene were replaced by the corresponding exons of the desmin gene. The 9.5 kb *Bam*HI–*Bgl*II fragment harboring the first six exons of vimentin was subcloned into the *Bam*HI site of the pUC19. A 3.4 kb *Eco*RI–*Stu*I fragment containing the last three exons of desmin was fused to the 3′ end of the vimentin fragment via the *Eco*RI site in the polylinker region. The complete pVVim2 hybrid gene is present on a 13 kb *Bam*HI fragment. As hybridization probe specific for hamster vimentin M13 phage E49 was used, which contained a *Sau*3A fragment covering the region between 150 nucleotides upstream to 370 nucleotides downstream from the cap site (Quax *et al.*, 1983). For hamster desmin the M13 phages X54 (a *Sau*3A insert, containing the last 25 nucleotides of exon 8, intron 8 and the first 350 nucleotides of exon 9) and AA85 [a *Pst*I–*Taq*I insert corresponding to nucleotides 165 to 357 downstream from the stop codon (Quax *et al.*, 1985)] were used.

Cell culture

Cell cultures from ear fibroblasts were established as described (Bloemendal *et al.*, 1980).

Transgenic mice

Fertilized mouse eggs were recovered in cumulus from oviducts of superovulated (CBA × C57Bl/LiA) F1 females that had mated with F1 males several hours earlier. Approximately 200 copies of the pVVim2 hybrid gene construct (without plasmid sequences) were microinjected in the most accessible pronucleus. Microinjected eggs were implanted into oviducts of 1-day pseudopregnant MA or F1 foster mothers and carried to term. Total genomic DNA was prepared from tail biopsies 3–4 weeks after birth. For Southern blot analysis, 8 μg of total genomic DNA was digested with *Bam*HI or *Stu*I, run on a 0.6% agarose gel and transferred to nitrocellulose. The filter was hybridized to ^{32}P-labeled probes as described (Cuypers *et al.*, 1984).

Northern blot analysis

Fifteen μg of total cellular RNA, prepared by the LiCl–urea method (Auffray and Rougeon, 1980) was separated on a 1% agarose gel in formaldehyde and transferred to nitrocellulose.

Immunohistochemical analysis

The intermediate filament protein expression of tissues and cell lines was assayed by indirect immunofluorescence and Western blot analysis. The antisera used for this purpose include: (i) an affinity purified polyclonal antibody directed against human skin keratins (poly-Ker); this antibody reacts with virtually all epithelial tissues but not with non-epithelial cells (Ramaekers *et al.*, 1983a); (ii) an affinity purified polyclonal antibody to bovine lens vimentin (poly-Vim) (Ramaekers *et al.*, 1983a); (iii) a rabbit antibody to chicken gizzard muscle desmin (poly-Des) (Ramaekers *et al.*, 1983b); (iv) monoclonal antibodies RV202 and RV203 directed against bovine lens

vimentin (Broers *et al.*, 1986) and (v) a mouse monoclonal antibody RD301 to desmin raised against chicken gizzard desmin (Broers *et al.*, 1986). As second antibodies for indirect immunofluorescence either fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG or FITC-conjugated rabbit anti-mouse IgG were used at a dilution of 1:25 (Nordic, Tilburg, The Netherlands). For double-label immunofluorescence FITC-conjugated goat anti-rabbit IgG was combined with Texas Red-conjugated sheep $(Fab')_2$ anti-mouse Ig (New England Nuclear, Boston, MA, USA). In control experiments PBS was substituted for the primary antibody. Cells on coverslips and 5 μm thick frozen sections of mouse tissues were fixed in methanol for 5 s (−20°C) and acetone (3 × 5 s), air dried and incubated with the primary antibody for 30−45 min at room temperature. Further processing for indirect immunofluorescence was performed as described (Broers *et al.*, 1986).

Gel electrophoresis and immunoblotting assays

Cytoskeletal preparations of eye lens were made as follows: lenses were suspended in PBS containing 1% Triton X100, 1 mM phenylmethylsulphonylfluoride (PMSF, Merck,Darmstadt) and 1 mM EDTA for 10 min at 0°C. After centrifugation (3000 *g* for 10 min) and washing (PBS) the pellet was dissolved by boiling during 5 min in SDS sample buffer. For the crude desmin preparation from mouse skeletal muscle, frozen 5 μm thick sections were first extracted with 1.5 M KCl, 0.5% Triton X100, 5 mM EDTA, 0.4 mM PMSF, and 10 mM Tris−HCl, pH 7.2 and processed as described above. One- and two-dimensional SDS gel electrophoresis and immunoblotting was performed as described (Broers *et al.*, 1986).

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