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# Expression of intermediate filament proteins during development of *Xenopus laevis*

## I. cDNA clones encoding different forms of vimentin

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### Summary

**To provide a basis for studies of the expression of genes encoding the diverse kinds of intermediate-filament (IF) proteins during embryogenesis of *Xenopus laevis* we have isolated and characterized IF protein cDNA clones. Here we report the identification of two types of *Xenopus* vimentin, Vim1 and Vim4, with their complete amino acid sequences as deduced from the cloned cDNAs, both of which are expressed during early embryogenesis. In addition, we have obtained two further vimentin cDNAs (Vim2 and 3) which are sequence variants of closely related Vim1. The high evolutionary conservation of the amino acid sequences (Vim1: 458 residues; $M_r \sim 52\,800$; Vim4: 463 residues; $M_r \sim 53\,500$) to avian and mammalian vimentin and, to a lesser degree, to desmin from the same and higher vertebrate species, is emphasized, including conserved oligopeptide motifs in their head domains. Using these cDNAs in RNA blot and ribonuclease protection assays of various embryonic stages, we observed a dramatic increase of vimentin RNA at stage 14, in agreement with immunocytochemical results obtained with antibody VIM-3B4. The significance of very weak mRNA signals detected in earlier stages is discussed in relation to negative immunocytochemical results obtained in these stages. The first appearance of vimentin has been localized to a distinct mesenchymal cell layer underlying the neural plate or tube, respectively. The results are discussed in relation to programs of *de novo* synthesis of other cytoskeletal proteins in amphibian and mammalian development.**

Key words: *Xenopus laevis*, oocytes, cytoskeleton, intermediate filaments, vimentin, mesoderm.

### Introduction

In embryonic development, cell differentiation and morphogenesis are correlated with – and probably dependent on – programs of expression of cell type-specific proteins. This is especially evident for the diverse kinds of cytoskeletal proteins which contribute to the formation of major cell structures involved in cell and tissue shaping, in addition to other functions. Most cytoskeletal proteins, notably those forming the various kinds of filamentous structures, exist as multigene families whose members are differentially expressed during development in patterns specific for certain routes of cell and tissue differentiation. In amphibia, the best studied examples are the different actins (Sturgess *et al.* 1980; Vanderkerckhove *et al.* 1981; Mohun *et al.* 1984; Gurdon *et al.* 1985*a*) and the large family of proteins that form either cytoplasmic intermediate-sized filaments (IFs) or the nuclear lamina (Franz *et al.* 1983; Benavente *et al.* 1985; Dawid *et al.* 1985; Godsave *et al.* 1986; Wylie *et al.* 1986).

The cytoplasmic IFs are formed by a highly complex group of polypeptides that are expressed in cell type-characteristic patterns (for reviews see Anderton, 1981; Franke *et al.* 1982*b*; Lazarides, 1982). (1) Cytokeratins constitute a group of about 30 polypeptides that includes the approximately 20 polypeptides forming the typical IFs of epithelial cells ('epithelial cytokeratins'; Franke *et al.* 1982*b*; Moll *et al.* 1982) and at least 10 different polypeptides characteristic of hair- and nail-forming cells ('trichocytic cytokeratins'; cf. Heid *et al.* 1988). (2) Typically, mesenchymally derived tissues contain IFs formed by vimentin (Franke *et al.* 1978) which, however, can also be coexpressed in some other cell types, including certain epithelia, with one of the other IF protein types (e.g. Franke *et al.* 1979, 1982*b*; Lazarides, 1982; Czernobilsky *et al.* 1985; and references cited therein). (3) Desmin is an IF protein typically induced during myogenesis (for reviews see Lazarides, 1982). (4) The glial filament protein is a polypeptide synthesized typically, but not exclusively, in astrocytes. (5) The neurofilament polypeptides NF-L, NF-M and NF-H are typically found in cells of neuronal differentiation (e.g. Liem *et al.* 1978; Ander-

ton, 1981; Dahl & Bignami, 1985; Lasek *et al.* 1985).

Because of their abundance and cell type-restricted expression, the IF proteins are valuable candidates in studies of developmental changes of protein expression, in relation to cell differentiation and tissue formation. Various authors have used IF protein antibodies to identify the developmental appearance of the specific protein, by using immunocytochemistry alone or in combination with gel electrophoretic separations of cytoskeletal proteins. During mammalian development, cytokeratins 8 and 18, which form the first IFs, have been detected in mouse embryogenesis as early as the morula–blastocyst transition (Brûlet *et al.* 1980; Jackson *et al.* 1980, 1981; Franke *et al.* 1982*a*,*b*) or even one or two cell generations earlier (Oshima *et al.* 1983; Duprey *et al.* 1985; Johnson *et al.* 1986; Chisholm & Houliston, 1987). Lehtonen *et al.* (1983*a*; see also Lehtonen, 1985, 1987) have reported that some cytokeratin already exists in the oocyte and the egg, although in a nonfibrillar, perhaps 'soluble' form (for divergent reports from other mammalian species see also Czernobilsky *et al.* 1985; Gall, LeGuen & Hunneau, 1988). In contrast, vimentin IFs have first been seen at day 8 of mouse embryogenesis, in primary mesenchymal cells (Franke *et al.* 1982*a*) and in some distal (parietal) endoderm cells that coexpress cytokeratins and vimentin (Franke *et al.* 1983; Lane *et al.* 1983; Lehtonen *et al.* 1983*b*). IFs containing desmin or the two neural types of IF proteins have not been detected in these early stages (e.g. Jackson *et al.* 1981; Raju *et al.* 1981; Schnitzer *et al.* 1981; Bignami *et al.* 1982; Bignami & Dahl, 1984; for avian embryogenesis see Tapscott *et al.* 1981). These observations suggest that in mammalian development early embryogenesis is dominated by the synthesis of an epithelial type IF cytoskeleton, in agreement with the polar architecture and the presence of typical desmosomes of ectodermal and endodermal cells.

In developmental research, amphibian embryogenesis, notably that of *Xenopus laevis*, is a 'classical' system for various reasons, not the least being the rapidity of early cleavages, blastula formation and gastrulation, which apparently is made possible by large maternal supplies of proteins and mRNAs. If one accepts the widely believed hypothesis that no transcription of zygote genes takes place before the 'mid-blastula transition (MBT) point' (for reviews see Kirschner *et al.* 1985; see, however, also Nakakura *et al.* 1987), one has to conclude that the early differentiation of epithelial cell layers, including the formation of cytokeratin IFs, that occurs before this point of development, must use maternally supplied cytokeratins and cytokeratin mRNAs. Indeed, cytokeratins of the 'simple epithelium type', i.e. the amphibian equivalents to human cytokeratins 8, 18 and 19, and the corresponding mRNAs have been identified in oocytes, eggs and early embryos of *Xenopus laevis* (Franz *et al.* 1983; Gall *et al.* 1983; Godsave *et al.* 1984*b*; Wylie *et al.* 1985, 1986; Franz & Franke, 1986; Klymkowsky *et al.* 1987), whereas other kinds of cytokeratins are synthesized after gastrulation (for details see Fouquet *et al.* 1988).

With respect to vimentin synthesis, controversial immunocytochemical results have been reported. Franz *et al.* (1983) have not detected vimentin IFs in *Xenopus* oocytes of various stages but only in adjacent interstitial cells. In contrast, Godsave *et al.* (1984*a*) have reported reactions with vimentin antibodies in oocytes, eggs and throughout embryogenesis, suggesting that the protein is continually present but not specifically induced *de novo* at a certain developmental time. On the other hand, in this species, the synthesis of various myogenic proteins, including cardiac and skeletal muscle $\alpha$-actins, has been elucidated in great detail, and it has been shown that their genes are postgastrulationally induced specifically in cells committed to a myogenic expression program (e.g. Mohun *et al.* 1984; Wilson *et al.* 1986; Mohun & Garrett, 1987; for a recent review see, Gurdon, 1987). To provide a basis for detailed examinations of the embryogenic appearance, in space and time, of the specific IF proteins we have isolated cDNA clones of several IF proteins. In the present study, we describe cDNA clones for *Xenopus* vimentin which we have used for a study of their first expression, in combination with immunocytochemistry using a vimentin-specific monoclonal antibody, VIM-3B4.

## Materials and methods

### *Animals*

Females of *Xenopus laevis* were kept as described (Krohne *et al.* 1981). After injection of female frogs with human chorion-gonadotropin (Predalon, Organon, Oberschleissheim, FRG), eggs were stripped and fertilized *in vitro*. Embryos were incubated in 5% DeBoers medium (100% medium is: 110 mM-NaCl, 1·2 mM-KCl, 0·44 mM-$CaCl_2$, pH 7·2; cf. Wolf & Hedrick, 1971) and staged according to Nieuwkoop & Faber (1967). After dejellying with 1·5% cysteine–HCl (adjusted with NaOH to pH 7·8) in the same medium, the embryos were frozen in liquid nitrogen. For preparation of cytoskeletal material various embryonal stages, whole tadpoles (stage 42), dissected material from adult animals and cultured *X. laevis* kidney epithelial cells (XLKE cells, line A6) were used (Franz *et al.* 1983).

### *Cytoskeletal preparations and gel electrophoresis*

Cytoskeletal proteins were prepared from cultured cells and oocytes as described (Franz *et al.* 1983), except that 3 mM-PMSF and 1 mM-EGTA were included in the high-salt extraction buffer, and analysed by two-dimensional gel electrophoresis and immunoblotting. Vimentin antibodies were raised in guinea pigs (cf. Franke *et al.* 1979) against purified bovine lens vimentin commercially available from Progen, Biotechnics (Heidelberg, FRG). Monoclonal antibody PK V1 against human vimentin (Lehtonen *et al.* 1983*b*), which has been characterized on mammalian cells (Lehtonen *et al.* 1983*b*; Franke *et al.* 1984), was kindly provided by Dr I. Virtanen (University of Helsinki, Finland). Monoclonal antibody anti-IFA reacting with most IF proteins (Pruss *et al.* 1981) was prepared in our laboratory from cultured supernatants of hybridoma cells purchased from American Type Culture Collection (Rockville, Maryland, USA). Monoclonal murine antibody VIM-3B4 (IgG2a) produced from mice immunized with purified bovine lens vimentin is available from Progen Biotechnics (Heidelberg, FRG) and Boehringer (Mannheim,

FRG). In all species examined (human, bovine, rat, mouse, hamster, chicken, *Xenopus laevis*) it reacts specifically, in immunoblot tests and in binding assays using purified antibodies, only with vimentin and does not cross-react with any other IF protein. The antibody is strongly positive on sections of frozen tissues as on sections of formalin-fixed, paraffin-embedded tissue samples.

Soluble and cytoskeletal fractions of unfertilized eggs were prepared in two different ways. For the preparation of 100 000 *g* supernatants, 200 dejellied eggs were homogenized by pipetting up and down in 300 μl '5:1' medium (Callan & Lloyd, 1960) containing 3 mM-phenylmethylsulphonyl fluoride (PMSF). After 10 min centrifugation at 3500 *g* the pellet was saved, and the resulting supernatant was centrifuged for 1 h at 100 000 *g* to separate particulate material ('high speed pellet'; HSP) from the corresponding supernatant fraction (HSS). At each step of preparation, aliquots of the fractions obtained were taken for SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

For further fractionation, low speed pellets were extracted with buffers containing high salt and 1 % Triton X-100 (Franz *et al.* 1983). Alternatively, dejellied eggs were taken up directly in a 100 mM-morpholinoethane sulphonic acid buffer containing 0·8 M-NaCl and 1 % Triton X-100 (Herrmann & Wiche, 1983), resuspended and centrifuged briefly at 800 *g* or 10 000 *g*. Pellets and supernatants were processed for gel electrophoresis. All steps were carried out at 4 °C on ice.

Samples were boiled in SDS-containing buffer and examined by SDS–PAGE (Laemmli, 1970) or by two-dimensional gel electrophoresis according to O'Farrell (1975), using the modifications of Garrels (1979). Immunoblotting reactions on nitrocellulose sheets were visualized by $^{125}$I-labelled antibodies (for refs see Achtstätter *et al.* 1986) or by the alkaline phosphatase method, following the technical manual of the supplier (Promega, Madison, WI, USA).

### *Preparation and* in vitro *translation of RNA*

RNA was prepared as described and used for *in vitro* translation (Magin *et al.* 1983; Franz & Franke, 1986; Fouquet *et al.* 1988). Translation products were characterized by two-dimensional gel electrophoresis, using cytoskeletal proteins from XLKE-A6 cells for coelectrophoresis.

### *RNA blot analysis*

Poly(A)$^+$ or total RNA was analysed by electrophoresis on formaldehyde/agarose gels (Davis *et al.* 1986) or on agarose gels after denaturation with glyoxal (for refs see Jorcano *et al.* 1984). After transfer to nitrocellulose paper, hybridization was carried out with $^{32}$P-DNA from clone pXenVim1 (for details see below) obtained by labelling of purified restriction fragments with [$\alpha$-$^{32}$P]dATP using the random primed labelling kit (Boehringer Mannheim, FRG) or with $^{32}$P-anti-sense RNA obtained by transcription with T3 polymerase of *Bam*HI-restricted pXenVim1, yielding a probe representing the complete clone. For calibration, a commercially available RNA molecular weight marker set was used (Bethesda Research Laboratories, Gaithersburg, MD, USA).

### *Isolation and characterization of cDNA clones*

For isolation of cDNA clones encoding *Xenopus* vimentin, we screened a cDNA library in λgt10 which had been prepared with poly(A)$^+$ RNA from stage-17 embryos (kindly provided by Dr D. A. Melton, Harvard University, Cambridge, MA, USA). As hybridization probe, a gel electrophoretically purified (*Sph*I–*Afl*II) insert of the hamster vimentin cDNA clone, pVim 2 (Quax-Jeuken *et al.* 1983; kindly provided by Dr W. Quax, University of Nijmegen, Netherlands) was used. The start of this fragment corresponds, in the protein, to a position immediately in front of the $\alpha$-helical rod domain (for nomenclature see Weber & Geisler, 1984; Steinert *et al.* 1985) and ends 61 nucleotides downstream of the stop codon. Under our conditions, randomly-primed labelled fragments with average lengths of 80–120 bp were obtained, resulting in a mixed pool of probes representing various regions of the insert. Hybridization conditions for Southern blot analysis were adopted from those used by Quax *et al.* (1984) with genomic *X. laevis* DNA, employing a pVim2-related, overlapping hamster vimentin clone as probe (pVim1; Quax-Jeuken *et al.* 1983). The insert size of the positive clones obtained was determined by a mini-preparation method (Lewis & Cowan, 1986), followed by Southern blot analysis of *Eco*RI-digested DNA with the pVim2 probe (see above). From 18 positive clones, 4 with very strong (pXenVim1-4) and 4 clones with strong signals, all containing inserts larger than 1·8 kb, were chosen and used for large-scale preparation of DNA.

For isolation of cDNA clones encoding human vimentin, we screened a human testis cDNA library in λgt11 (Clontech, Palo Alto, CA, USA) essentially as described above, employing the hamster clone pVim2.

### *Subcloning and DNA sequencing*

The *Eco*RI fragments of the clones obtained as described above were purified by gel electrophoresis on low melting point agarose gels. After phenol extraction of gel slices, the DNA fragments were inserted into the *Eco*RI site of the Bluescript M13$^+$ plasmid (Stratagene, La Jolla, CA, USA). The polypeptides encoded by these subclones were characterized by the hybrid-selection–translation method (for details see Jorcano *et al.* 1984; Fouquet *et al.* 1988) and by *in vitro* transcription from these vectors as described by the manufacturer, followed by *in vitro* translation of the transcripts (see above). For each clone, all *Pst*I fragments were subcloned into the *Pst*I site of the M13mp9 vector and sequenced by the dideoxynucleotide sequencing method (Sanger *et al.* 1977). Deduced amino acid sequences were used for computer-aided comparison with available IF sequences, using a special program of the German Cancer Research Center. Clones of interest were fully sequenced, using appropriate restriction enzyme fragments in M13 vectors and the exonuclease III/S1 nuclease deletion method of Henikoff (1984). In addition, at least one strand was completely sequenced by the chemical degradation method (Maxam & Gilbert, 1977).

### *Ribonuclease protection assays*

The procedure of Melton *et al.* (1984) was used with minor modifications. Sense RNA transcripts were synthesized with T7 polymerase from pXenVim1 cloned into the Bluescript plasmid after linearizing with *Cla*I. Uniformly labelled anti-sense RNA was made from the same construct with T3 polymerase after cutting with *Hin*fI yielding a polynucleotide probe of 424 residues, including 69 nucleotides of polylinker, using [$^{32}$P-$\alpha$]CTP or GTP (800 Ci mmol$^{-1}$). A second probe, containing the region coding for the conserved TYRKLLE-GEE domain ('TYRKLEGE-probe'), was obtained by exonuclease III deletion of pXenVim1 linearized by *Kpn*I and *Cla*I. The S1 nuclease-treated, blunted and religated clone contained nucleotides 1–1300. After transcription with T3 RNA polymerase the *Hin*fI-restricted plasmid yielded a $^{32}$P-labelled, 'anti-sense' RNA of 263 nucleotides, including 21 nucleotides transcribed from the plasmid. The RNA probes were purified by urea–polyacrylamide gel electrophoresis and $1\times10^5$ cts min$^{-1}$ (~1 fmole) were hybridized overnight at 45 to 65 °C with 5 μg total RNA each or with 10 μg tRNA in a

solution containing 80% formamide, 40 mM-Pipes (pH 6·4), 400 mM-NaCl and 1 mM-EDTA. In an other series of experiments, we used 50% formamide instead of 80% (Cho *et al.* 1988). The mixtures were digested with 10 $\mu$g ml$^{-1}$ RNase A and 20 i.u. ml$^{-1}$ RNase T1 (both obtained from Boehringer) at 30°C for 1 h. To test if this amount of probe was in excess of the RNA to be quantified, various amounts (approximately 1, 2·5, 5 and 12·5 $\mu$g) from stage-39 embryos were each protected with $1\times10^5$ cts min$^{-1}$ of probe, and the recovered RNA was quantified by liquid scintillation counting. A linear increase was obtained, indicating that the amount of probe used was sufficient to protect all vimentin transcripts present in 12·5 $\mu$g RNA of stage-39 embryos. Sense RNA transcripts were synthesized from the corresponding clones after linearizing at unique polylinker sites followed by transcription with the appropriate polymerase.

### *Expression of proteins in* E. coli

The *Xenopus* vimentin clones pXenVim1 and pXenVim4 were ligated into the *Eco*RI site of the expression vector 17A/B (Magin *et al.* 1987) which was kindly provided by H. Bujard (Center for Molecular Biology, Heidelberg, FRG), and recombinant vimentin was isolated from inclusion bodies.

### *Immunofluorescence microscopy*

Cryostat sections through snap-frozen tissues or whole embryos were processed for immunofluorescence microscopy as described by Jahn *et al.* (1987), using murine monoclonal antibodies VIM-3B4 to vimentin and lu-5 to cytokeratins (Franke *et al.* 1987) or guinea pig antibodies to vimentin (cf. Franz *et al.* 1983). Alternatively, ovarian tissue and embryos were fixed for 2 h in 2% trichloroacetic acid (TCA) as described by Godsave *et al.* (1984*a*,*b*), frozen and cryostat-sectioned. Sections were either air-dried or briefly (approx. 1 min) treated with 2 M- or 3 M-urea (in PBS) in order to produce partial loosening of IF structures and 'unmasking' (Franke *et al.* 1983*b*; Godsave *et al.* 1984*a*,*b*). Further procedures were as described elsewhere (Jahn *et al.* 1987).

## Results

### (A) *Identification of vimentin in* Xenopus *with specific antibodies*

Monoclonal antibody VIM-3B4 to vimentin, which in immunoblot tests reacts specifically only with vimentin, detected characteristic fibrillar arrays in cultured epithelial A6 cells of *X. laevis* (Fig. 1A). Upon colcemid treatment, these vimentin-positive filaments collapsed into the typical perinuclear aggregates (Fig. 1B,C) previously shown for vimentin IFs of various cultured cells of mammalian and avian origin (for refs see Bennett *et al.* 1978; Franke *et al.* 1978; Lazarides, 1982). This antibody was very specific and sensitive, as also shown by the fact that it allowed detection of the typical bundles of vimentin IFs present in the nucleated red blood cells (Fig. 1D,E), a cell-type known to contain vimentin IFs in amphibia (Gambino *et al.* 1984). Essentially identical results were obtained with our guinea pig antisera against bovine vimentin (data not shown).

The protein component responsible for this antibody reaction was identified as a cytoskeletal polypeptide of $M_r \sim 55\,000$, as estimated from SDS–PAGE and immunoblot results using cytoskeletons of cultured XLKE-A6 cells, stage-42 embryos (tadpoles) and erythrocytes from adult animals (Fig. 2A–C). In direct SDS–PAGE comparison, this polypeptide migrated slightly faster than vimentin from several mammalian cells (e.g. Fig. 2A), confirming the observation of Nelson & Traub (1982). On two-dimensional gel electrophoresis, the immunoreactive protein from *Xenopus* erythrocytes was resolved into a group of three isoelectric variants slightly more acidic than $\alpha$-actin (Fig. 2D). A nearly identical pattern was obtained with high-salt-buffer- and detergent-resistant residues from XLKE-A6 cells (Fig. 2F; at the loading shown here, except that the more basic spot was not split into two separate components). However, in XLKE-A6 cytoskeletons, the

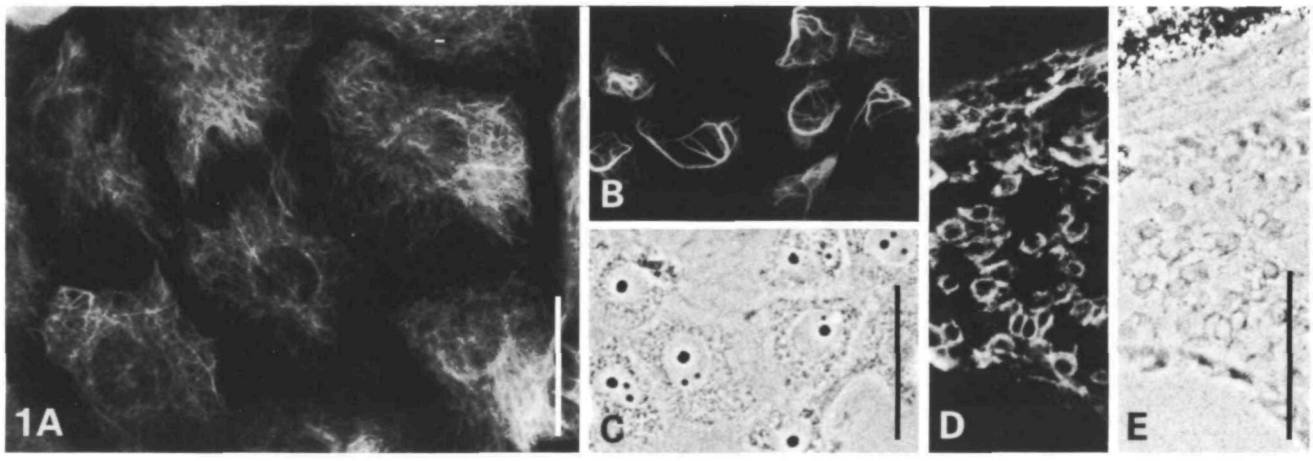



**Fig. 1.** Immunofluorescence microscopy showing reactions of monoclonal antibody VIM-3B4 to vimentin on cultured kidney epithelial (XLKE) cells of line A6 (A–C) and a frozen section of ovary tissue of *Xenopus laevis* (D,E). (A) Typical fibrillar networks of vimentin IFs are seen in XLKE-A6 cells. (B,C) Epifluorescence (B) and phase-contrast (C) pictures of the same field of an XLKE-A6 cell monolayer after treatment with $10^{-7}$ M-colcemid for 4 h, showing aggregates of collapsed IF in the perinuclear cytoplasm, as is typical for vimentin IF. (D,E) Epifluorescence (D) and phase contrast (E) pictures of a cryostat section of ovarian tissue, showing bright staining of interstitial cells as well as endothelial cells and erythrocytes of a blood vessel. Bars, 50 $\mu$m.

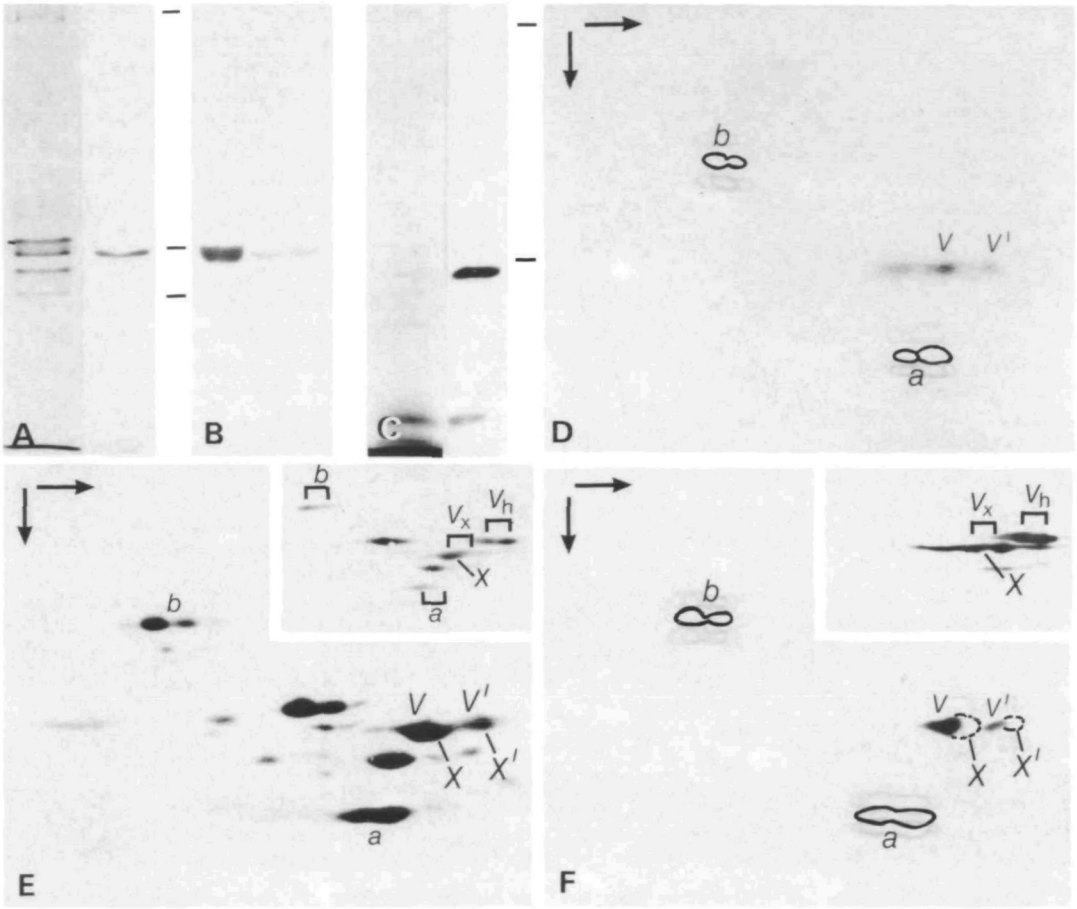



**Fig. 2.** Identification of vimentin in preparations of high-salt-buffer- and detergent-resistant cytoskeletal proteins of cultured kidney epithelial cells (XLKE-A6) and erythrocytes of *X. laevis* by gel electrophoresis and immunoblotting. (A) Coomassie brilliant blue staining (left lane) of XLKE-A6 cytoskeletal proteins and immunoblot analysis using monoclonal antibody VIM-3B4 (right lane). Positions of $M_r$ reference proteins, from hamster CHO cells, as determined in parallel, are indicated by bars (from top to bottom): $M_r$ ~300 000 (IF-associated protein, plectin; Herrmann & Wiche, 1987), $M_r$ ~57 000 (vimentin), $M_r$ ~43 000 (actin). (B) Immunoblot of cytoskeletal proteins from XLKE-A6 cells (left) in comparison with those of whole stage-42 embryos (the equivalent of 10 tadpoles is loaded in the right-hand lane) with antibody VIM-3B4, showing reactivity with larval vimentin. (C) Coomassie brilliant blue staining (left lane) and immunoblot using monoclonal antibody VIM-3B4 (right lane) of total proteins of *Xenopus* erythrocytes. Freshly harvested erythrocytes were directly lysed in hot SDS–electrophoresis sample buffer (Laemmli, 1970) containing 5 mM-EDTA to minimize proteolysis. Bars indicate (from top to bottom) the positions of spectrin and vimentin. (D) Immunoblot analysis of total red blood cell proteins separated by two-dimensional gel electrophoresis with antibody VIM-3B4 (first dimension: isoelectric focusing, direction denoted by horizontal arrow; second dimension: SDS–PAGE, vertical arrow). Proteins were prepared as in C and diluted with lysis buffer according to Garrels (1979) before isoelectric focusing. Positions of coelectrophoresed reference proteins are indicated: *a*, actin; *b*, bovine serum albumin; *V*, vimentin; *V'*, major acidic vimentin variant. (E,F) Coomassie brilliant blue staining (D) and corresponding immunoblot reaction (E) of salt-resistant cytoskeletal proteins XLKE-A6 separated by two-dimensional gel electrophoresis. In E, proteins transferred to nitrocellulose paper were detected by Ponceau S staining after the immunoreaction in order to allow correct designation of polypeptide spots immunoreactive with antibody VIM-3B4. Note that only polypeptide spots designated *V* and *V'* are positively identified by the vimentin antibody whereas the components designated *X* and *X'* are not recognized. The inserts demonstrate coelectrophoresis of vimentin from *Xenopus* ($V_x$) and hamster ($V_h$).

immunoblot reaction did not fully coincide with the Coomassie blue staining (Fig. 2E), as was best revealed by staining immunoblots with alkaline phosphatase-conjugated secondary antibodies followed by protein staining with Ponceau S. From the major, more basic Coomassie blue-stained spot only the left part was positive (designated *V* in Fig. 2E,F) whereas the more acidic variant (*X* in Fig. 2E,F) was completely negative. The more acidic, immunoreactive variant (*V'* in Fig. 2D–F) was positioned between *X* and *X'* (Fig. 2E) whereas spot *X'* was negative with the vimentin antibody. These results were confirmed by immunoblots with guinea pig antiserum to vimentin, followed by $^{125}$I-protein A reaction and Ponceau S-protein staining. Reprobing these blots with antibody VIM-3B4 showed a superposition of both reaction sites (data not shown).

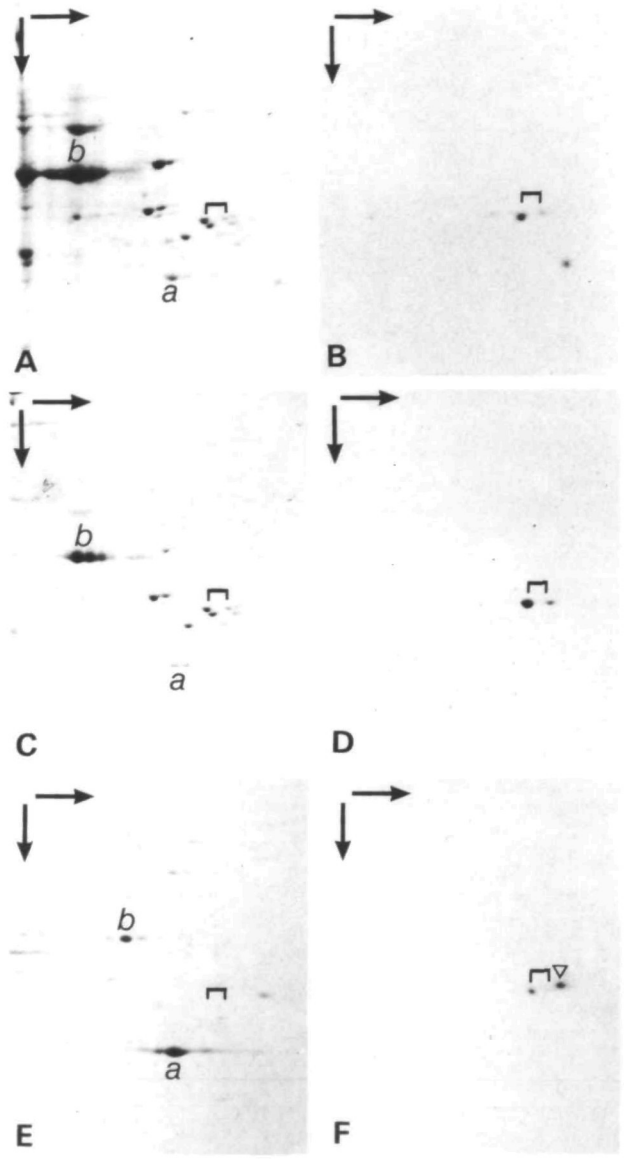



**Fig. 3.** Identification of the polypeptides encoded by the cDNA clones pXenVim1 and pXenVim4. Coelectrophoresis of the high-salt-buffer-resistant cytoskeletal proteins of XLKE-A6 cells (same system and symbols as in Fig. 2C,D) with products of *in vitro* translation from hybrid-selected embryonal stage-18 mRNA (A,B) and from mRNA obtained by *in vitro* transcription/translation of pXenVim1 (C,D). Coelectrophoresis of the mixed products obtained from *in vitro* transcription/translation of pXenVim1 and pXenVim4, respectively (E,F). (A,B) Coomassie-brilliant-blue-stained gel (A) and corresponding autoradiograph (B), showing that the [$^{35}$S]methionine-labelled *in vitro* translation products from hybrid-selected mRNA comigrate with unlabelled vimentin (bracket) of XLKE-A6 cells. (C,D) Coomassie-brilliant-blue-stained gel (C) and corresponding autoradiograph (D) of the product obtained by *in vitro* transcription and translation, showing that the [$^{35}$S]methionine-labelled translation product comigrates with unlabelled vimentin (bracket) of XLKE-A6 cells. Note that some modification, probably phosphorylation, also takes place in the rabbit reticulocyte assay used for *in vitro* translation, resulting in the appearance of a minor, more acidic variant. (E,F) Coomassie-brilliant-blue-stained gel (E) and corresponding autoradiograph (F) of mixed *in vitro* transcription/translation products of *Xenopus* vimentin clones pXenVim1 and pXenVim4. As revealed from parallel gels of individual [$^{35}$S]methionine-labelled translation products, pXenVim4 is translated into a polypeptide slightly more acidic and less mobile (triangle) than pXenVim1 (bracket). Fluorography was for 4 h. After prolonged exposure acidic variants were also visible.

Our results also show that in XLKE-A6 cytoskeletons two non-vimentin polypeptides with similar electrophoretic properties occur which might represent a yet unidentified IF protein, probably a type I cytokeratin (cf. Franz & Franke, 1986).

On two-dimensional coelectrophoresis of XLKE-cytoskeletal proteins with hamster vimentin, *Xenopus* vimentin displayed a slightly higher electrophoretic mobility in SDS and a lower isoelectric point (inserts in Fig. 2E and F; note that the antibody VIM-3B4 recognizes vimentin from both species equally well). Moreover, two-dimensional gel electrophoresis of partly degraded vimentin from both *Xenopus* and hamster further showed that this antibody reacted with the $M_r \sim 38\,000$ $\alpha$-helical rod fragment (data not shown). This indicates that the epitope recognized by VIM-3B4 lies in a highly conserved region of the vimentins that is not present in desmin or other IF proteins.

(B) *Characterization of cDNAs for* Xenopus *vimentin*

Using a cDNA for hamster vimentin, pVim2 (Quax-Jeuken *et al.* 1983), we screened a library in $\lambda$gt10 from *Xenopus* stage-17 embryonal RNA. From 18 positive plaques carried through three rounds of screening, two groups of clones were distinguished: one group comprising 11 clones with intense reactions and another group of 7 clones with less intense but significant signals. One clone (pXenVim1) from the first group with an insert of approximately 1·8 kb was subcloned into the Bluescript vector and used in hybrid selection experiments with total RNA from stage-18 *Xenopus* embryos. The clone selected a mRNA encoding a polypeptide indistinguishable from authentic XLKE-A6 *Xenopus* vimentin on two-dimensional gel electrophoresis (Fig. 3A,B). Moreover, the polypeptide obtained from this clone by transcription and translation *in vitro* comigrated with the major Coomassie blue-stained spot of XLKE-A6 vimentin upon two-dimensional gel electrophoresis (Fig. 3C,D). Unexpectedly, one of the four clones selected (pXenVim4) yielded a translation product that, on two-dimensional gel electrophoresis, was slightly more acidic (Fig. 3F) and slightly less mobile, compared to the pXenVim1-coded product (Fig. 3F). This indicated that at least two different vimentins exist in *Xenopus laevis* that are both coexpressed in stage-17 embryos. Since these clones yielded nearly identical restriction maps with various restriction enzymes, *Pst*I fragments of these clones were subcloned into M13 vectors and sequenced. Partial sequence comparison with the hamster cDNA sequence confirmed that we had isolated *X. laevis* vimentin cDNA clones.

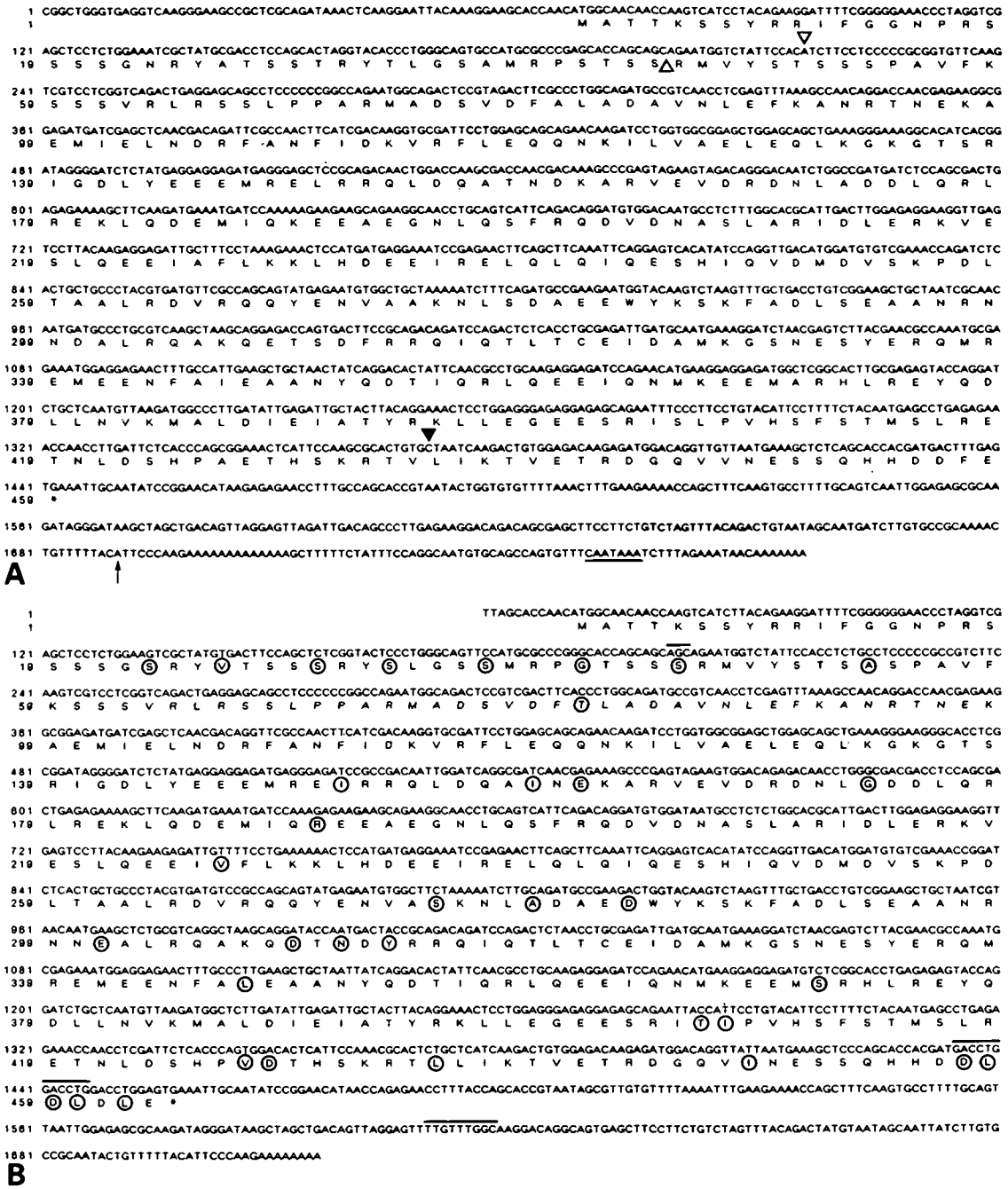


**Fig. 4.** Nucleotide sequence and deduced amino acid sequence of cloned *Xenopus laevis* vimentins. (A) Combined sequence of clones pXenVim1, containing nucleotides 1–1688 (indicated by an arrow), and pXenVim3, containing nucleotide 1367 (indicated by an arrowhead) to the 3′-end of the clone. The clones are identical in the overlapping region. Differences found in the pXenVim2 sequence are indicated by an upward triangle (insertion of an AGC triplet coding for serine) and a downward triangle (substitution of T for C without change of the coded amino acid). (B) Sequence of clone pXenVim4. Major differences of the nucleotide sequence to pXenVim1 are overlined and the amino acid changes are encircled.

(C) *Nucleotide sequences and deduced amino acid sequences*

The two clones, pXenVim1 and pXenVim4, were fully sequenced. The cDNA sequence (1688 bp) of pXenVim1 and the deduced amino acid sequence are shown in Fig. 4A. The first ATG codon is found in the sequence AACATGG known to be optimal for initiation by eukaryotic ribosomes (Kozak, 1986). The reading frame ends after 1374 nucleotides at a TGA codon, thus defining a polypeptide of 458 amino acids, including the initial methionine, corresponding to a total molecular weight of 52 812. This value is somewhat lower than that estimated from SDS–PAGE, as observed for all other vimentins sequenced thus far (Quax *et al.* 1983; Ferrari *et al.* 1986; Zehner *et al.* 1987). The sequence of a second clone, pXenVim2, was determined in parts. At

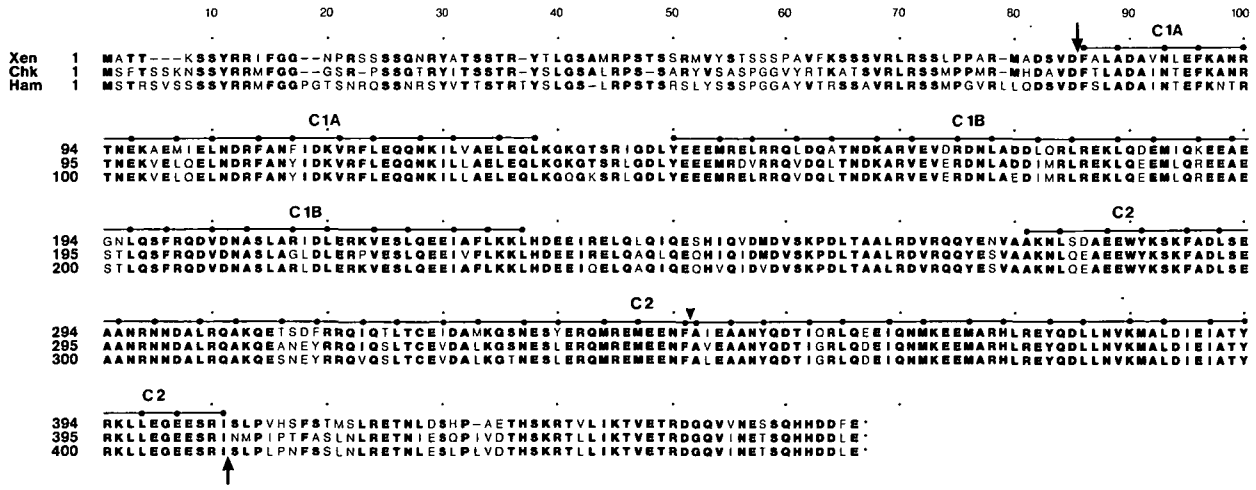



**Fig. 5.** Amino acid sequence comparison of *Xenopus* vimentin Vim 1 (Xen), chicken vimentin (Chk; taken from Zehner *et al.* 1987) and hamster vimentin (Ham; taken from Quax *et al.* 1983). Bold-faced letters denote amino acids identical in *Xenopus* and at least one of the other two species. Amino acid sequences have been aligned for maximal homology, insertions introduced for this purpose are denoted by horizontal bars. The downward arrow demarcates the start and the upward arrow the end of the $\alpha$-helical rod domain. The dots represent positions a and d of the heptade convention to maximize coiled-coil configuration. The rod domain contains two non-$\alpha$-helical interruptions of 11 and 43 amino acids, respectively, giving rise to coiled-coil subdomains 1A (C1A), 1B (C1B) and 2 (C2). The arrowhead indicates an alteration in the heptade pattern that probably results in a 'stutter' in coil 2.

the 5'-end, this clone starts 30 bp more downstream than pXenVim1, contains an additional AGC codon (42 codons after the ATG; Fig. 4A, upward triangle) and a conservative base change from T to C 18 bases downstream from this AGC (see Fig. 4A, downward triangle). In this clone, the 3'-untranslated region is 58 bp longer but identical with pXenVim1 in the overlapping sequence. Otherwise, the two clones were identical for several hundred base pairs (data not shown). Another clone, pXenVim3, was found to be identical with pXenVim1 and pXenVim2 in the 3'-end up from the nucleotide marked by an arrowhead (Fig. 4A) but extended further downstream to a AATAAA polyadenylation signal, which was followed 14 nucleotides further by seven adenosine residues assumed to represent the begin of the poly(A)tract. As this clone was identical with pXenVim1 in all nucleotides comparable, we have combined the two sequences to complete the vimentin sequence as shown in Fig. 4A.

In contrast, clone pXenVim4 differed considerably from pXenVim1. Clone pXenVim4 contained the whole coding sequence, starting 11 nucleotides before the presumptive initiation codon. The amino acid sequence of Vim4 varied in a total at 31 positions (encircled in Fig. 4B) which were spread rather evenly throughout the molecule: 8 in the head, 16 in the rod, and 7 in the tail. Nearly all changes were conservative. In addition, vimentin Vim4 contained a 4-amino-acid insertion three residues before the carboxy terminus. Overall, this vimentin, Vim4, is 92% identical to Vim1 at the amino acid level, but only 62% homologous to *Xenopus* desmin (compare Herrmann *et al.* 1988). Furthermore, its sequence is identical with the recently published 74 amino acid sequence derived from the genomic clone XIF1 (Sharpe, 1988), in contrast to our XenVim1 sequence which differs from XIF1 in two positions. In the 3'-untranslated region pXenVim1 and pXenVim4 are nearly identical, except for a 9/18 nucleotide mismatch (overlined in Fig. 4B) and several single base substitutions.

Comparison of the deduced amino acid sequence of pXenVim1 with the sequences of vimentin of chicken (Zehner *et al.* 1987) and hamster (Quax *et al.* 1983) revealed that *Xenopus* vimentin is highly homologous to the avian and mammalian proteins (Fig. 5): 85 and 86% of the amino acids of the $\alpha$-helical rod domain of *Xenopus* vimentin Vim1 (Fig. 5) are identical to those of hamster and chicken vimentin, respectively, and most of the changes are conservative. The tail domain shows 73 and 65% identity, compared to chicken and hamster, and the head 57 and 58%, respectively. Within the $\alpha$-helical rod, extended regions were identical in all three species, for example a stretch of 44 amino acids before the end of the helix. Moreover, the number of amino acids in the rod is absolutely conserved within all three species. Compared to hamster vimentin, the head of the *Xenopus* vimentin Vim1 is shorter by 6 amino acids and the tail is shorter by one. In total, *Xenopus* vimentin Vim1 is 7 amino acids, and Vim4 two amino acids, smaller than hamster vimentin, in agreement with their slightly higher SDS-PAGE mobility.

### (D) *Conservation of sequences in the 3'-untranslated region*

It has been previously noted that the 3'-untranslated regions of the hamster and chicken vimentin genes share a high degree of identical sequences, with a polyadenylation signal approximately 300 nucleotides after the stop codon (Quax *et al.* 1983). However, this

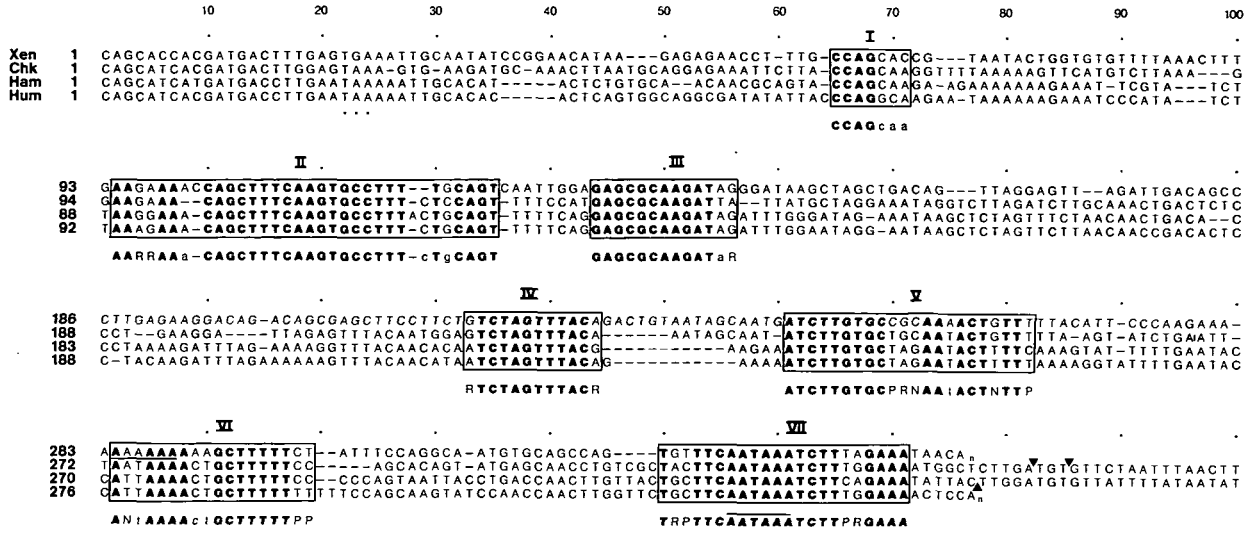


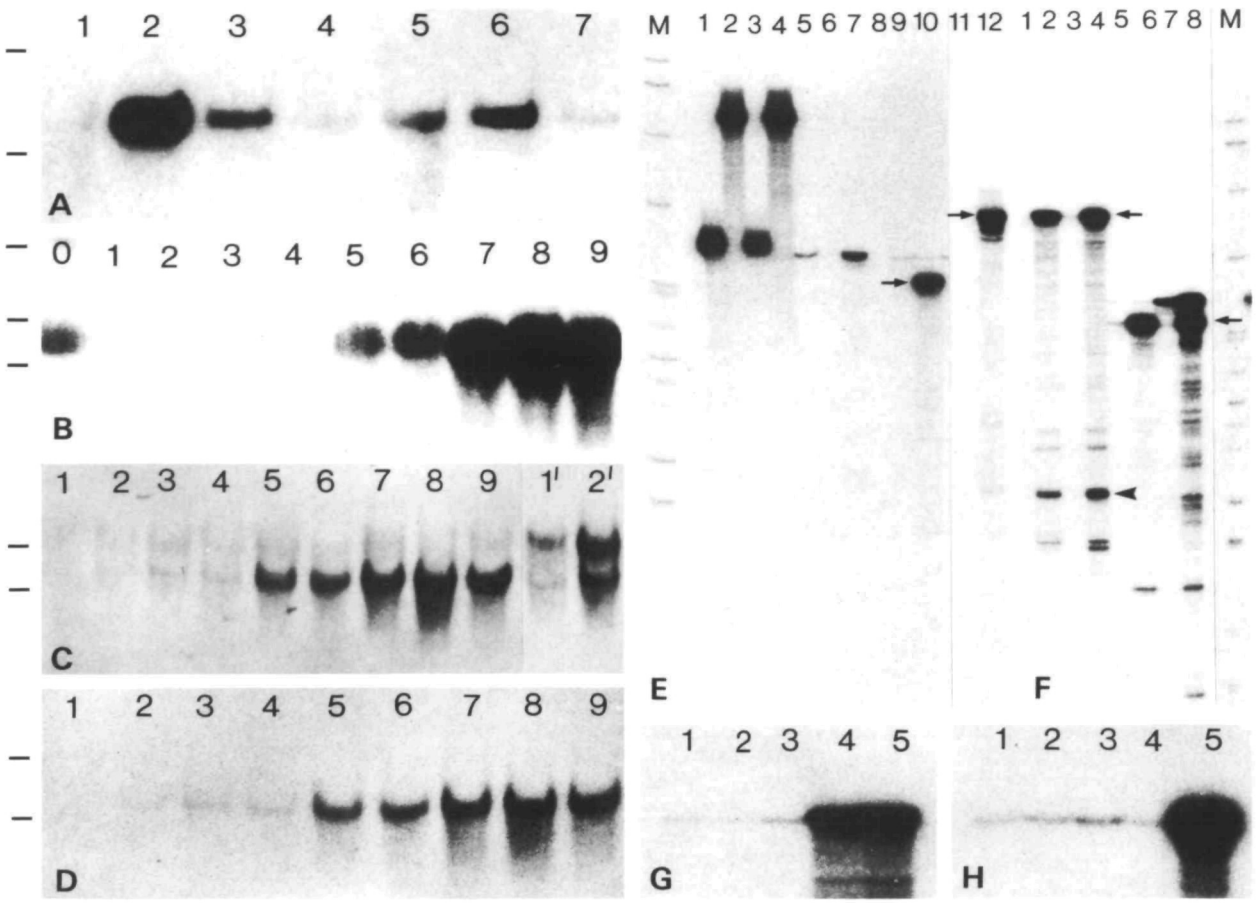
**Fig. 6.** Comparison of the 3′-untranslated sequences of vimentin mRNAs of *Xenopus* (Xen), chicken (Chk; from Zehner *et al.* 1987), hamster (Ham; from Quax *et al.* 1983) and human (Hum; this study) as determined from cDNAs and genes, respectively. Sequences have been aligned for maximal homology and insertions introduced for this purpose are denoted by horizontal bars. The three stars mark the ends of the coding region. Regions of relatively high homology are boxed and numbered by roman numerals. Bold-faced letters denote nucleotides present in all four sequences. The apparent consensus sequences are indicated underneath each box; Lower case letters indicate the presence of at least three identical nucleotides. P, pyrimidine nucleotide; R, purine nucleotide. The arrowheads indicate the probable polyadenylation sites in the genomic sequences for chicken (Zehner & Paterson, 1983) and hamster vimentin (Quax *et al.* 1983). Note that the presumptive polyadenylation signal (overlined) in box VII is flanked by a highly conserved sequence.

type of homology was not found in the human vimentin sequence reported by Ferrari *et al.* (1986) in which a region of 57 nucleotides precedes a poly(A) tail without a classical polyadenylation site at the approximate distance. Since the 3′-untranslated region of the *Xenopus* vimentin clones showed regions of sequence homology with the chicken and hamster genes (Fig. 6), including the distance between stop codon and polyadenylation site, we screened a human cDNA library and sequenced several vimentin-positive clones. All of them (a representative, pHumVim1, is shown in Fig. 6) differed in their 3′-untranslated region from the sequence described by Ferrari *et al.* (1986) in that they were highly homologous to the other three species (Fig. 7). On closer comparison, several clusters of high homology became apparent in all species (boxes I–VII in Fig. 6). Remarkably, the putative poly(A)-addition signal, which lies within a consensus sequence (box VII) TRPyTTCAATAAATCTTPyRGAAA, is much further downstream from the end of the human clone reported by Ferrari *et al.* (1986) who considered the AATAAA around the stop codon as the polyadenylation signal. Consensus box VI is also highly conserved and contains a second poly(A)-addition signal in the chicken sequence which, however, seems not to be used (Zehner & Paterson, 1983; cf. Capetanaki *et al.* 1983).

### (E) *Expression of vimentin in tissues and during embryogenesis*

Using Northern blot analysis with a 5′-end-specific probe for *Xenopus* vimentin, we detected a single ~2·1 kb band of vimentin mRNA in all tissues and cell cultures examined, although at very different intensities of reaction (Fig. 7A). This size is larger than that of the two cDNA clones (see above), probably due to the absence of most of the poly(A) sequence and also some untranslated 5′ sequence in the latter. The signals were very low in tissue samples from oesophagus (lane 1), skeletal muscle (lane 4) and ovary (lane 7), markedly higher in skin (lane 3), cardiac tissue (lane 5) and liver (lane 6), and most prominent in XLKE-A6 cell cultures (lane 2).

Northern blot analysis was also done with RNAs from various stages of *Xenopus* embryos using glyoxal for denaturation and an antisense riboprobe corresponding to the complete pXenVim1 and very stringent washing conditions (72 °C; Fig. 7B). Under these stringency conditions, no significant reaction was obtained with RNAs from unfertilized eggs and early embryos (stages 6·5, 9 and 11; Fig. 7B, lanes 1–4). From stage 14 on (lane 5), a signal was found which increased in intensity up to stage 39 (lanes 5–8) and then remained constant in swimming tadpoles (lane 9). When these embryonic RNA samples were examined at somewhat lower stringency (65 °C) using formaldehyde for RNA denaturation, very weak signals at the position of vimentin mRNA were detected in stage-6·5 to -11 embryos (Fig. 7C, lanes 2–4) and, on longer exposure, also in eggs (Fig. 7C, lanes 1 and 1′). However, the intensities of the signals at the position of vimentin mRNA that were obtained with eggs and stage-6·5 to -11 embryos were similar to those seen at the positions of 28S rRNA present in the same gels (Fig. 7C, lanes 1–4 and 1′, 2′). Therefore, we treated the blots exten-




sively with RNase A, upon which only the reaction at 2·1 kb, i.e. the position of vimentin mRNA, was retained in mRNA samples from stages 6·5 to 11 (Fig. 7D, lanes 2–4).

For further clarification and quantification we also performed RNase protection experiments, using a 3'-specific vimentin probe as well as a probe representing nucleotides 1061 to 1302 of pXenVim1, i.e. the nucleotide sequence most highly conserved among the diverse IF protein genes ('TYRKLEGE-probe'). At low and moderate stringency (45° and 60°C) both probes were specific for vimentin mRNA under the conditions used (Fig. 7E, lanes 10 and 12). Notably, *in vitro* synthesized mRNA for cytokeratin 1/8, which is known to be abundantly present in eggs and early embryos (Franz & Franke, 1986), was not protected at all (Fig. 7E, lanes 9 and 11). With both vimentin probes we observed protection by RNA of eggs (Fig. 7F, lanes 1, 3, 5, 7) and stage-18 embryos (lanes 2, 4, 6, 8). Compared to egg RNA, however, a more than hundredfold higher protection was found with stage 18 RNA, as estimated from determinations of the radioactivity of the protected bands. These results were confirmed and extended by two sets of experiments performed at higher stringency (65°C; 50 and 80% formamide), which showed very weak but significant protection bands in oocytes, unfertilized eggs and embryos of stage-6·5 and -9 embryos (Fig. 7G, lanes 1–3 and Fig. 7H, lanes 1–4). These reactions, however, were more than hundredfold lower than those obtained with mRNAs from stage-18 (Fig. 7G, lane 4), and -28 (lane 5 in Fig. 7G,H) embryo. In addition, we noticed a conspicuous protected band of approximately 160 nucleotides (Fig. 7F, lane 4, arrowhead) which probably presents the intercept of nucleotides 1447 and 1608 of pXenVim4 (cf. Fig. 4) flanked by mismatch regions between Vim1 and Vim4.

(F) *Localization of vimentin in ovaries and early embryos*

Immunofluorescence microscopy on frozen sections of several tissues of adult *Xenopus laevis* showed, with monoclonal antibody Vim-3B4 and the vimentin-specific guinea pig antibodies, positive reactions in cells of connective tissue, endothelium and erythrocytes, whereas skeletal and cardiac muscle as well as various smooth muscles were negative (Fig. 1D and data not shown). In ovarian tissue, the interstitial cells, including endothelial cells and erythrocytes, were strongly stained (Fig. 8A–D). In contrast, vimentin was not detected either in vitellogenic oocytes (Fig. 8A,B) or in previtellogenic oocytes (Fig. 8C,D). Essentially, the same results were obtained when the immunofluorescence reaction was performed on TCA-fixed ovaries (data not shown). Monoclonal antibody PK V1 which reacts with mammalian vimentin in immunoblots and in perimitotic filament configurations (cf. Lehtonen *et al.* 1983; Franke *et al.* 1984), was totally negative on *Xenopus* tissues (data not shown).

**Fig. 7.** Detection of vimentin-specific mRNA in various tissues and cells of adult animals and in embryonic stages of *Xenopus laevis* by RNA blot analysis (A–D) and RNase protection assays (E–H). (A) RNA was prepared from various tissues (Davis *et al.* 1986), and 20 $\mu$g total RNA (lanes 1, 4 and 5) or 1 $\mu$g poly(A)$^+$ RNA (lanes 2, 3, 6 and 7) were applied for formaldehyde/agarose gel electrophoresis. Hybridization was carried out with a random-primed, $^{32}$P-labelled ($0{\cdot}8\times10^6$ cts min$^{-1}$ ml$^{-1}$), 5′-end-specific probe of pXenVim1. Lane 1, oesophagus; lane 2, XLKE-A6 cells; lane 3, skin; lane 4, skeletal muscle; lane 5, cardiac muscle; lane 6, liver; lane 7, ovary. Horizontal bars indicate the positions of *Xenopus* 28 S and 18 S rRNA. Exposure: 24 h. (B) RNA was prepared from ovary of adult frogs and embryos of the indicated developmental stages, 20 $\mu$g total glyoxylated RNA were loaded per lane. RNA was blotted to Genescreen plus and hybridized with a $^{32}$P-labelled ($3\times10^6$ cts min$^{-1}$ ml$^{-1}$) anti-sense RNA probe from pXenVim1. The final washes were with 0·1×SSC, 0·5 % SDS at 72 °C. Lane 0, ovary; lane 1, unfertilized eggs; lane 2, stage 6·5 (morula); lane 3, stage 9 (fine cell blastula); lane 4, stage 11 (gastrula); lane 5, stage 14 (neural plate stage); lane 6, stage 18 (neural groove stage); lane 7, stage 28; lane 8, stage 39; lane 9, stage 42 (swimming tadpole). Horizontal bars indicate the positions of bovine 28 S, *E. coli* 23 S and bovine 18 S rRNAs. Exposure: 4 h. Because of the higher activity of the riboprobe, the signal obtained for ovarian tissue appears enhanced, compared to that shown in A, lane 7). (C) RNA as loaded in B was run on a formaldehyde/agarose gel, blotted to nitrocellulose and hybridized with $^{32}$P-labelled probe as in B. The final washes were with 0·1×SSC, 0·1 % SDS at 65 °C. Lane 1, unfertilized eggs; lane 2, stage 6·5; lane 3, stage 9; lane 4, stage 11; lane 5, stage 14; lane 6, stage 18; lane 7, stage 28; lane 8, stage 39; lane 9, stage 42. Horizontal bars indicate the positions of *Xenopus* 28S and 18S rRNA. Exposure: 5 h. After 64 h exposure, the signals were considerably enhanced as shown in lanes 1′ and 2′ which correspond to lanes 1 and 2. Note the reaction with 18S and 28S rRNAs. (D) Blot shown in C after ribonuclease A treatment (20 $\mu$g ml$^{-1}$ for 20 min at 25 °C followed by 65 °C washes with 0·1×SSC, 0·1 % SDS). Exposure: 10 days. Note that the signal at the position of the rRNA disappeared whereas that at the position of vimentin mRNA persisted (lanes 2–9). (E) Autoradiogram of a ribonuclease protection assay with 1 $\mu$g sense RNA generated by *in vitro* transcription from pXenVim1 (lanes 10 and 12) and from the cytokeratin clone pXenCK 1/8 (lanes 9 and 11), showing that the sense RNA of pXenVim1, but not that of pXenCK1/8, specifically protects the $^{32}$P-labelled anti-sense vimentin probes (arrows in lanes 10 and 12). Hybridizations shown here were carried out at 45 °C (lanes 1, 2, 7 and 8) or 60 °C (all other lanes) for 15 h with $1\times10^5$ cts min$^{-1}$ (~1 fmole) of the uniformly labelled 3′-specific probe (lanes 2, 4, 6, 8, 11 and 12) or the 'TYRKLEGE-probe' (see Materials and methods; lanes 1, 3, 5, 7, 9 and 10). To control for stringency, the probes were hybridized with 10 $\mu$g tRNA (lanes 1–8) and either subjected to RNAse treatment (lanes 5–8) or processed further without RNAse treatment (lanes 1–4). Lane M shows *Hpa*II-restricted pBR 322 size markers (from top to bottom: 622, 527, 403, 309, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90 nucleotides). Exposure: 1 h. (F) Autoradiogram of a ribonuclease protection assay with 5 $\mu$g total *Xenopus* RNA from unfertilized eggs (odd numbers) and stage 18 (even numbers) using the 3′-specific probe (lanes 1–4) or the 'TYRKLEGE probe' (lanes 5–8). Hybridization was at 45 °C (lanes 3, 4, 7 and 8) or 60 °C (lanes 1, 2, 5 and 6). Arrows mark the fully protected probes without polylinker sequences. The arrowhead in lane 4 marks a prominent protected fragment of 160 nucleotides. Exposure: 3 days. (G) Autoradiogram of a ribonuclease protection assay with 5 $\mu$g total RNA from oocytes (lane 1), unfertilized eggs (lane 2), stage-6·5 (lane 3), stage-18 (lane 4) and stage-28 embryos (lane 5) hybridized to the 3′-specific probe at 65 °C. Exposure: 3 days. (H) Autoradiogram of a ribonuclease protection assay with 5 $\mu$g total RNA from oocytes (lane 1), unfertilized eggs (lane 2), stage-6·5 (lane 3), stage-9 (lane 4) and stage-28 embryos (lane 5) hybridized to the 3′-specific probe at 65 °C in buffer containing only 50 % formamide as compared to 80 % as used in panels E–G. Exposure: 3 days.

When embryonic stages were examined by immunofluorescence microscopy, cells with significant vimentin immunostaining were not seen before gastrulation. In stage-14 ('neural plate') embryos, groups of cells positive for vimentin were detected that appeared to belong to two thin cell layers located below the neural plate (Fig. 9) whereas the ectoderm, the notochord, the neural plate and the presumptive somite cells were negative. At this point of development practically all cells of the embryo were positive for cytokeratins (Fig. 9B), including the vimentin-positive cells. In stage-18 (Fig. 10A) embryos, the vimentin-positive cells were still restricted to a mesenchymal cell layer underlying the neuroectoderm of the neural groove and tube. All other tissues such as somites, notochord, ectoderm, endoderm and the neural groove tissue were negative for vimentin but positive for cytokeratin (Fig. 10B).

### (G) *Screening of egg cell fractions for vimentin*

In view of the obvious discrepancy between our negative findings and the positive reports of vimentin in oocytes and eggs (Godsave *et al.* 1984*a*), we also used immunoblotting to examine the possible existence of this protein in various protein fractions of unfertilized eggs. Specifically, we studied the 'high speed supernatant' (HSS), in which some vimentin might exist in a soluble form (e.g. Blikstad & Lazarides, 1983; Soellner *et al.* 1985), and the corresponding pellet in which IF protein polymers would accumulate. When proteins of HSS fractions were analysed directly (Fig. 11A, lane 2) or after concentration by precipitation with acetone (Fig. 11A, lane 1) no immunoreaction was detected with monoclonal VIM-3B4 antibody (Fig. 11B, lanes 1 and 2) and with guinea pig antibodies to vimentin (data not shown). In controls, *Xenopus* vimentin from XLKE-A6 cells was readily detected (Fig. 11B, lanes 7). The high-speed pellet, which was applied as a sample corresponding to the material from 100 eggs, was also negative for vimentin.

High-salt-buffer- and detergent-resistant residues of low speed pellets (Fig. 11A, lane 3) contained three major Coomassie blue-stained bands with $M_r$ values of

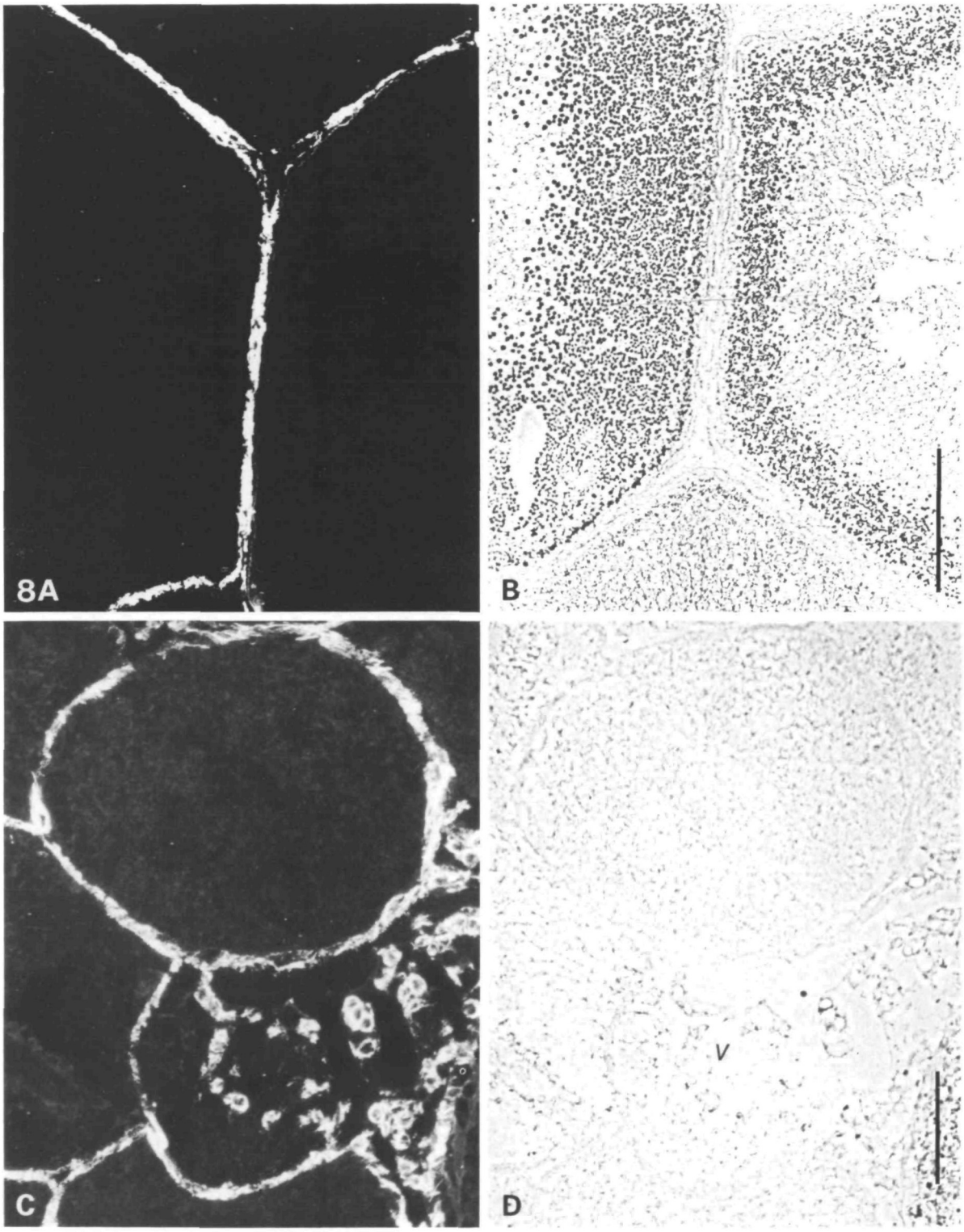



**Fig. 8.** Immunofluorescence microscopy performed on cryostat sections of frozen ovarian tissue from *Xenopus laevis* using monoclonal antibody VIM-3B4. (A,B) The same field is shown in epifluorescence (A) and phase-contrast (B) optics. Note that only the interstitial cells show a bright fluorescence, whereas the oocytes are negative. (C,D) In a region with previtellogenic oocytes (C, epifluorescence; D, phase contrast) the oocytes are negative whereas the interstitial cells, the endothelial cells and the erythrocytes of the blood vessel show intense fluorescence. *v*, blood vessel. Bars, 50 μm.

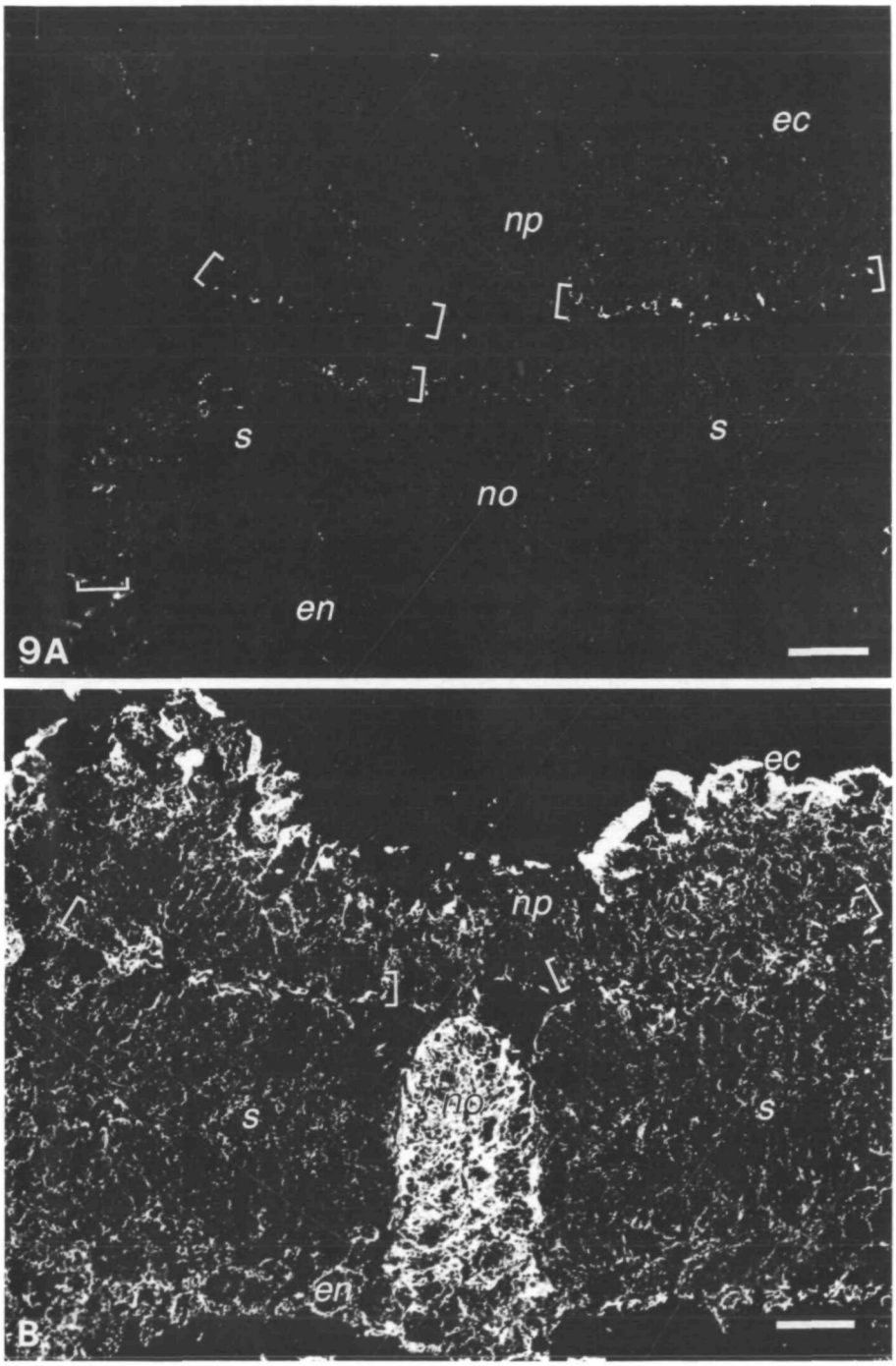



**Fig. 9.** Survey picture montage of immunofluorescence micrographs of sections through snap-frozen stage-14 embryos (neural plate stage) of *Xenopus laevis* (whole embryos were sectioned and documented in overlapping micrographs). (A) Reaction of monoclonal antibody VIM-3B4, showing positive reaction only in a distinct and thin mesenchymal cell layer (demarcated by brackets) whereas the neural plate (*np*), notochord (*no*) and somites (*s*) are negative. *ec*, ectoderm; *en*, endoderm. (B) Reaction of monoclonal cytokeratin antibody lu-5, showing an area corresponding to that shown in A in a step section. Note that all cell layers are positive for cytokeratins. In particular, the notochord (*no*) and the ectoderm (*ec*) are intensely stained. The mesenchymal cell layer (brackets) shown to be positive for vimentin in A, is also positive for cytokeratin. Symbols are as in A. Bars, 50 $\mu$m.

~66 000, 58 000 and 43 000 (see also Franz *et al.* 1983) but, at the position of *Xenopus* erythrocyte vimentin, no Coomassie blue-stained band was detected (data not shown). Similar results were obtained when unfertilized eggs were lysed directly in buffers containing high salt and detergent and pellets obtained at 800 *g* (Fig. 11A, lane 5), at 10 000 *g* (lane 4) from the resulting supernatant, and at 10 000 *g* without any precentrifugation (lane 6) were examined. Immunoblot analyses of these fractions (Fig. 11B, lanes 3–6) did not reveal any vimentin, even after extensive development of the colour reaction, resulting in unspecific staining of yolk proteins (Fig. 7B). The corresponding soluble fractions were also vimentin-negative (data not shown).

In parallel, we determined the sensitivity of this assay for vimentin using even recombinant *Xenopus* vimentin produced by *E. coli* after transformation with an expression vector carrying XenVim1. As seen in Fig. 11C and D, vimentin can be diluted from 1 $\mu$g (Fig. 11C, lane 2, and D, lane 1) to 5 ng and still be detected with the colour reaction produced by the Ig-coupled alkaline phosphatase (Fig. 11D, lane 6). This shows that the egg cytoskeletons contain less than 0·25 ng vimentin per egg, if any. The corresponding

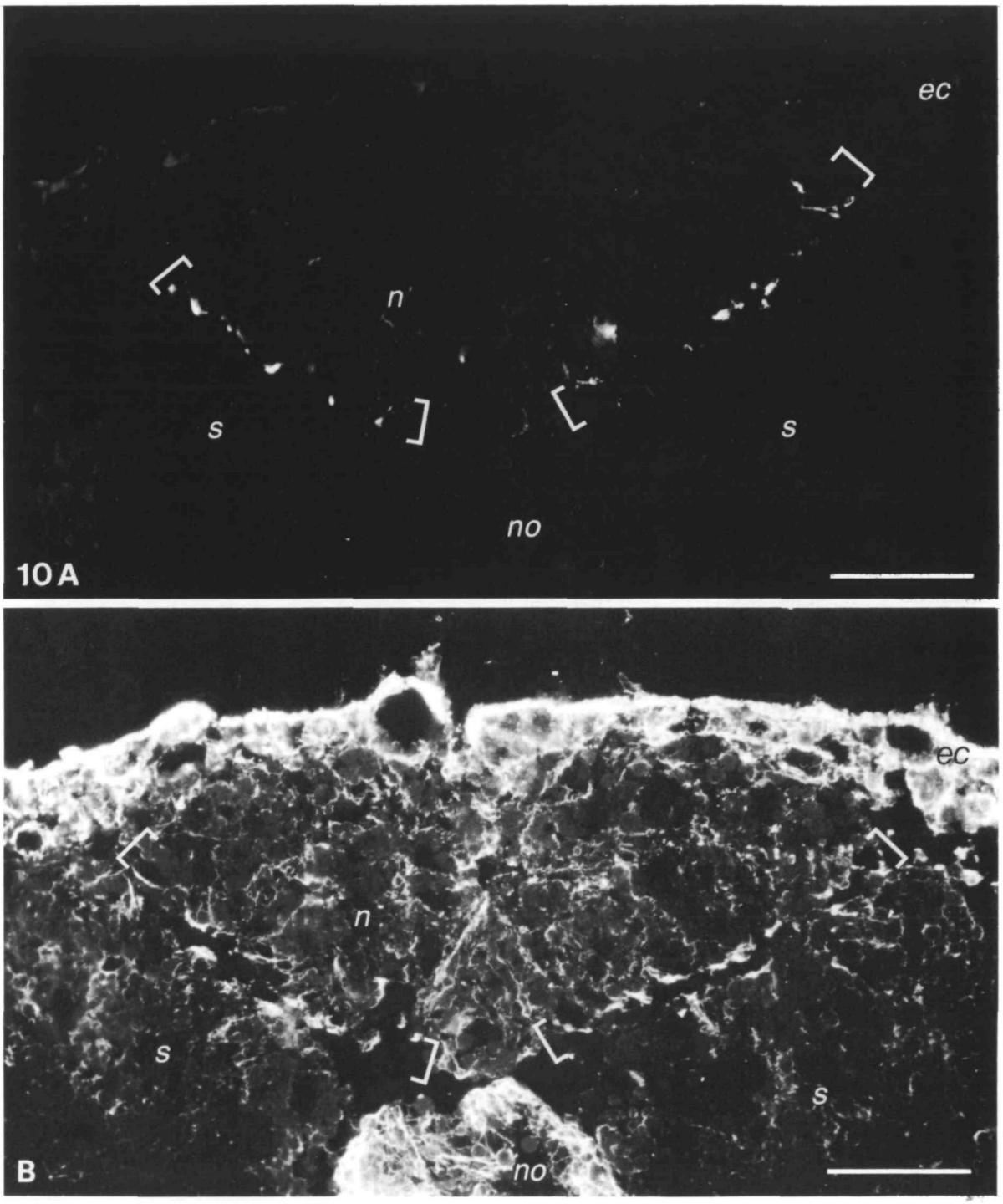



**Fig. 10.** Immunofluorescence microscopy performed on cryostat sections of snap-frozen stage-18 (neural groove stage) embryos of *Xenopus laevis* using monoclonal antibodies to vimentin (A) and cytokeratin (B). (A) Immunofluorescence micrograph of the neural groove region of a stage-18 embryo, showing the reaction of monoclonal antibody VIM-3B4. The reaction is restricted to a single cell layer (brackets) beneath the neural groove (*n*). All other cell layers are negative. *ec*, ectoderm; *s*, somite; *n*, neural groove; *no*, notochord. (B) Reaction of monoclonal antibody lu-5, on a step section of the same region shown in A. Note that all cell layers show a positive reaction, the notochord (*no*) and the ectoderm (*ec*) being brightly stained and that the thin layer of cells shown to be positive for vimentin in A is also positive for cytokeratin (brackets). Symbols are as in A. Bars, 50 $\mu$m.

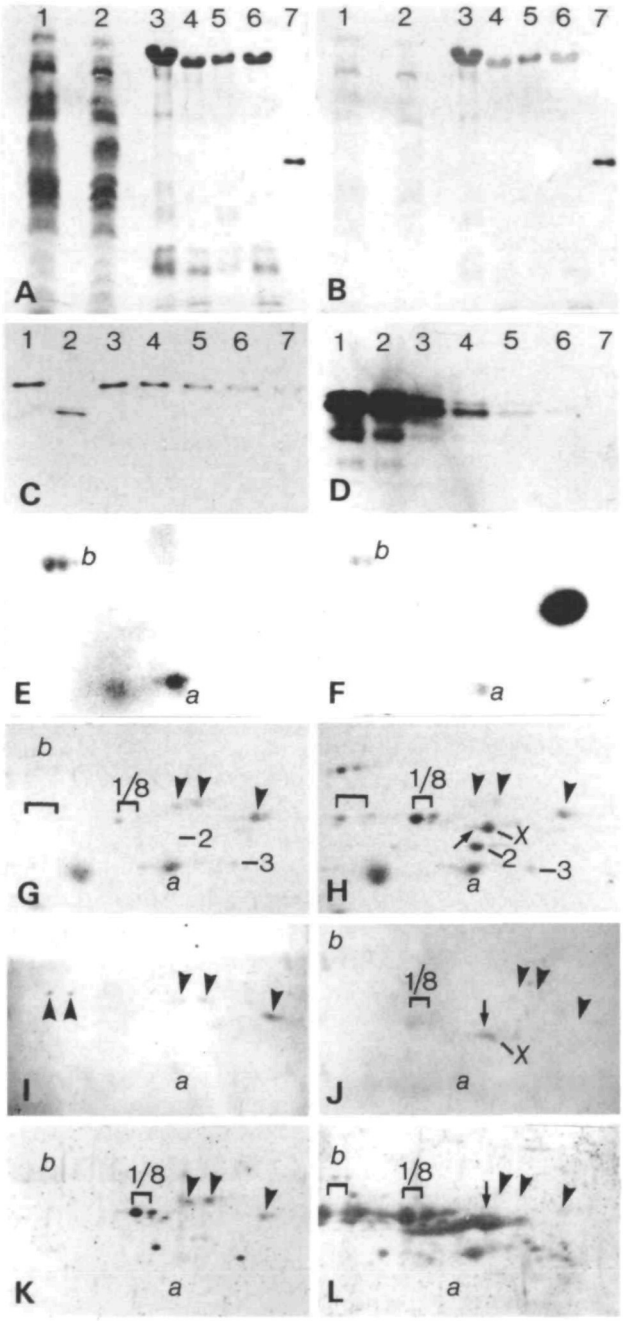



**Fig. 11.** Analysis of various fractions from oocytes and unfertilized eggs of *Xenopus laevis* for the presence of vimentin by SDS–PAGE and immunoblotting with different antibodies. (A) Proteins transferred to nitrocellulose were stained by Ponceau S after the alkaline phosphatase immunostaining reaction shown in B. Lane 1, proteins recovered in the 100 000 *g* supernatant from total egg extracts (HSS proteins), concentrated by precipitation with acetone, 30-egg equivalents; lane 2, HSS proteins directly loaded, 12-egg equivalents; lane 3, high-salt-buffer-resistant fraction of the low-speed pellet from egg homogenates; lane 4, insoluble fraction from high-salt-buffer-extracted material pelleted at 10 000 *g* after an initial 800 *g* centrifugation; lane 5, high-salt-buffer-resistant material pelleted at 800 *g*; lane 6, high-salt-buffer-resistant material pelleted at 10 000 *g*. Lanes 3–6, 20-egg equivalents. The prominent high molecular weight band in lanes 3–6 contains the abundant major yolk protein. Lane 7, total cytoskeletal proteins of XLKE-A6 cells, 2 μg, representing approximately 0·2 μg vimentin (see Fig. 2A). (B) Immunoreaction corresponding to A, developed until background staining appeared, as shown by the unspecific reaction of the $M_r$ ~100 000 yolk protein band (lanes 3–6) which was also seen when blots were only incubated with second antibody. Specific vimentin reaction is only seen in XLKE-A6 cells (lane 7). (C,D) Calibration of the sensitivity of antibody VIM-3B4 for the detection of *Xenopus* vimentin. (C) For quantification, *Xenopus* vimentin synthesized in *E. coli* transfected with clone pXenVim1 (cf. Magin *et al.* 1987) was dissolved in electrophoresis sample buffer, and a 10 μl sample was applied to the gel (lane 2). For comparison, bovine serum albumin was loaded as follows: lane 1, 1·4 μg; lane 3, 1·2 μg; lane 4, 1 μg; lane 5, 0·8 μg; lane 6, 0·6 μg; lane 7, 0·4 μg. As estimated after staining with Coomassie brilliant blue, the applied amount of vimentin corresponded to approximately 1 μg of bovine serum albumin. (D) Immunoblot analysis of recombinant *Xenopus* vimentin diluted to various degrees: lane 1, 1 μg; lane 2, 500 ng; lane 3, 100 ng; lane 4, 50 ng; lane 5, 10 ng; lane 6, 5 ng; lane 7, 1 ng. Note faint reaction in lane 6, showing that 5 ng are detectable. The band of somewhat higher SDS–PAGE mobility is proteolytically trimmed vimentin of $M_r$ ~38 000. Blots shown in B and D have been processed in parallel. (E,F) Examination of cytoskeletal fraction from unfertilized eggs with guinea pig antibodies to vimentin (E), in comparison with reference vimentin (F). (E) Autoradiogram of an immunoblot analysis of proteins from 15 unfertilized eggs separated by two-dimensional gel electrophoresis, followed by incubation of nitrocellulose blots with guinea pig antibodies to vimentin and $^{125}$I-labelled protein A. (F) Parallel experiment to that shown in E with cytoskeletal proteins from Chinese hamster ovary fibroblasts containing approximately 1 μg vimentin. Both blots were exposed for 3 days at 70°C using intensifying screens. (G–L) Examination of the presence of vimentin in oocytes of *Xenopus* by two-dimensional gel electrophoresis, followed by immunoblotting. (G) Cytoskeletal proteins from 150 mg oocytes were separated by two-dimensional gel electrophoresis, transferred to nitrocellulose and stained with Ponceau S. The bracket denotes the major oocytes type II cytokeratin 1/8, the three arrowheads denote a triplet of unidentified cytoskeletal proteins of $M_r$ 56 000–60 000. Positions of cytokeratin 2 and 3, corresponding to human cytokeratin 18 and 19 and a group of basic cytokeratins of $M_r$ ~56 000 are also indicated. (H) The same amount of protein as shown in G was mixed with cytoskeletal proteins from XLKE-A6 cells for the identification of the position of *Xenopus* vimentin (arrow) relative to the oocyte proteins. Symbols as in G. (I) Immunoblot analysis of cytoskeletal oocyte proteins with monoclonal antibody PK V1. Note absence of staining at the position of vimentin (arrows in H and J). Weak staining of five polypeptides of $M_r$ 56 000–60 000 is indicated by arrowheads. (J) Immunoblot analysis of a mixture of cytoskeletal proteins from oocytes and XLKE-A6 cells with monoclonal antibody PK V1. Note staining of cytokeratin 1/8 and vimentin but not component X. (K) Immunoblot analysis of cytoskeletal oocyte proteins with antibody anti-IFA. Note staining of cytokeratin 1/8 but absence of staining at the position of vimentin. Note weak staining of triplet polypeptides of $M_r$ 56 000–60 000. (L) Immunoblot analysis of a mixture of cytoskeletal proteins from oocytes and XLKE-A6 cells with anti-IFA. Vimentin as well as several cytokeratins are heavily stained, thus demonstrating that anti-IFA reacts with *Xenopus* vimentin. Comparison of L and K shows absence of detectable vimentin in K.

value for HSS fractions was even lower (≤0·17 ng per egg). The guinea pig antibodies to vimentin also did not react with any protein of cytoskeletal fractions from unfertilized eggs, even after extremely prolonged exposure (Fig. 11E,F).

To examine these negative results obtained with monoclonal antibody VIM-3B4 and with guinea pig antibodies to vimentin, we used two further monoclonal antibodies recommended for the detection of vimentin, i.e. antibodies PK V1 and anti-IFA. Fig. 11G–L show the immunoblot results obtained with cytoskeletal proteins from oocytes (Fig. 11G,I,K; results with egg cytoskeletons are similar, not shown) in comparison with mixtures of oocytes and XLKE-A6 cytoskeletal proteins (Fig. 11H,J,L). Of the various cytoskeletal proteins present in such preparations (Fig. 11G and H show the Ponceau S-stained nitrocellulose-blotted polypeptides), antibody PK V1 reacted only weakly with authentic vimentin of XLKE-A6 cells (Fig. 11J, arrow) but this reaction was not specific as certain cytokeratins were also stained (Fig. 11J) and at prolonged exposure on oocyte polypeptides additional components were stained, including some with only slightly lower electrophoretic mobility than vimentin (Fig. 11I, arrowheads). Anti-IFA also reacted with vimentin of XLKE-A6 cytoskeletons as well as with many cytokeratins and some unidentified, cytoskeletal components, including cytokeratin 1/8 and a pair-spot component of about 60 000 (Fig. 11K,L). Both antibodies do not react with an oocyte polypeptide located at the position of vimentin. These results make clear that, in studies of *Xenopus* cells and tissues, both antibodies, PK V1 and anti-IFA, cannot be considered exclusive and sensitive probes for the unequivocal demonstration of *Xenopus* vimentin.

## Discussion

The *Xenopus laevis* cDNA clones encoding various forms of vimentin should provide valuable tools for studies of cell and tissue differentiation during amphibian development, particularly those dealing with the formation of mesenchymal and myogenic tissues. In addition, our results have allowed the unequivocal gel electrophoretic identification of the two major vimentin polypeptides of this species and a comparison of these proteins in taxonomically distant vertebrate species such as amphibia, birds and mammals. Clearly, all forms of *Xenopus* vimentin identified in this study are considerably more acidic (see also Franz *et al.* 1983) than the component tentatively identified as vimentin by Nelson & Traub (1982), which probably was one of the many cytokeratin polypeptides present in XLKE-A6 cells.

The comparison of the amino acid sequences of the *Xenopus* vimentins with those of higher vertebrates indicates a remarkably high degree of sequence conservation during vertebrate evolution. On the basis of the relatively low number of amino acid exchanges, *Xenopus* vimentin appears equally distant from the chicken and the mammalian protein, both showing 79 % identical amino acid residues. Remarkably, the sequence homology is not confined to the α-helical rod domain (86 % and 85 % identity between avian and hamster vimentin) but extends to both the head (58 % and 57 % identical residues) and the tail (65 % and 73 %). Our study also reveals the conservation, in all three species, of nucleotide sequence elements of considerable lengths (up to 32 residues) in the 3′- and, more restrictedly, the 5′-untranslated regions of the vimentin mRNAs, suggesting that important regulatory functions, in translation or transcription, are located in these regions (for previous comparisons of avian and mammalian vimentin see Quax *et al.* 1983; Zehner & Paterson, 1985; Ferrari *et al.* 1986; Zehner *et al.* 1987).

The genes encoding vimentin have been reported to be single copy genes in other species (e.g. Quax *et al.* 1984, 1985*a*; Zehner & Paterson, 1985; Zehner *et al.* 1987; Ferrari *et al.* 1986). Without doubt, our sequence data show the existence of at least three different *Xenopus laevis* vimentin forms representing different genes, which is somewhat at variance with an earlier conclusion of Quax *et al.* (1984) made on the basis of Southern blot experiments probing *Xenopus* DNA with the hamster sequence. The sequence differences observed in *Xenopus* between different cDNAs for the same kind of IF protein, as shown for vimentin (this study) and several cytokeratins (Hoffmann *et al.* 1985; Miyatani *et al.* 1986), are difficult to interpret. Probably, the three different forms of vimentin identified in the present study represent three of at least four vimentin alleles that are expected in a tetraploid species such as *Xenopus laevis* (cf. Kobel & DuPasquier, 1986).

Using immunological reactions with the sensitive and vimentin-specific monoclonal antibody VIM-3B4 and guinea pig antisera to vimentin, we have not detected significant concentrations (≥0·25 ng) of vimentin in oocytes of various stages, in eggs and in pregastrulation embryos (for reactivity of antibody VIM-3B4 with embryonic vimentin see Figs 2B, 9A and 10A). This is in agreement with our present and previous immunofluorescence observations (Franz *et al.* 1983) but seems to be at variance with Godsave *et al.* (1984*a*) who reported positive immunocytochemical and immunoblot results for vimentin in oocytes, eggs and stage-10 embryos (see also Wylie *et al.* 1986). While this article was under review, a report by Tang *et al.* (1988) appeared in which positive vimentin immunoblot results have been reported for skeletal muscle tissue and oocytes using monoclonal antibodies PK V1 and anti-IFA, which is in contrast to our results with the same and other antibodies. In contrast, we have found that PK V1 and anti-IFA also react with several other cytoskeletal polypeptides of slightly lower electrophoretic mobility but different isoelectric points. Surprisingly, the polypeptide identified as vimentin by Tang *et al.* (1988) is slightly more basic than desmin, which is also in contrast to our two-dimensional gel electrophoretic separations (see also Herrmann *et al.* 1989). At present, we cannot resolve this controversy. We have to emphasize, however, that our quantitative comparisons show that the maximal amount of vimentin that may

possibly exist in oocytes and eggs (≤0·25 ng) is more than two orders of magnitude less than the total cytokeratin present (approx. 100 ng).

Our results with Northern blot and nuclease protection assays show that the amounts of vimentin mRNAs in *Xenopus* embryos increase drastically, i.e. approximately hundredfold, upon gastrulation, i.e. between stage 9 on the one hand and stage 14 on the other. The very weak but significant reaction of a band with the same electrophoretic mobility as vimentin mRNA in RNA samples from oocytes, eggs and pregastrulation embryos suggests the occurrence of trace amounts of vimentin mRNA in these cells, in basic agreement with Tang *et al.* (1988), although the relative postgastrulation increase of vimentin mRNA appears to be lower in the latter study. The biological significance of the finding of such low concentrations of vimentin mRNA in eggs (≤0·08 pg per μg RNA, i.e. ~0·3 pg mRNA per cell) and in early embryonic development is difficult to assess, notably in relation to our findings of the presence of very little, if any, detectable protein. Similarly, trace levels of mRNAs in *Xenopus* eggs and early embryos have recently also been reported for the cell adhesion protein, N-CAM (Kintner & Melton, 1987), as well as for the acetylcholine receptor protein and cardiac α-actin, both usually considered to be muscle-specific proteins (Baldwin *et al.* 1988).

The great increase of the synthesis of vimentin mRNA, and the observed restriction of vimentin to certain cell layers, at approximately 16 h after fertilization, i.e. at the neural plate stage, indicates that the vimentin genes are among those that are transcribed with much greater intensity and frequency after gastrulation in cell-type-specific ways (Carrasco *et al.* 1984; Jonas *et al.* 1985; Dworkin-Rastl *et al.* 1986; Harvey *et al.* 1986; Kintner & Melton, 1987; Fouquet *et al.* 1988). Remarkably, this increase of vimentin is almost coincident with that of several major proteins typical of myogenesis (Mohun *et al.* 1984; Gurdon *et al.* 1985*a*,*b*; Wilson *et al.* 1986; Gurdon, 1987; Kay *et al.* 1987; Mohun & Garrett, 1987).

By immunofluorescence microscopy the first appearance of vimentin in *Xenopus* embryos is not seen in all cells, but is detected only in certain mesodermal cells of layers subjacent to the neural groove and tube. Interestingly, most if not all cells of this mesodermal layer are positive for vimentin whereas the adjacent notochord and the presumptive somite tissue are negative. Apparently, these mesenchymal cells producing vimentin are also positive for cytokeratins, similar to other situations in which these two kinds of IFs have been found to coexist in certain embryonic and fetal cells of other vertebrate species (e.g. Lane *et al.* 1983; Lehtonen *et al.* 1983*b*; Czernobilsky *et al.* 1985; Erickson *et al.* 1987; Jahn *et al.* 1987). At present we cannot decide whether the cytokeratins found in these mesenchymal cells, as in all other cells of embryos of these stages, represent residual proteins synthesized in the oocyte or in pregastrulation stages, or whether they are actually synthesized at this stage in these specific cells. Clearly, simultaneous synthesis of vimentin and cytokeratins takes place in certain adult non-epithelial tissues of *Xenopus laevis* such as endothelia and certain smooth muscles (Godsave *et al.* 1986; Jahn *et al.* 1987).

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