A whole-mount immunocytochemical analysis of the expression of the intermediate filament protein vimentin in *Xenopus*

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Summary

We have developed a whole-mount immunocytochemical method for *Xenopus* and used it to map the expression of the intermediate filament protein vimentin during early embryogenesis. We used two monoclonal antibodies, 14h7 and RV202. Both label vimentin filaments in *Xenopus* A6 cells. RV202 reacts specifically with vimentin (M_r, 55x10^3) on Western blots of A6 cells and embryos. 14h7 reacts with vimentin and a second, insoluble polypeptide of 57x10^3 M_r found in A6 cells. The 57x10^3 M_r polypeptide appears to be an intermediate filament protein immunochemically related to vimentin. In the whole-mount embryo, we first found vimentin at the time of neural tube closure (stage 19) in cells located at the lateral margins of the neural tube. By stage 26, these cells, which are presumably radial glia, are present along the entire length of the neural tube and in the tail bud. Cells in the optic vesicles express vimentin by stage 24. Vimentin-expressing mesenchymal cells appear on the surface of the somites at stage 22/23; these cells appear first on anterior somites and on progressively more posterior somites as development continues. Beginning at stage 24, vimentin appears in mesenchymal cells located ventral to the somites and associated with the pronephric ducts; these ventral cells first appear below the anterior somites and later appear below more posterior somites. The dorsal fin mesenchyme expresses vimentin at stage 26. In the head, both mesodermally-derived and neural-crest-derived mesenchymal tissues express vimentin by stage 26. These include the mesenchyme of the branchial arches, the mandibular arch, the corneal epithelium, the eye, the meninges and mesenchyme surrounding the otic vesicle. By stage 33, vimentin-expressing mesenchymal cells are present in the pericardial cavity and line the vitelline veins. Vimentin expression appears to be a marker for the differentiation of a subset of central nervous system cells and of head and body mesenchyme in the early *Xenopus* embryo.

Key words: *Xenopus*, whole-mount immunocytochemistry, vimentin, mesoderm, gliogenesis.

Introduction

Because the intermediate filament proteins of vertebrates are expressed in a cell-type-specific manner, they are useful markers of differentiation (Traub, 1985; Franke, 1987; Steinert & Roop, 1988). Seven classes of intermediate filament subunit proteins have been characterized. The nuclear intermediate filament proteins, the nuclear lamins, are found in all somatic and some germ cell types. Specific lamin proteins appear to be expressed in specific cell types (Krohn & Benavente, 1986). Of the cytoplasmic intermediate filament proteins, cytokeratins are typically expressed in epithelia, desmin in muscle, glial fibrillary acidic protein (GFAP) in astrocytic glia and the neurofilament proteins in neurones (Lazarides, 1982). A newly recognized cytoplasmic intermediate filament protein, 'clone 73', appears to be expressed in rat PC12 cells and in both the peripheral and central nervous system of the rat (see Leonard et al. 1988 and references therein).

Vimentin is characteristic of mesenchymal cells. In addition, it has been found to be coexpressed with desmin in some adult muscles (Osborn et al. 1981), with GFAP in some astroglia (Schnitzer et al. 1981), with cytokeratins in human amniotic cells (Cremer et al. 1981) and in cells of the parietal endoderm of the early mouse embryo (Lane et al. 1983). In cultured cells, vimentin is often aberrantly coexpressed with the intermediate filament protein characteristic of the tissue of origin (Franke et al. 1979; Virtanen et al. 1981).

Vimentin expression has been examined in several vertebrate embryos (Bennet, 1987). In the chick, vimentin is expressed in the neural plate before neural fold formation (Bennet, 1987). Its expression continues in these cells until it is replaced by neurofilament proteins in postmitotic neuroblasts (Tapscott et al. 1981a) or augmented by GFAP in astroglial cells (Tapscott et al. 1981b). In the mouse, vimentin expression replaces cytokeratin expression in the embryonic mesoderm shortly after these cells separate from
the ectoderm (Franke et al. 1982). After neural tube closure, vimentin is expressed throughout the mouse central nervous system (CNS; Cochal & Paulin, 1984; Bovolenta et al. 1984) and is eventually replaced by neurofilament protein expression in neurones and by GFAP expression in astroglia (Schnitzer et al. 1981; Bovolenta et al. 1984; Cochal & Paulin, 1984). Neural crest cells in both quail and mouse express vimentin (Zillier et al. 1983; Cochal & Paulin, 1984) as do the neural-crest-derived Schwann cells in rat and chick (Shaw et al. 1981; Tapscoitt et al. 1981b).

In Xenopus, there is some disagreement concerning the initial appearance of vimentin during embryogenesis. Godsave et al. (1984) and Tang et al. (1988) report that vimentin and its mRNA are present in the oocyte and throughout early embryogenesis. On the other hand, Franz et al. (1983) failed to find vimentin in the oocyte. In later embryogenesis, Godsave et al. (1986) reported that vimentin was absent in the stage-17 embryo neural tube, but present in significant amounts in a subset of neural tube cells at stage 25/26.

As part of our studies on the function of intermediate filaments, we developed a monoclonal antibody that reacts with Xenopus vimentin (Klymkowsky et al. 1987). To use this antibody to disrupt vimentin organization in the embryo, we needed a method of visualizing vimentin organization in the embryo. Section-based methods, while adequate for visualizing the distribution of specific proteins within the embryo and within specific cells, are largely inadequate for visualizing the fine details of intermediate filament organization (for example, see Klymkowsky et al. 1987). Because vimentin is expressed in cells located in the interior, rather than on the surface, of the Xenopus embryo, our previous cortical whole-mount immunocytochemical method was not applicable. Spurred on by the development of an improved clearing agent by Andrew Murray & Marc Kirschner (personal communication), we developed a whole-mount immunocytochemical method for Xenopus oocytes and embryos. We describe the development of this method and illustrate its usefulness by using it to map the expression of vimentin in the developing Xenopus embryo. In the course of these studies, we have found evidence for a 57×10^6 M, polypeptide immunologically related to vimentin. Our results indicate that vimentin is a useful marker for several early differentiation events in Xenopus.

**Materials and methods**

**Monoclonal antibody characterization**

The mouse monoclonal antibody RV202 (IgG) ascites fluid was a gift from R. O. Hynes (U. Nijmegen, The Netherlands). The mouse monoclonal antibody 14h7 (IgG) was generated in mice immunized with cell residues derived from the Xenopus kidney epithelial cell line A6 as described in Klymkowsky et al. (1987). Rabbit anti-vimentin antiserum (Hynes & Destree, 1978, see also Godsave et al. 1983, 1986) was supplied by R. O. Hynes (MIT). Both 14h7 and RV202 were initially characterized by indirect immunofluorescence microscopy of normal and nocodazole-treated methanol-fixed A6 cells. For nocodazole treatment, cells were cultured for 24 h in the presence of 10 μg ml⁻¹ nocodazole (Sigma); the effects of nocodazole were visualized using the anti-β-tubulin antibody E7 (Chu & Klymkowsky, 1987). Immunofluorescence microscopy was carried out as described in Klymkowsky et al. (1983).

Both 14h7 and RV202 were characterized by Western immunoblot analysis of whole A6 cells, A6 cell residues and embryo residues. A6 cells were grown as described by Klymkowsky et al. (1987). Whole-cell samples were prepared by washing cultures three times with cold phosphate-buffered saline (PBS) containing 10 mm-EGTA; the cells were then scraped from the plate, collected by centrifugation and solubilized in Laemmli (1970) sample buffer (SDS-sample buffer). A6 cell residues were prepared as described previously (Klymkowsky et al. 1987). Embryo residues were prepared from stage 32/35 embryos (all embryo stages according to Nieuwkoop & Faber, 1967). Embryos were homogenized in XEX buffer (XEX: 1.5 M-KCl, 0.3 M-sucrose, 10 mm-Pipes, 10 mm-β-mercaptoethanol, 5 mm-magnesium chloride, 1 mm-EGTA, 0.5% NP-40, 0.2% sodium azide, 10 μg ml⁻¹ cytochalasin B and 5 μg ml⁻¹ leupeptin (Sigma; pH7.0). The homogenate was centrifuged at 11,000 g for 15 min at 4°C. Care was taken to remove the supernatant and the yolk. The pellet was resuspended in XEX buffer, collected by centrifugation and then solubilized in SDS-sample buffer.

SDS–polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970) using a 5% stacking and separating gel and an 8 and 9% separating gel. Both stacking and separating gels contained 0.4% SDS; the running buffer contained 0.1% SDS. Gels were 0.75 mm thick and were run for 5 h at 20 mA (constant current). Proteins were electrophoretically transferred to nitrocellulose paper as described in Klymkowsky et al. (1987). For immunoblot analysis, RV202 ascites fluid was diluted 1:200 and 14h7 tissue culture supernatant was used neat. Bound primary antibody was visualized using affinity-purified goat anti-mouse immunoglobulin antibody conjugated to either horseradish peroxidase diluted 1:2000 or to alkaline phosphatase diluted 1:1000–1:2000 (Biorad). Peroxidase-conjugated antibodies were visualized as described previously (Klymkowsky et al. 1987), alkaline-phosphatase-conjugated antibodies were visualized using either Vector laboratories reagent kits or the p-nitrotoerazolium blue (NBT; 0.1 mg ml⁻¹)/5-bromo-4-chloro-3-indolylphosphate (BCIP; 0.05 mg ml⁻¹) reaction in 50 mm-glycine, 4 mm-MgCl₂ (pH9.6).

During the course of developing our whole-mount immunocytochemical method, we used the mouse monoclonal anti-lamin II/III antibody 1494 (IgG; anti-lamin), generated from a mouse immunized with A6 cell residues (K. M. M. Swords & M. W. Klymkowsky, unpublished data), and the anti-β-tubulin antibody E7 (IgG; anti-tubulin; Chu & Klymkowsky, 1987).

**Whole-mount immunocytochemistry**

Eggs were stripped from hormonally stimulated females and artificially fertilized as described previously (Klymkowsky et al. 1987). Shortly after fertilization, embryos were dejellied using 2% cysteine (pH8.0) and allowed to develop in 20% Ringers' supplemented with 50 μg ml⁻¹ gentamycin sulphate. At appropriate stages, embryos were fixed in 20% dimethyl sulfoxide (DMSO); 80% methanol (Dent's fixative). After 2–12 h, the embryos were transferred to 10% (final concentration) hydrogen peroxide diluted in fixative and incubated at room temperature for 2 days. This effectively bleached the pigment of the embryo and destroyed endogenous peroxidase activity. After bleaching, embryos can be stored at −20°C in 100% methanol indefinitely.
For staining, bleached embryos were washed twice with Tris-buffered saline (TBS) for five minutes each. They were then incubated overnight at 4°C in primary antibody diluted into 20% newborn calf serum/TBS. RV202 ascites fluid was diluted 1:100 and 14h7 tissue culture supernatant was diluted 1:10. After this incubation, the embryos were washed five times in TBS; each wash lasted longer than 1 h. The embryos were then placed into secondary antibody, either affinity-purified goat anti-mouse immunoglobulin antibody conjugated to either horseradish peroxidase (1:200) or alkaline phosphatase (1:2000). Secondary antibodies were diluted into 20% newborn calf serum/TBS and were incubated with the embryos overnight at 4°C. The embryos were then washed as described above. Bound peroxidase-conjugated antibody was visualized using 0.5 mg ml⁻¹ diaminobenzidine (DAB)/0.02% hydrogen peroxide in TBS; reactions were carried out for 2–6 h at room temperature. Embryos stained with alkaline-phosphatase-conjugated antibody were washed twice (5 min each) with 50 mM-phosphate buffer (pH 9.2) and then reacted with the Vector laboratories phosphatase II substrate kit for 1–3 h. The endogenous phosphatase inhibitor levamisole (1 mM; Sigma) was included in the reaction mixture. Reactions were stopped by dehydrating the embryos in 100% methanol (2 times, 5–15 min each). Embryos were cleared by placing them in a 1:2 mixture of benzyl alcohol:benzyl benzoate (BABB). Throughout the staining, washing, reaction, dehydration and clearing steps, embryos were gently rocked in 1.5 ml microfuge tubes. In the absence of primary antibody, we found no non-specific staining of oocytes; in embryos, there was occasional non-specific staining of the lining of the archenteron, the cement gland and the surface of the embryo (see Results).

The cleared embryos were mounted in BABB in specially constructed brass slides that allow coverslips to be mounted with a 1 mm spacing between them. The coverslips were stuck to these slides using melted paraffin wax. Since transverse optical sections of embryos are of poor quality, the embryos were sectioned. To cut sections, whole-mount stained embryos were transferred from BABB into 95% ethanol and then embedded in 99% polyethylene glycol 400 diastearate, 1% cetyl alcohol (Aldrich) according to the method of Steedman (1957). After hardening, the embryos were sectioned on a SLEE cryostat at room temperature. Sections were collected onto glass slides and mounted in BABB under a coverslip. All microscopy was done on Zeiss IM35 inverted microscopes using x2.5, x6.3 plan-neofluar, x16, x25 plan-neofluor, x40 and x63 planachromatic lenses. Photographs were taken on Kodak Plus-X Pan film (ASA 125).

**Results**

Andrew Murray (UCSF) developed the improved clearing solution (BABB) for the *Xenopus* embryo that made whole-mount immunocytochemistry feasible. Since autofluorescence from yolk remains in cleared embryos, we have developed enzyme-based immunocytochemistry. Using enzyme-based reagents, we found that diaminobenzidine (DAB) but not 4-chloro-1-naphthol was stable to the clearing solution. We also tested alkaline-phosphatase-based secondary reagents. Of the Vector laboratories alkaline phosphatase reagents kits (I, II and III) only reagent II was stable to clearing. Nitro-blue tetrazolium/bro-mo-chloro-indolylphosphate, naphthol-AS-MX-phosphate/fast red TR and naphthol-AS-BI-phosphate/new fuchsin reagents (Malik & Daymon, 1982) were not stable to the clearing agent.

The *Xenopus* oocyte and early embryo are pigmented and this pigment interferes with visualizing the enzyme-based reaction products. We found that we could eliminate this problem and destroy endogenous peroxidase activity by bleaching the embryos in 10% hydrogen peroxide; bleaching did not appear to affect the ability of any of the antibodies that we have tested to react specifically with embryos.

Fixation with a 1:4 mixture of DMSO:methanol gave the best penetration of antibody into the embryo. In DMSO:methanol-fixed embryos, all nuclei were stained by the anti-lamin antibody 14a9 and mitotic spindles throughout the volume of the embryo were labelled by the anti-β tubulin antibody E7 (not shown). IgM antibodies were also found to penetrate throughout the volume of DMSO:methanol-fixed oocytes (not shown). While antibodies can penetrate throughout the embryo, we did find that staining was often weaker in the yolkiest parts of the embryo. In the absence of primary antibody, we occasionally found weak non-specific labelling by the secondary antibody of the cement gland, the surface of the archenteron and the embryo (not shown). This non-specific staining was readily recognized and in all cases control embryos, reacted with only the secondary antibody, were examined to distinguish specific and non-specific labelling.

During the course of this work, we found significant differences in the effective working dilutions between different commercially available conjugates. It is therefore critical to titre all conjugates in the presence and absence of primary antibody to determine the appropriate working dilution for each secondary antibody. In addition, we consistently found that the level of non-specific background staining was much higher with alkaline-phosphatase-conjugated secondary antibodies than with peroxidase-conjugates. All whole-mount stained embryos shown in this paper were generated by using peroxidase-conjugated secondary antibodies and DAB as a substrate.

**Characterization of anti-vimentin antibodies**

The specificity of the monoclonal antibodies RV202 and 14h7 was initially determined in the *Xenopus* adult kidney epithelia-derived cell line A6; A6 cells express both cytokeratin and vimentin-type intermediate filaments. Immunofluorescence microscopy of methanol-fixed A6 cells showed that both RV202 and 14h7 stained a filamentous network (Fig. 1A–C). We showed that these filaments are distinct from both microtubules and cytokeratin filaments by treating A6 cells with the microtubule-depolymerizing drug nocodazole. In nocodazole-treated (10 µg ml⁻¹ for 20 h) cells, both RV202 and 14h7 specifically stained the collapsed vimentin filament system (Fig. 1D,E); microtubules were depolymerized (Fig. 1B) and the cytokeratin filament system remained extended (not shown).

In *Xenopus*, vimentin has a reported relative molecular mass of 55×10³ (Nelson & Traub, 1982; Godsall et al., 1984, 1986; Tang et al. 1988). Under our gel
conditions, both 14h7 and RV202 reacted with a polypeptide of \(55 \times 10^3\) Mr found in \(A_6\) cell residues and total cell protein (Fig. 2B,D). In addition, 14h7 reacted with a polypeptide of approximately \(57 \times 10^3\) Mr (Fig. 2C,D). In earlier experiments (see Klymkowsky et al. 1987, figure 1), we had found only a single 14h7-reactive protein and subtle changes in electrophoretic conditions may be critical to resolving these two polypeptides. In Western immunoblot analyses of insoluble residues derived from stage-35 embryos, both RV202 and 14h7 reacted with a \(55 \times 10^3\) Mr polypeptide (Fig. 2E–G). The \(57 \times 10^3\) Mr 14h7-reactive polypeptide also appeared to be present in the embryo, but in relatively small amounts (Fig. 2G). Occasionally, RV202 appeared to react with a set of high molecular weights in the embryo (not shown).

Given the specificity of staining by 14h7 and RV202 in \(A_6\) cells, it appears that the \(55 \times 10^3\) Mr polypeptide is vimentin. The \(57 \times 10^3\) Mr 14h7-reactive polypeptide is insoluble and is recognized by the monoclonal antibody IFA (Fig. 3A–C), an antibody that recognizes an epitope characteristic of intermediate filament subunit proteins (Pruss et al. 1981; Osborn & Weber, 1987). It therefore appears to be an intermediate filament subunit protein in its own right. Surprisingly, when \(A_6\) cell residues were probed with a rabbit anti-vimentin antiserum (Hynes & Destree, 1978), we found that this antiserum reacted preferentially with the \(57 \times 10^3\) Mr polypeptide (Fig. 3E,F). Under conditions of whole-mount immunocytochemistry, this anti-vimentin antiserum reacted only weakly with the embryo, although its general pattern was consistent with that seen with RV202 and 14h7 (not shown). We are currently further characterizing the relationship between the 57 and \(55 \times 10^3\) Mr polypeptides. In whole-mount immunocytochemistry of embryos, both RV202 and 14h7 gave identical staining patterns. This staining is presumably due to their common reaction with vimentin (\(55 \times 10^3\)).

**Embryonic vimentin expression**

**Central nervous system**

We first detected vimentin within a subset of neural tube cells shortly after the closure of the neural tube (stage 19) (Fig. 4A). The location of these cells is similar to the first vimentin-positive cells identified by Godsave et al. (1986). Vimentin expression begins in the centre of the neural plate where the neural folds first meet and spreads anteriorly and posteriorly as the neural tube closes. Individual vimentin-positive cells

![14h7 and RV202 staining of A6 cells. Methanol-fixed A6 cells were stained with anti-tubulin (A,B), 14h7 (C,D) or RV202 (E,F). In control cells (A,C,E), both 14h7 and RV202 stain an extended filamentous system, distinct from the microtubule system. Cells treated with nocodazole have depolymerized microtubules (B) and collapsed filament systems stained by 14h7 and RV202 (D,F). Cytokeratin filaments remain extended in nocodazole-treated cells (not shown). Bar in A marks 15 μm.](image-url)
Vimentin expression in Xenopus

Fig. 2. Western immunoblot analysis of A6 cells and embryo residues. Whole cell and detergent-insoluble residues of A6 cells were separated by SDS–polyacrylamide gel electrophoresis and either stained with Coomassie brilliant blue (A) (wc, whole cell; cr, cell residue) or were electrophoretically transferred to nitrocellulose paper and probed with the monoclonal antibody RV202 (B) or 14h7 (C). RV202 labelled a single band of 55×10^3 in both whole cell (wc) and cell residue (cr) lanes. Occasionally a band below the 55×10^3 polypeptide was labelled (asterisk), this is presumably a proteolytic degradation product of the 55×10^3 polypeptide. Typically 14h7 appears to react weakly with a 55×10^3 polypeptide in whole cell, and with polypeptides of 55×10^3 (large arrow) and 57×10^3 (small arrow) in cell residue (C). To illustrate that RV202 and 14h7 react with the same 55×10^3 polypeptide, a single lane of A6 cell residue was cut down the middle (D) and probed with 14h7 (left side) or RV202 (right side); the labelled 55×10^3 polypeptides align perfectly. To define the specificity of 14h7 and RV202 in embryos, insoluble residues from stage-35 tadpoles were separated by SDS–polyacrylamide gel electrophoresis and either stained with Coomassie brilliant blue (E) or transferred to nitrocellulose and probed with either RV202 (F) or 14h7 (G). RV202 reacted with polypeptide of 55×10^3, 14h7 reacted with this same polypeptide (large arrow), and very weakly with a polypeptide of 57×10^3. A minor band that presumably represents a proteolytic degradation product of the 55×10^3 polypeptide was also labelled by both antibodies (asterisk in F and G). Dash marks on the left side of A and E mark the position of the molecular weight markers phosphorylase (97×10^3), bovine serum albumin (66×10^3), ovalbumin (45×10^3) and carbonic anhydrase (29×10^3).

are visible from a lateral perspective as a punctate pattern of staining along the neural tube (Fig. 5A). Examination of transverse physical sections indicated that vimentin expression was mostly at the margins (outer surfaces) of the neural tube. Some faint vimentin staining was found in cells bordering the ventricle (Fig. 5B). Serial sections also show that vimentin-expressing cells are restricted to the lateral surfaces of the neural tube and are absent from both ventral and dorsal surfaces (Fig. 5B). Vimentin-expressing cells of the cephalic neural tube begin to elongate by stage 22 (Fig. 4B). By stage 26, vimentin-expressing cells in the cephalic and anterior trunk neural tube have vimentin-containing processes that extend into the neural tube toward the ventricular zone (Figs 6B, 7A–B). In transverse physical sections of the head at stage 35, processes from vimentin-expressing cells extend from the pial (outer) to the ventricular (inner) surface of the neural tube (Fig. 8A). The degree of elongation of these vimentin-expressing cells from stage 22 until at least stage 35 decreases in a gradient toward the posterior neural tube (Fig. 6B). Vimentin-expressing cells appear in the tail bud as soon as it forms at stage 24 (not shown – for stage 26, see Fig. 6A).

Vimentin-expressing cells are not present in the budding optic vesicle (stage 22; Fig. 4B). However, by stage 24, vimentin can be seen in the optic vesicle in a punctate pattern of expression, similar to that seen in the early neural tube (Fig. 4C). The vimentin-expressing cells of the optic vesicle do not appear to have vimentin-containing processes in physical sections of stage-35 retinas (Fig. 8A).

Somitic and trunk mesenchyme

Outside the CNS, vimentin-expressing cells first appear at stage 22/23 on the ventral and outer lateral surfaces of the first two to three anterior trunk somites (not shown). By stage 25/26, the outer lateral surfaces of the anteriormost third of the somites are covered with an even monolayer of vimentin-expressing mesenchymal cells (Fig. 6A). The somitic mesenchyme can also be seen by stage 26 on the ventral and inner surfaces of the somites (Fig. 7B). As more somites form, vimentin-expressing cells appear in association with increasingly more posterior somites (not shown). The density of these somitic mesenchymal cells is constant over the period from stage 25 to 35, with new vimentin-positive cells covering the increasing surface area of the growing somites. Thus, at stage 26, there are 25–30 cells on the...
outer surface of the most anterior somite; by stage 35, the outer surface of this somite is covered with 45–50 cells (compare Figs 6A, 9D).

Soon after the vimentin-expressing somitic mesenchymal cells appear (stage 24), vimentin-expressing cells appear ventral to the somites (Fig. 6A). These cells are located between the endoderm and the ectoderm and first appear ventral to the anterior somites. They are more densely packed than the somitic mesenchymal cells although, like the somitic mesenchymal cells, they are found in increasingly posterior positions as development proceeds (Fig. 9A). In addition, these ventral mesenchyme cells accumulate and extend increasingly more ventrally during development (compare Figs 6A, 9D,E). Sections of stage-35 embryos show that these vimentin-expressing cells are part of the nephrotomic mesoderm (metamere) ventral to the pronephric ducts (Fig. 8B). There are also mesenchymal cells associated with the pronephric ducts, although the duct epithelium itself does not appear to express vimentin (Fig. 8B). In a lateral view of stage-33 to -35 embryos, the vitelline veins stain for vimentin (Fig. 9A,E). Transverse sections of the trunk of stage-35 embryos show that vimentin-expressing mesenchyme coats the wall of the vitelline veins (Fig. 8C). Mesenchymal cells can also be seen in the pericardial cavity surrounding the presumptive heart by stage 33 (Fig. 9C).

**Dorsal and ventral fins**

Vimentin-expressing cells are present in the forming dorsal fin at stage 26 (out of focus in Fig. 6A) and, by stage 30, morphologically similar cells are found in the ventral fin (for stage 35 see Fig. 9A,B). The density of these cells did not increase appreciably during the period up to stage 35. Immediately before and during formation of the dorsal fin, there are vimentin-expressing mesenchymal cells dorsal to the neural tube (Fig. 7B).
Vimentin expression in Xenopus

Fig. 5. Vimentin expression in stage 20 and 22 embryos. In a lateral optic section of a stage-22 embryo stained with RV202 (A), vimentin-expressing cells of the anterior neural tube (nv) are more elongate. Somites (s) are beginning to form beneath the anterior neural tube; vimentin staining can be seen on their surfaces in different focal planes. In a transverse physical section through the trunk of a stage-20 embryo stained with 14h7 (B), the cells at the outer margin of the neural tube stain intensely and those on the ventricular surface stain weakly (nv). The stained cells on the outer margin of the neural tube are restricted to the lateral neural tube and are absent from both dorsal and ventral regions. c, cement gland; n, notochord; nt, neural tube; ov, optic vesicle. Bars in A, 150 μm; in B, 100 μm.

Cranial mesenchyme

Beginning at stage 25/26, increasing numbers of vimentin-positive mesenchymal cells appear throughout the cranial region (Fig. 6A). At stage 26, only a few of the head mesenchyme cells express vimentin. These are primarily found lateral to the mesencephalon and around the mandibular arch (Fig. 7A). By stage 35, virtually all of the head mesenchyme expresses vimentin (Fig. 8A). As well as the head mesenchyme which is dispersed between the brain and the optic cups, vimentin is expressed in the mesenchyme of the corneal endothelium, in the meninges, in the branchial arches, in the mandibular arch and around the otic vesicle (Figs 8A, 9A). Vimentin-expressing mesenchyme is found between the optic cup and the lens (Fig. 8A).

Discussion

For the characterization of tissue-specific protein expression, whole-mount methods offer a number of advantages over conventional section-based methods. First, in contrast to section-based methods, whole-mount immunocytochemistry provides a global view of the specimen, which makes it much easier to appreciate the overall pattern of protein expression. Second, a rather large number of specimens can be examined in a relatively short time, making it feasible to compare accurately the variations between specimens and to follow protein expression through the course of development. Third, it is possible to visualize details of tissue-specific expression (for example see Fig. 9D,E) that are not readily apparent in serial-section-based analyses. A modified version of our basic method has been applied to chick embryos with good results (J. B. Miller, personal communication).

While whole-mount immunocytochemistry is clearly valuable, some care should be taken in its application. Most importantly, it is likely that the immunoperoxidase reaction is not as sensitive as immunofluorescence methods. This lower sensitivity arises from the nature of the signal generated, a light-absorbing product compared with a light-emitting source, together with the fact that the high-resolution/short-working-distance lenses commonly used in immunofluorescence microscopy are not useful in examining whole-mount specimens of the Xenopus embryo. This is because their working distance barely allows them to penetrate the approximately 0.5- to 1 mm-thick early embryo. In addition, out-of-focus information confuses much of the in-focus information obtained with even longer-working-distance lenses. We have found that in most cases objective magnification over ×16 to ×25 produces no significant new information. In many cases, ×2.5 and ×6.3 objective lenses are optimal.

Vimentin in the Xenopus oocyte

The initial appearance of vimentin in the Xenopus embryo is problematic at present. Using conventional immunocytochemical methods, Godsave et al. (1984, 1986) found vimentin in the oocyte, egg and early
Fig. 6. Vimentin expression in the stage-26 embryo. A lateral optic section of a stage-26 embryo stained with RV202 (A) reveals that vimentin-expressing cells (nv) are present in the neural tube, from the head to the tailbud. Vimentin-expressing somitic mesenchyme (sm) is present on the eight anteriormost trunk somites. Vimentin-expressing mesenchymal cells are found in the head where the branchial arches are forming (hm) as well as below the anterior trunk somites where the pronephros are forming (pm). An oblique frontal/lateral physical section through posterior trunk region of the neural tube and somites of a stage-26 embryo stained with RV202 (B) reveals that the vimentin-expressing cells of the neural tube (nv) become gradually less elongated toward the tail. ant, anterior; pos, posterior; c, cement gland; nt, neural tube; ov, optic vesicle; s, somite. Bars, 350 μm in A and 150 μm in B.

This observation has been strengthened by the recent report by Tang et al. (1988) that sequences homologous to vimentin are present in both oocytes and eggs. On the other hand, Franz et al. (1983), using two-dimensional gel analysis and immunocytochemistry, found no evidence for vimentin in the oocyte or the egg. We have made some observations that may shed some light on this discrepancy. In oocytes, both 14h7 and RV202 react with vimentin-positive thecal cells of the follicle, but only 14h7 reacts with the oocyte itself (Dent & Klymkowsky, 1989). Different anti-vimentin antibodies recognize the 55 and 57×10^3 M_r polypeptides with different affinities as illustrated by the high degree of selectivity of RV202 for the 55×10^3 M_r polypeptide (vimentin) (Figs 2D,3D), the approximately equal reactivity of 14h7 with both polypeptides (Figs 2D, 3A,B) and the selectivity of a rabbit anti-vimentin antibody (Hynes & Destree, 1978) for the
Vimentin expression in the Xenopus embryo

In their immunocytochemical characterization of intermediate filament expression in the CNS of the developing Xenopus embryo, Godsave et al. (1986) found that vimentin was absent at stage 17 and present in the neural tube of stage-25 embryos. Our results are consistent with this finding. Using whole-mount immunocytochemistry, vimentin first appears at stage 19 in a subpopulation of cells within the neural tube. Based on our results and those of Godsave et al. (1986), the pattern of vimentin expression in Xenopus differs significantly from that found in the mouse or chick. In the mouse, vimentin appears first in the embryonic mesoderm (primary mesenchyme) soon after its original formation (Jackson et al. 1981; Franke et al. 1982). While vimentin is expressed in mesodermally derived tissues in Xenopus, e.g. the somitic mesenchyme and the meninges (Figs 8A, 9D), its expression occurs relatively late (stage 22/23), long after the embryonic mesoderm has differentiated (stage 12). In chick, vimentin is found in some developing muscles (Gard & Lazarides, 1980; Lazarides et al. 1981). We find no evidence that vimentin is expressed in the myotome of the somites of Xenopus at any stages examined (Figs 7B, 8B). Desmin, the muscle-specific intermediate filament protein, appears in somitic tissue by stage 19/20 (Chu, McMillan & Klymkowsky, unpublished data).

In chick, mouse and Xenopus, vimentin is expressed in the early CNS. In chick, vimentin is expressed in the neural plate before closure of the neural tube (Bennet, 1987). Subsequently, all cells of the neuroepithelium...
express vimentin until it is replaced by neurofilament proteins in neuroblasts, or supplemented by GFAP in astrocytes (Tapscott, 1981a, b). In mouse, vimentin is first expressed in cells of the neuroepithelium after neural tube closure. Similar to chick, vimentin is replaced by neurofilament protein in neurones and by GFAP in astrocytes in the mouse CNS. In *Xenopus*, on the other hand, vimentin is expressed very early in the neural tube but in only a small subset of the neuroepithelial cells. Furthermore, based on the distribution of neuronal cell types in the neural tube of *Xenopus* (Roberts & Clarke, 1982) and by comparison with whole-mount immunocytochemical studies using antibodies against neuronal markers such as acetylated tubulin and the neurofilament proteins (Chu & Klymkowsky, submitted), it appears that vimentin is not

Fig. 8. Transverse sections of stage-35 embryos stained for vimentin. In a transverse section of the head stained with 14h7 (A), vimentin is found in most of the head mesenchyme (hm) surrounding the brain, in the meninges (mn), in the corneal endothelium (ce) and in the mandibular arch (ma). There is also some mesenchyme (em) in the eye. Vimentin is expressed in cells of the mesencephalon and diencephalon (nv). These cells exhibit the characteristic morphology of mature radial glia, i.e. they are very thin and extend from the margin to the ventricular surface. The staining of the ectoderm is artifactual. A transverse section through an anterior somite of the dorsal trunk (B) reveals that vimentin is expressed in the dorsal fin mesenchyme (dm). Vimentin is also found in the mesenchyme associated with the outer surface of the somites (sm) and in mesoderm below the pronephric ducts (pm). Vimentin-expressing cells are present in the neural tube and extend from the pial to the ventricular surface (nv). In the area of the embryo ventral to the region illustrated in B (C) are found the lateral body wall and the yolky endoderm ventral to the pronephros. The pronephric mesoderm is at the top (pm) and below it, between the mesoderm and the endoderm are the vitelline veins (vv) which contain vimentin-expressing mesenchyme. c, cement gland; de, diencephalon; df, dorsal fin; e, eye; me, mesencephalon; n, notochord; nt, neural tube (spinal chord); p, pharynx; pd, pronephric duct; s, somite; vv, vitelline veins. Bars, 50 μm in A & B, 25 μm in C.
Fig. 9. Vimentin staining in the stage-35 embryo. In a lateral optic section of stage-35 embryo stained with RV202 (A), vimentin can be found in the neural tube, in the dorsal fin (df) and on the somites (the somitic mesenchyme is mostly out of focus in this picture). Vimentin is expressed in the pronephric mesenchyme (pm) below the first six somites with progressively less mesenchyme below the more posterior somites. The reticulated pattern of the vitelline veins (vv) can be seen throughout the belly around the yolky endoderm. Vimentin-expressing mesenchyme is present on the branchial arches (ba), around the otic vesicle (ot), covering the eye (e) and on the surface of the brain (b). A close-up optical section of the dorsal fin (B) reveals that the morphology of individual dorsal fin mesenchymal cells (dm) can be distinguished by the vimentin staining. In a lateral optical section midway through the embryo (C), vimentin-expressing mesenchyme surrounds the heart in the pericardial cavity (cm). A close lateral optical section of the anterior somites (D) reveals individual, vimentin-expressing mesenchymal (sm) cells. Individual pronephric mesenchymal cells (pm) are visible below the somites. A close lateral view of the region ventral to the anterior somites is shown in D. (E) reveals that the pronephric mesenchyme (pm) expresses vimentin, as do cells of the vitelline veins (vv). b, brain; ba, branchial arch; c, cement gland; df, dorsal fin; e, eye; nt, neural tube; ot, otic vesicle; s, somite; vv, vitelline veins. Bars, 250 μm in A, 50 μm in B, 20 μm in C, 100 μm in D and 20 μm in E.
expressed in significant amounts by neurones of the *Xenopus* embryo.

Radial glia are defined as bipolar glial cells that stretch from the ventricular to the pial surface of the early neural tube (Ramon y Cajal, 1909; Tapscott *et al.* 1981b; Levitt & Rakic, 1980). Several lines of evidence suggest that the vimentin-expressing cells in the neural tube of *Xenopus* are radial glia and that vimentin is therefore a specific marker of early radial glial differentiation. First, the vimentin-expressing cells have a morphology and time of appearance typical of radial glia. Second, radial glia have been observed by histological methods and electron microscopy in the spinal chord of *Xenopus* tadpoles at stage 60 (Michel & Reier, 1979). Third, in other organisms, radial glia typically express vimentin as their only cytoplasmic intermediate filament protein (Tapscott *et al.* 1981b; Bovolenta *et al.* 1984; however, see Levitt & Rakic, 1980 for a discussion of the Rhesus monkey). Radial glia are thought to act as guides for the migration of neurones from the ventricular to the pial layers of the CNS (Rakic, 1972).

In contrast to the neural tube, vimentin expression is delayed in the optic vesicles until stage 24. Whether this reflects a lower level of vimentin expression by these cells or a delay in their differentiation remains unclear. The vimentin-expressing cells of the optic vesicle may be Müller glia, cells characteristic of vertebrate retina. The Müller glia extend across the retina by stage 46 in *Xenopus* (Wets & Fraser, 1988). In the rat, they also express vimentin as their only cytoplasmic intermediate filament protein (Shaw & Weber, 1983). It is not clear to us whether these cells have processes at the stages that we examined.

Later in development, vimentin is expressed in a number of different cell populations located outside the CNS. The first of these peripheral, vimentin-expressing, cell types to appear are the mesenchymal cells associated with the somites at stage 22/23. This somitic mesenchyme is presumably derived from the dermatome on the dorsolateral somitic surface and the sclerotome on the ventral somitic surface (Hamilton, 1969). Vimentin expression in these cells occurs in an anterior–posterior progression lagging behind the progression of somite maturation.

The vimentin-expressing cells in the dorsal fin are derived from the trunk neural crest (DuShane, 1935; Bodensteine, 1952). Neural crest cells have been shown to be necessary and sufficient for the formation of a dorsal fin in amphibia if the overlying ectoderm is competent (Bodenstein, 1952). The vimentin-expressing cells dorsal to the neural tube (Fig. 7B) may be precursors of the dorsal fin mesenchyme. Melanocytes also sit on the dorsal surface of the neural tube at this stage and may express vimentin.

The vimentin-expressing mesenchyme ventral to the pronephric ducts appears to be derived from the nephrotome (metameres) of the lateral mesoderm. It is not clear what function these cells serve though they may contribute to the vimentin-expressing mesenchyme in the pronephric ducts. The vimentin-expressing mesenchyme in the vitelline veins presumably arises in situ from the splanchnic mesoderm.

The mesenchymal tissues of the head arise from both mesoderm and ectoderm (neural crest). Cephalic neural crest gives rise to a population of mesenchymal cells that eventually form part of the skull (Noden, 1986). Neural crest cells also migrate into the gut and generate the enteric nervous system and some mesenchyme (Le Douarin, 1982). The paraxial mesoderm contributes to head mesenchyme in the form of the meningeal layer that surrounds the brain as well as contributing to the bones of the head (Noden, 1986; Sadaghiani & Thiebaud, 1987). Our results show that by stage 26, neural-crest-derived mesenchyme present in the mandibular arch as well as mesodermally derived mesenchyme lateral to the mesencephalon express vimentin. By stage 35, vimentin-expressing mesenchymal tissues include the meningeal mesenchyme (mesodermal), the corneal mesenchyme (neural crest), the mandibular arch mesenchyme (neural crest) and the branchial arch mesenchyme (mesodermal & neural-crest derived) (Sadaghiani & Thiebaud, 1987). Cells located with the vitreous humor are of unknown origin. Thus, head mesenchyme of both mesodermal and ectodermal origin express vimentin concurrently.

Migration of cephalic neural crest in *Xenopus* begins at stage 17 (Sadaghiani & Thiebaud, 1987) and these cells do not appear to express vimentin until after stage 25/26. Thus, even though some neural crest cells appear to be restricted to form mesenchyme before they migrate (Newth, 1954), they do not express vimentin, a marker of mesenchymal differentiation, until after migration has begun. This observation is consistent with experiments showing that neural crest needs to interact with substrate tissues in order to form cartilage and bone (Newsome, 1976; Bee & Thorogood, 1980). Vimentin may be a useful marker of mesenchymal differentiation for studies of induction of cartilage and bone in neural crest tissue.

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**References**


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