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The appearance and distribution of intermediate filament proteins during differentiation of the central nervous system, skin and notochord of *Xenopus laevis*

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SUMMARY

Antibodies against various intermediate filament proteins have been used to follow cell differentiation in the early *Xenopus* embryo. Three new monoclonal antibodies against *Xenopus* cytokeratins raised against Triton-insoluble material from tadpoles (RD35/2a, RD35/3a and D3/3a), two antibodies against mammalian cytokeratins (LE65 and LP3K), monoclonal anti-(rat 200 K neurofilament protein), rabbit anti-(rat glial filament acidic protein), and rabbit antibodies to hamster and calf vimentin were used. We show that cytokeratins are present in the early central nervous system (CNS) and persist in the ependymal cells of the adult CNS. We also show that the notochord contains cytokeratin. The ontogeny of intermediate filament protein appearance in the CNS, skin and notochord between neural fold stage and swimming tadpole stage are described. These results are discussed in particular with regard to the use of the antibodies as differentiation markers.

INTRODUCTION

Almost all cell types are now known to contain intermediate filaments, which are polymers of a number of distinct, tissue-restricted polypeptides (Anderton, 1981; Lazarides, 1980, 1982; Osborn & Weber, 1982). However, the functions of intermediate filaments remain obscure, as does the reason for the tissue-specific distribution of the different polypeptide subunits.

Even now, few studies have been carried out on the ontogeny of the tissue-specific expression of intermediate filament polypeptides. In mouse embryos, only cytokeratins are thought to be expressed before the primitive streak stage (Lehtonen *et al.* 1983; Jackson *et al.* 1980, 1981; Paulin, Babinet, Weber & Osborn, 1980; Brulet, Babinet, Kemler & Jacob, 1980; Kemler *et al.* 1981; Oshima *et al.* 1983). After this, cytokeratin is replaced by vimentin in primary mesenchyme cells (Franke *et al.* 1982*a*). In embryos from several species, it has also been shown that the other intermediate filament proteins, desmin (Franke *et al.* 1982*a*), neurofilament protein and glial filament acidic protein (Schnitzer, Franke & Schachner,

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1981; Raju, Bignami & Dahl, 1981; Bignami & Dahl, 1975; Bignami, Raju & Dahl, 1982; Tapscott *et al.* 1981; Bovolenta, Liem & Mason, 1984), are not expressed until differentiation of the cell types concerned (muscle, neurones and glial cells, respectively).

In *Xenopus laevis*, intermediate filaments have been found in oocytes and early embryos (Franz *et al.* 1983; Gall, Picheral & Gounon, 1983; Godsave, Wylie, Lane & Anderton, 1984*a*; Godsave, Anderton, Heasman & Wylie, 1984*b*; Winkles *et al.* 1985; Jonas, Sargent & Dawid, 1985). In previous work we have reported that cytokeratin-containing filaments appear during early oogenesis, change their distribution during oocyte differentiation and further change during maturation to form a fertilizable egg (Godsave *et al.* 1984*a*). We also described an abundant cytoplasmic filament system which cross reacts with mammalian anti-vimentins (Godsave *et al.* 1984*b*). This also changes its distribution during oogenesis and again during maturation. Using a different approach Sargent & Dawid (1983) have prepared a cDNA library which selects out RNA molecules present in the *Xenopus* gastrula but not in the egg. These include several RNA molecules that code for embryonic epidermal keratins (Jonas *et al.* 1985; Winkles *et al.* 1985).

In this paper, we describe the ontogeny of intermediate filament proteins in the skin, notochord and developing nervous system of the *Xenopus* embryo. We demonstrate amongst other things that the central nervous system of the early swimming tadpole contains several classes of cells that can be distinguished by their content of intermediate filament polypeptides, including a class of cytokeratin-containing cells.

The different distributions of the various intermediate filament polypeptides should prove useful, both in the study of function of these molecules and as markers in studies of cell determination in *Xenopus* embryos. In the short term, this latter point may prove important. Several recent papers have followed the progeny of cells grafted into early embryos in order to assess their capacity to differentiate into cell types that they would not normally form (Heasman, Wylie, Hausen & Smith, 1984; Slack, Dale & Smith, 1984; Gimlich & Cooke, 1983). It should be of great benefit to work such as this, if reliable histologically identifiable cell markers can be identified.

MATERIALS AND METHODS

Embryos

Adult *Xenopus laevis* (from Xenopus Ltd) were induced to mate by injection of human chorionic gonadotrophin (Profasi, 600 i.u. per female, 400 per male) into the dorsal lymph sac. Embryos were dejellied by gentle swirling in 2% cysteine–HCl adjusted to pH 7·6 with NaOH and grown in 1/10th strength modified Barth saline (N/10 BX, Hamburger, 1960; Elsdale, Gordon & Fischberg, 1960) and staged according to Nieuwkoop & Faber (1967).

Antibodies used

Mouse monoclonal antibodies were prepared using Triton-insoluble preparations (prepared as in Wood & Anderton, 1981) from tissue culture cells or *Xenopus* swimming tadpoles at

stage 48. Screening of antibodies was carried out on frozen sections of ethanol-, or 2% w/v TCA-fixed, gelatin-embedded tadpoles. Three antibodies thus obtained, designated RD35/2A, RD35/3A and D3/3A, were used in this study. Their specificities are described in detail below. Two mouse anti-cytokeratin monoclonals raised against PtK1 cell cytoskeleton preparations (LE65 and LP3K) were kindly provided by Dr E. B. Lane (ICRF, London) and have been used previously on *Xenopus* tissues (Godsave *et al.* 1984*a*). Rabbit antibodies against hamster and calf vimentins were kind gifts from Drs R. O. Hynes (see Hynes & Destree, 1978) and F. Ramaekers (see Ramaekers *et al.* 1982), respectively. The anti-hamster vimentin was a purified IgG preparation. Rabbit anti-glial filament acidic protein (anti-GFAP) was an antiserum raised against the GFAP band cut from preparative SDS gels of a rat brain Triton-insoluble fraction (Wood & Anderton, 1981). The mouse monoclonal antibodies RT97 and 147, which react with the 200 K neurofilament protein were raised against rat brain Triton-insoluble material (Anderton *et al.* 1982). Anti-IFA is a monoclonal antibody which recognizes all types of intermediate filament protein (Pruss *et al.* 1981).

Immunocytochemistry

Embryos and adult *Xenopus* tissues were fixed either in 2% w/v TCA for 2 h or absolute ethanol for 48 h, at room temperature. Fixed material was impregnated with sucrose, embedded in gelatin, sectioned and stained with antibodies as described previously (Godsave *et al.* 1984*a*). In some staining reactions, horse serum was used instead of rabbit serum to block non-specific antibody binding.

Incubations with primary antibody solutions were for 16 h at 4°C with anti-cytokeratins and 1 h at room temperature with other antibodies. Staining of early embryo sections was weak unless the longer incubation time was used. Control sections were incubated with tissue culture medium, or with rabbit serum diluted appropriately. The second antibodies, tetra-methyl rhodamine isothiocyanate-coupled rabbit anti-mouse or goat anti-rabbit antisera (Nordic Ltd) were used at 1:50 dilution in phosphate-buffered saline containing 1% w/v bovine serum albumen, and reacted with tissue sections for 1 h.

Immunoblotting

Whole tadpole homogenates and Triton-insoluble fractions of other tissues and tissue culture cells were separated electrophoretically in 10% w/v SDS–polyacrylamide slab gels, transferred to nitrocellulose sheets, treated with anti-intermediate filament antibodies and then stained either with ^{125}I antibodies as described previously (Pruss *et al.* 1981) or with HRP-labelled second antibodies (Miles), diluted 1:1000 in PBS containing 3% BSA and 1% goat serum, for 1 h. After washing with PBS, blue reaction product was produced by 1–5 min incubation in 0·5 mg ml^{-1} diaminobenzidene, 0·025% w/v cobalt chloride, 0·02% w/v ammonium nickel sulphate, 0·013% hydrogen peroxide (100 vols, Fisons). Blots were rinsed several times in distilled water before drying.

Electron microscopy

Dorsal regions from the trunks of swimming tadpoles were dissected out and prepared for electron microscopy as described previously (Godsave *et al.* 1984*b*).

RESULTS

Analysis of antibody specificity by immunoblotting

Antibody binding to gel blots was visualized indirectly in two different ways for these experiments. In some cases second antibodies labelled with ^{125}I were used and the blots were then autoradiographed. In other cases we used HRP-conjugated second antibodies and obtained a coloured reaction product after treatment of the immunoblot with diaminobenzidene. With the former method it

is possible to allow the signals from individual tracks to develop to the desired intensity. This is useful for experiments where antigens from different species are compared. However, the definition of bands is much clearer using the peroxidase method of labelling.

In order to examine the full spectrum of intermediate filament proteins in various frog tissues, we used anti-IFA to stain tissue extracts on immunoblots. This antibody binds to all types of intermediate filament protein (Pruss *et al.* 1981) including those of *Xenopus* (Godsave *et al.* 1984*b*). Anti-IFA was used to stain Triton-insoluble material from swimming tadpoles, adult *Xenopus laevis* spinal cord, rat brain and HT29 cells. These last cells are a human colon carcinoma-derived epithelial cell line (Fogh & Trempe, 1975). The reactions of anti-IFA with *Xenopus* tadpoles and rat brain have been described previously (Godsave *et al.* 1984*b*; Pruss *et al.* 1981). As can be seen in Fig. 1A the major proteins stained by anti-IFA in Triton-insoluble material from rat brain are GFAP (relative molecular mass, M_r, 50×10^3, abbreviated 50 K), vimentin (M_r 58 K), the neurofilament (NF) triplet (M_r 68 K, 155 K and 200 K) and a 66 K M_r protein which is detected by anti-IFA in many tissues (Pruss *et al.* 1981) and may be an intermediate-filament-associated protein (Pachter & Liem, 1985; Napolitano, Pachter, Chin & Liem, 1985).

Figs 1–9. Nitrocellulose blotting of extracts of *Xenopus* and mammalian tissues with anti-intermediate filament protein antibodies. All values are $M_r \times 10^{-3}$. Figs 1, 2, 8, 9 and 10 show autoradiographs of ^{125}I-labelled immunoblots. In these cases the molecular weights marked are those of rat brain and HT29 cell intermediate filament proteins known to react with anti-intermediate filament antigen (IFA) monoclonal antibody (Pruss *et al.* 1981). For Figs 3–7, immunoperoxidase labelling was used and photographs of the nitrocellulose are shown. The M_r markers shown are: carbonic anhydrase, 29 K; egg albumen, 45 K; bovine serum albumen, 66 K; phosphorylase b, 97·4 K; β-galactosidase, 116 K. Abbreviations: *NF*, neurofilament; *V*, vimentin; *GFAP*, glial filament acidic protein.

Fig. 1. Autoradiograph showing anti-IFA staining of Triton X-100 extracts of rat brain (lanes a and e), adult *Xenopus* spinal cord (lanes b and f), *Xenopus* swimming tadpoles (lane c), HT29 cells (lane d).

Fig. 2. Autoradiograph showing Triton-insoluble extracts of HT29 cells stained with RD35/2a (track a), RD35/3a (track b), D3/3a (track c).

Fig. 3. Anti-IFA staining of tadpole Triton-insoluble material (lane a) and whole tadpole protein (lane b).

Fig. 4. RD35/2a staining of tadpole Triton-insoluble material (lane a) and whole tadpole protein (lane b).

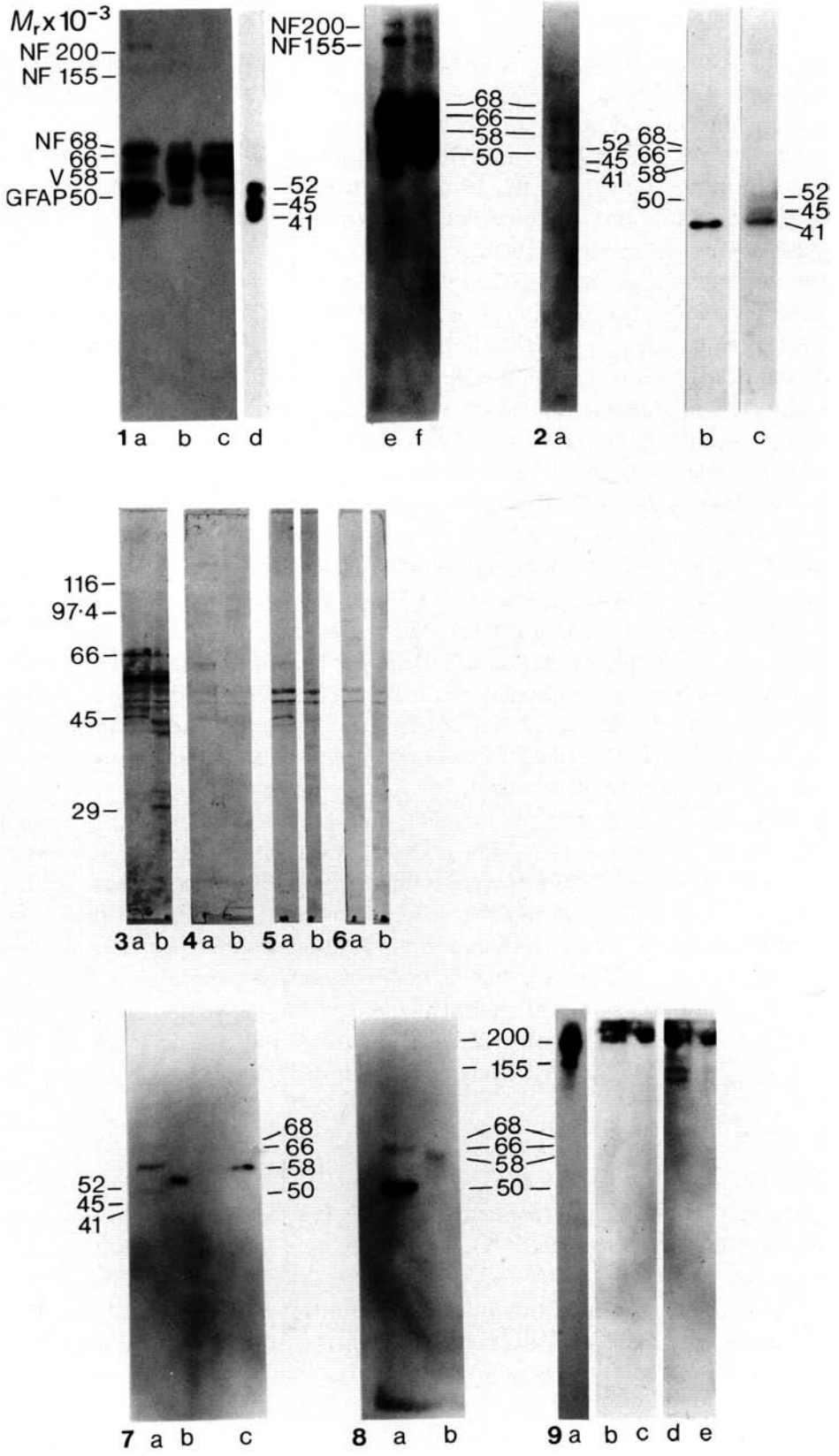
Fig. 5. RD35/3a staining of tadpole Triton-insoluble material (lane a) and whole tadpole protein (lane b).

Fig. 6. D3/3a staining of tadpole Triton-insoluble material (lane a) and whole tadpole protein (lane b).

Fig. 7. Rabbit anti-vimentin IgG staining of extracts of rat brain (lane a), adult *Xenopus* spinal cord (lane b), 3T3 cells (lane c).

Fig. 8. Rabbit anti-GFAP serum staining of extracts of rat brain (lane a), *Xenopus* spinal cord (lane b).

Fig. 9. Mouse monoclonal anti-neurofilament antibodies RT97 (lanes a–c) and 147 (lanes d and e): reaction with extracts of rat brain (lane a), *Xenopus* swimming tadpole (lanes b and d), adult *Xenopus* spinal cord (lanes c and e).


M_r x 10^{-3}
NF 200–
NF 155–
NF 68–
66–
V 58–
GFAP 50–
–52
–45
–41
1 a b c d
NF200–
NF155–
68
66
58
50
e f
52
45
41
2 a
68
66
58
50
b c
–52
–45
–41
116–
97·4–
66–
45–
29–
3 a b 4 a b 5 a b 6 a b
52–
45–
41
68
66
58
50
7 a b c
8 a b
200
155
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9 a b c d e

Anti-IFA

Anti-IFA stains a number of tadpole proteins. Western blots of Triton-insoluble proteins prepared from tadpoles were reacted with anti-IFA and both types of second antibody. Many more bands can be distinguished using peroxidase labelling (Fig. 3) than ^{125}I-labelling (Fig. 1). Tadpoles contain many intermediate filament proteins ranging in M_r from approximately 43 K to 66 K (Figs 1, 3). Several high M_r proteins can sometimes also be detected on ^{125}I-labelled blots after long exposures of the autoradiograph (not shown).

The complement of anti-IFA-reactive proteins observed on immunoblots of tadpole Triton-insoluble proteins (Fig. 3A) is very similar to that seen with whole tadpole homogenates (Fig. 3B), although there are several extra low M_r bands in the whole tadpole preparations which may be caused by proteolysis, to which some intermediate filament proteins are highly susceptible (Pruss *et al.* 1981).

Triton-insoluble material from *Xenopus laevis* spinal cord and HT29 cells have also been stained with anti-IFA on immunoblots. In spinal cord (Fig. 1B), at least seven bands are stained. Five of the bands have M_r values between 45 K and 68 K. The other two, which require longer exposure of the immunoblot, have slightly higher M_r values than the mammalian 155 K and 200 K neurofilament proteins, respectively (e.g. see Fig. 1E,F).

HT29 cells (Fig. 1D) contain three major cytokeratins corresponding to cytokeratins nos 8, 18 and, 19, with 52 K, 45 K and 41 K M_r, respectively (Moll *et al.* 1982). When Triton-insoluble material from HT29 cells is stained with anti-IFA, these three proteins are labelled strongly. Also a minor band at approximately 66 K M_r may be stained. Again this probably represents the 66 K protein detected by anti-IFA in many tissues.

Analysis of all intermediate filament proteins was carried out by running identical sets of samples on a gel which was transferred to a sheet of nitrocellulose. Such sheets were cut and pieces were stained with different antibodies. Anti-IFA was always used to stain a portion of each immunoblot so that the proteins stained with each anti-intermediate filament protein antibody could be compared with the total complement of intermediate filament proteins in a sample.

The three antibodies raised against tadpole Triton-insoluble material were all found to stain some HT29 cell proteins and tadpole proteins on blots. However, none stained either rat brain or *Xenopus* spinal cord (not shown).

RD35/2a

RD35/2a stains two of the three cytokeratins in HT29 cells, the 41 K and 52 K M_r proteins (and the 66 K M_r protein) (Fig. 2A). It also stains at least three tadpole Triton-insoluble proteins (Fig. 4A). Two are of approximately 60 K M_r while the other is approximately 48 K M_r.

To confirm that this antibody binds only to intermediate filament proteins on gels, the staining patterns of RD35/2a on Triton-insoluble tadpole proteins and whole tadpole preparations were compared on gel blots. No new bands were seen

Table 1. *Immunoblotting data*

Antibody	Tadpoles	HT29 cells			
		41 K	45 K	52 K	66 K
anti-IFA	many bands	+	+	+	+
RD35/2a	2 bands of approx. 60 K, 1 of 48 K	+		+	+
RD35/3a	52 K, 48 K, 43 K	+			
D3/3a	52 K, 48 K	+	+	+	

Data obtained using Triton-insoluble material from *Xenopus* tadpoles. Major bands stained by the antibodies are listed.

in whole tadpole preparations (Fig. 4B) showing that all the tadpole antigens recognized by RD35/2a are present in preparations enriched in intermediate filaments.

RD35/3a

RD35/3a stains only one of the HT29 cell cytokeratins, the 41 K M_r protein as can be seen in the autoradiograph shown in Fig. 2B. It also stains several tadpole proteins. It binds most strongly to two proteins of approximately 52 K and 48 K M_r in both Triton-insoluble preparations (Fig. 5A) and whole tadpole homogenates (Fig. 5B). Several other bands are also visible in Triton-insoluble preparations, including a strongly stained band of approximately 43 K M_r. The two bands at approximately 60 K M_r to which RD35/2a bind may also be very weakly stained by RD35/3a (Fig. 5A).

D3/3a

D3/3a stains the three cytokeratins of HT29 cells but not the 66 K M_r protein (Fig. 2C). In tadpoles (Triton-insoluble material and total protein preparations) this antibody stains proteins of approximately 52 K and 48 K M_r (Fig. 6A,B).

A summary of the anti-cytokeratin blotting data is shown in Table 1.

Anti-vimentin

One of the anti-vimentin antibodies was used to stain immunoblots of *Xenopus* spinal cord, rat brain and 3T3 cells (Fig. 7A–C). Rabbit anti-vimentin IgG (Hynes & Destree, 1978) stains the 58 K M_r protein of rat brain (Fig. 7A) and of 3T3 cell (Fig. 7C) preparations. In rat brain, there is also a band of lower M_r than vimentin, which is presumed to be the result of proteolysis, to which vimentin is known to be very susceptible (e.g. Nelson & Traub, 1982). Both of the anti-vimentin antibodies used in this study have previously been shown to stain a protein from *Xenopus* oocytes and tadpoles of slightly lower M_r than 3T3 cell vimentin (Godsave *et al.* 1984*b*) in agreement with the findings that *Xenopus* vimentin is smaller than mammalian vimentin with an M_r of approximately 55 K (Nelson & Traub, 1982). In Fig. 7B it can be seen that the only antigen recognized by anti-vimentin in *Xenopus* spinal cord is also of around 55 K M_r.

Other antibodies

The rabbit anti-GFAP used in this study recognizes the 50 K GFAP of mammalian brain cytoskeleton (Fig. 8A). This antibody also shows weaker binding to the 66 K protein of rat brain and to a protein of lower M_r than GFAP which may be a product of proteolysis. In *Xenopus* spinal cord (Fig. 8B), the rabbit anti-GFAP stains a band of M_r just below 66 K and several bands of lower M_r which are also probably caused by proteolysis. The major band stained by anti-GFAP in the *Xenopus* spinal cord corresponds to the major protein stained by anti-IFA in these samples (see Fig. 1B). This is also the case with rat brain where the 50 K protein is clearly the most abundant intermediate filament protein present. No staining of tadpole Triton-insoluble material by anti-GFAP is evident (not shown).

The two anti-neurofilament antibodies used, 147 and RT97 (Anderton *et al.* 1982), both recognize the 200 K NF protein of rat brain and proteins of similar M_r in *Xenopus* swimming tadpoles and adult *Xenopus* spinal cord (Fig. 9). Additional bands are also seen in adult *Xenopus* spinal cord with both antibodies. Those stained by RT97 may be nuclear proteins since this antibody is found to stain nuclei in immunofluorescence experiments. The major proteins stained by both 147 and RT97 in spinal cord have the same electrophoretic mobility in SDS–PAGE as the highest M_r protein recognized by anti-IFA in Triton-insoluble material from spinal cord (Fig. 1F).

Analysis of antibody specificities by immunofluorescence

All of the above antibodies were tested on tissue sections of adult *Xenopus* by immunofluorescence. Both ethanol- and TCA-fixed tissue gave the same staining pattern. Staining was weaker, but cytoarchitecture better, with TCA.

RD35/2a

RD35/2a fails to stain the epidermis of adult frog skin apart from a few cells that are arranged perpendicularly to the surface and some cells associated with glands (Fig. 10A). In the dermis, it only stains blood vessel linings (not shown). In adult *Xenopus* gut, this antibody stains the lining of the blood vessels in the wall of the gut (Fig. 10B) but not epithelium or connective tissue of the gut (Fig. 10B).

RD35/3a

In adult skin, RD35/3a stains the basal layer of the epidermis and the perpendicular epidermal cells stained by RD35/2a (Fig. 11). The dermis is unstained by this antibody.

D3/3a

D3/3a stains the basal layer of the epidermis (Fig. 12A) and the epithelial lining of the gut (Fig. 12B), both in a filamentous pattern. In both skin and gut the connective tissue is unstained.

Anti-cytokeratins and anti-vimentins

The staining patterns described above were compared with those of already characterized anti-cytokeratins LE65 and LP3K, and with the anti-vimentin.

LP3K in adult skin does not stain epidermis, but stains numerous glands (Fig. 13A).

LE65 is also negative on epidermis, but it binds to the linings of blood vessels in the dermis (Fig. 13B).

The *anti-vimentins* stain cells in the dermis as expected, but not epidermis or glands (Fig. 13C). Interestingly, the anti-vimentins also stain the lining cells of blood vessels (Fig. 13D), although resolution was not sufficient to establish whether vimentin and cytokeratin were coexpressed by each cell.

In the gut, LP3K and LE65 both stain all cells of the gut epithelium but not the connective tissue, while the reverse is true of anti-vimentin (Godsave *et al.* 1984*a*,*b*).

Table 2 shows a synopsis of immunofluorescence staining patterns of the cytokeratin antibodies used in this study. LE65 and LP3K are anti-cytokeratins that have been previously characterized (Lane, 1982; Lane, Hogan, Kurkinen & Garrels, 1983; Godsave *et al.* 1984*a*).

Appearance and distribution of intermediate filament proteins during early Xenopus *development*

(A) *The developing nervous system*

(1) *Neural fold stage*

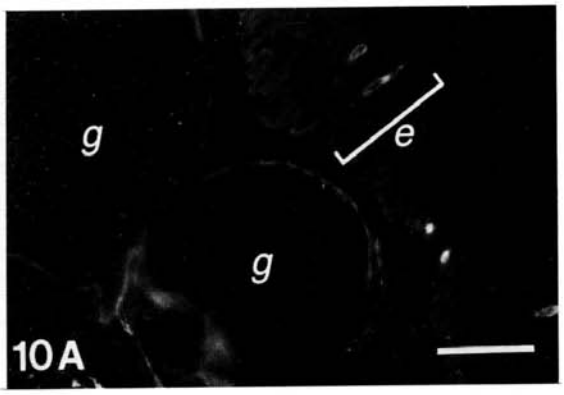
Cytokeratin. At the neural fold stage (stage 17/18), three of the anti-cytokeratins, LP3K (Fig. 14A), RD35/3a (Fig. 14B) and D3/3a (not shown) stained ectodermally derived tissues. LP3K stained both the epidermis and neural folds with approximately equal intensity. However, RD35/3a and D3/3a stained the epidermis strongly while showing significantly reduced staining in the neural folds (Fig. 14). Thus the intermediate filament constitution of the neural folds is already distinct from that of the embryonic epidermis. Interestingly, other differences between epidermis and presumptive neural tissue have been described at even earlier stages (Slack, 1984; Jones & Woodland, 1986).

Other intermediate filament antibodies fail to stain the neural folds.

(2) *Neural tube stage*

At stage 25/26, the neural folds have closed, but there is no obvious cell differentiation in the neural tube.

Cytokeratin. LP3K, RD35/3a and D3/3a continue to stain the epidermis (RD35/3a Fig. 14G) and LP3K also continues to stain neural cells particularly near the luminal surface of the tube. This area around the central canal is also stained by the anti-cytokeratins RD35/2a and LE65 (Fig. 14D) but not by control tissue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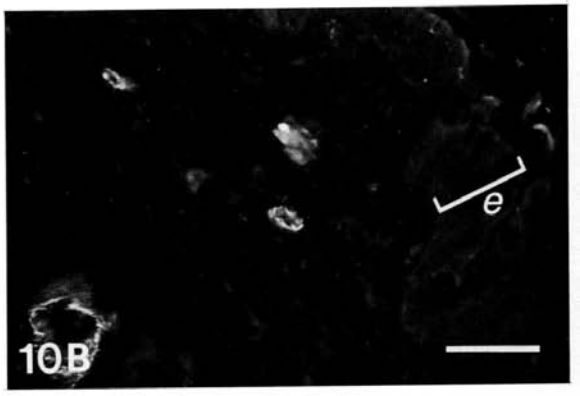
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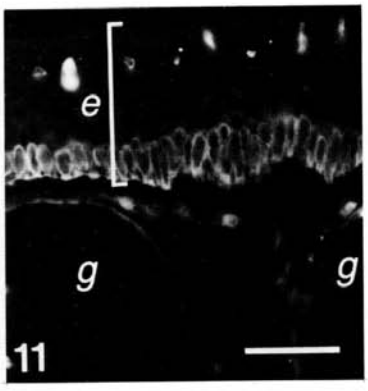
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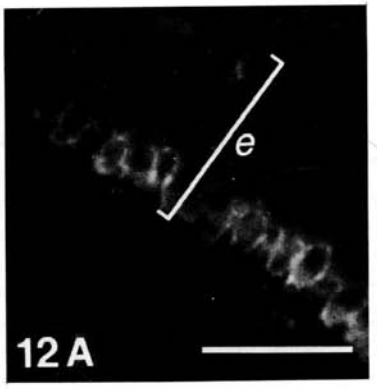
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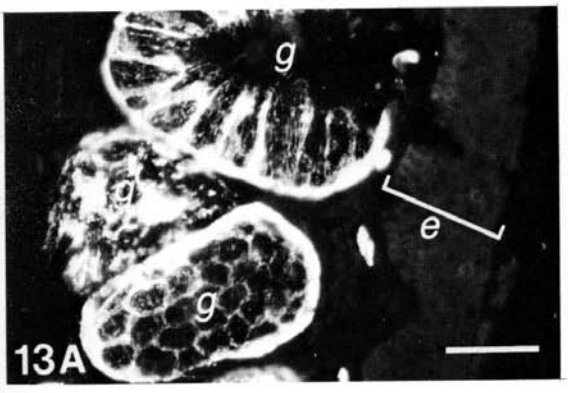
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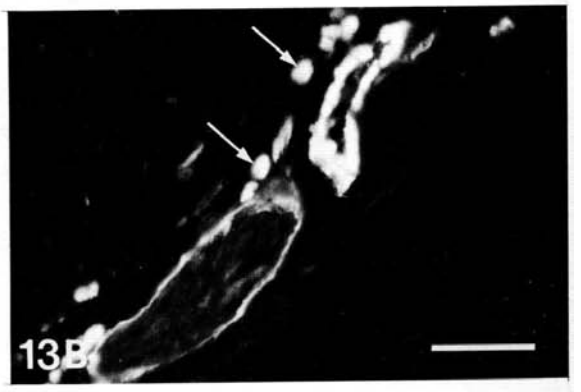
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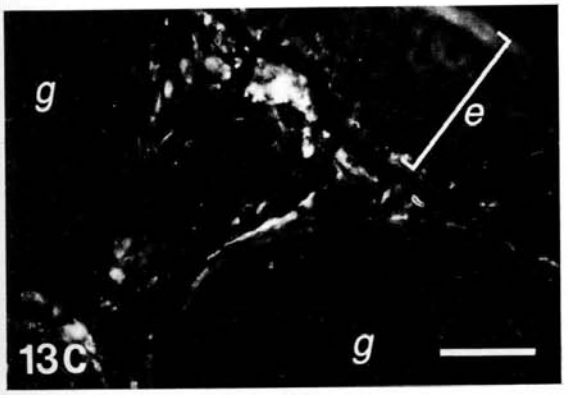
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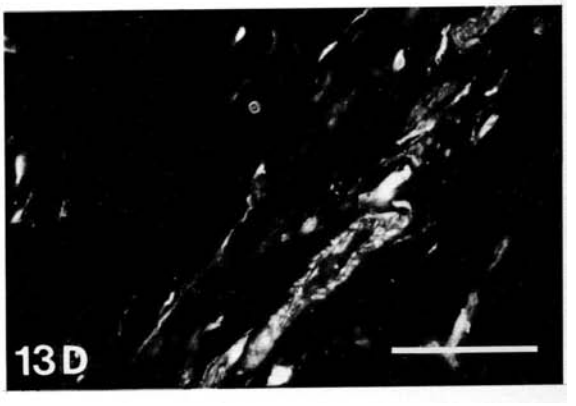

13D

culture medium (Fig. 14E). Some background staining of the epidermis is observed in control sections (Fig. 14E). This appears as a result of the long incubation period (16 h) used with anti-cytokeratin antibodies in order to observe labelling of weakly staining structures at early stages of development.

Vimentin. Anti-vimentins also stain discrete areas of the early neural tube, but at the opposite (outer) surface (Fig. 14F). Probably the cytokeratin-containing cells are those derived from the outer ectodermal layer of the gastrula while the inner layer of ectoderm provides the precursors of the vimentin-containing cells of the neural tube.

No neurofilament or GFAP staining is visible at the neural tube stage (not shown).

(3) *Late tailbud stage*

By stage 33/34 the neural tube in the trunk region is beginning to differentiate into a spinal cord, with developing neurone cell bodies and processes visible in the mantle and marginal zones, respectively.

Cytokeratins. Three anti-cytokeratins stain the stage 33/34 spinal cord, LE65, LP3K and RD35/2a. LE65 and RD35/2a stain in a similar pattern to that seen at stage 25/26. Strongest staining is seen in a line around the luminal surface. However, staining is also seen in cells that span the wall radially on its ventral side (Fig. 15A). LP3K gives weaker staining in spinal cord than LE65 and RD35/2a and it is present chiefly in dorsal median regions (not shown).

Vimentin & GFAP. Anti-vimentin and anti-GFAP both stain the most marginal (outermost) regions of the spinal cord (Fig. 15B,C).

There is no staining of the stage 33/34 spinal cord with the anti-neurofilament antibodies or with the epidermis-staining anti-cytokeratins, RD35/3a and D3/3a (Fig. 15D).

(4) *Swimming tadpole stage*

By the swimming larva stage (stage 48) the number of nerve fibres in the marginal zone has increased considerably, and meninges are beginning to appear around the spinal cord.

Figs 10–12. Indirect immunofluorescence labelling of sections of adult *Xenopus* skin and gut with anti-cytokeratin monoclonal antibodies. Abbreviations: *e*, epidermis; *g*, gland; *ge*, gut epithelium. Bars, 50 μm.

Fig. 10. RD35/2a staining of (A) skin and (B) gut.

Fig. 11. RD35/3a staining of skin.

Fig. 12. D3/3a staining of (A) skin and (B) gut.

Fig. 13. Indirect immunofluorescence labelling of adult *Xenopus* skin with (A) LP3K cytokeratin antibody; (B) LE65 cytokeratin antibody; (C,D) rabbit anti-vimentin IgG. B,D demonstrate the presence of cytokeratin and vimentin in blood vessels of the dermis. Arrows mark densely staining bodies which are also present in controls. Abbreviations: *e*, epidermis; *g*, gland. Bars, 50 μm.

Table 2. *Immunofluorescence*

	RD35/2a	RD35/3a	D3/3a	LE65	LP3K
(A) *Adult* Xenopus *tissues*					
skin					
epidermis	+*	+	+	−	−
blood vessels	−	−	−	+	+
connective tissue	−	−	−	−	glands
gut					
epithelium	−		+	+	+
blood vessels	+		−		
connective tissue	−		−	−	−
spinal cord	+†	−	−	+	+
(B) *Embryonic tissues*					
skin stages 17–48	−	+	+	−	+
CNS stage 25/26–48	+	−	−	+	+
notochord					
stage 25/26	+	−	−	+	+
stage 48	+	+	+	+	+

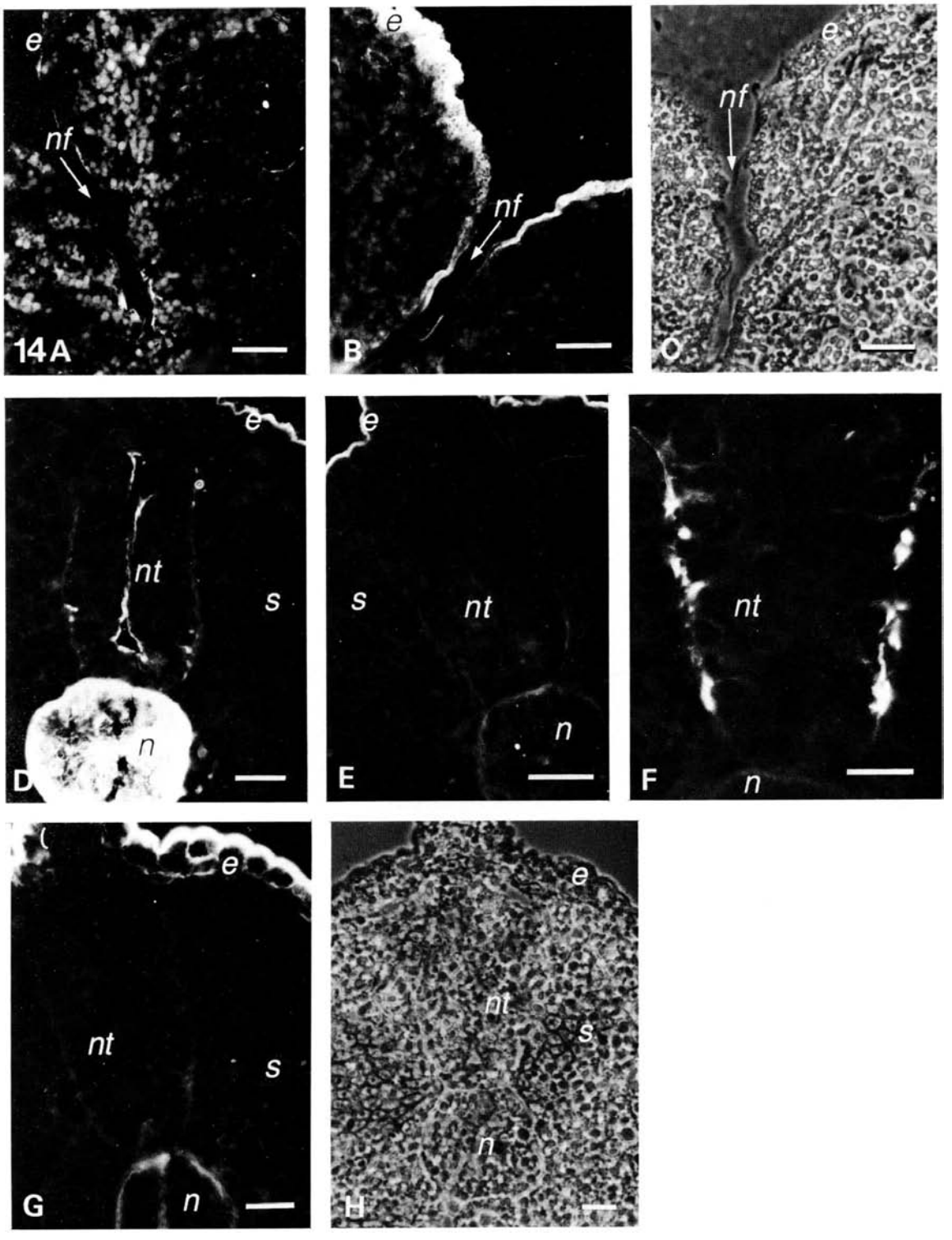
* Only a few perpendicular cells of the epidermis are stained by RD35/2a.
† Only blood vessels are stained.

Cytokeratins. At this stage, LE65 and RD35/2a show brighter staining, in a pattern similar to earlier stages but more obvious. Staining is concentrated around the central lumen and in a network throughout the spinal cord, particularly in radially arranged cells. The Mauthner neurones are conspicuously negative. The meningeal cells round the outside also stain with these antibodies (Fig. 16A), as well as with LP3K. LP3K still picks out a dorsal median section of the spinal cord (Fig. 16B).

Vimentin. Anti-vimentin stains a network of cells in the spinal cord including prominent radially arranged cells (Fig. 16C). This pattern is distinct from that picked out by the anti-cytokeratins LE65 and RD35/2a and probably several populations of cells are present.

GFAP and neurofilaments. Both anti-GFAP (Fig. 16D) and anti-neurofilament (Fig. 16E) now stain the spinal cord strongly. Both stain the marginal zone, made

Fig. 14. (A,B) Indirect immunofluorescence labelling of neural folds of stage 17/18 embryos with cytokeratin antibodies: (A) LP3K; (B) RD35/3a. (C) Phase contrast micrograph of stage 17/18 neural folds. Abbreviations: *e*, epidermis; *nf*, neural fold; Bars, 20 μm. (D–G) Indirect immunofluorescence labelling of the neural tube and notochord of stage 25/26 embryos with (D) LE65 cytokeratin antibody; (E) control culture medium; (F) rabbit antibodies to vimentin; (G) RD35/3a cytokeratin antibody. The fluorescence seen in the epidermis of the LE65 stained section (D) is thought to be non-specific since it was also observed in controls (E). Epidermis-staining anti-cytokeratins give much brighter staining (G). (H) Phase contrast micrograph of stage 25/26 neural tube. Abbreviations: *nt*, neural tube; *s*, somites; *n*, notochord; *e*, epidermis. Bars, 20 μm.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up of glial cells and nerve fibres. Most of the fibres stained appear to be in cross section (i.e. running in the craniocaudal axis) though anti-neurofilament picks out some fibres leaving the spinal cord towards the somites.

(5) *Adult spinal cord*

Cytokeratin. The adult *Xenopus* spinal cord is still stained by the three anti-cytokeratins that labelled embryonic spinal cord (i.e. LE65, RD35/2a and LP3K). However, RD35/2a now stains filaments in blood vessel walls only (Fig. 17A). The other two, LE65 and LP3K, also stain blood vessels as well as ependymal cells and radially arranged cells or cell processes running from the ependyma towards the ventral median fissure (Fig. 17B). They also give some staining in the rest of the white matter and the meninges (Fig. 17C).

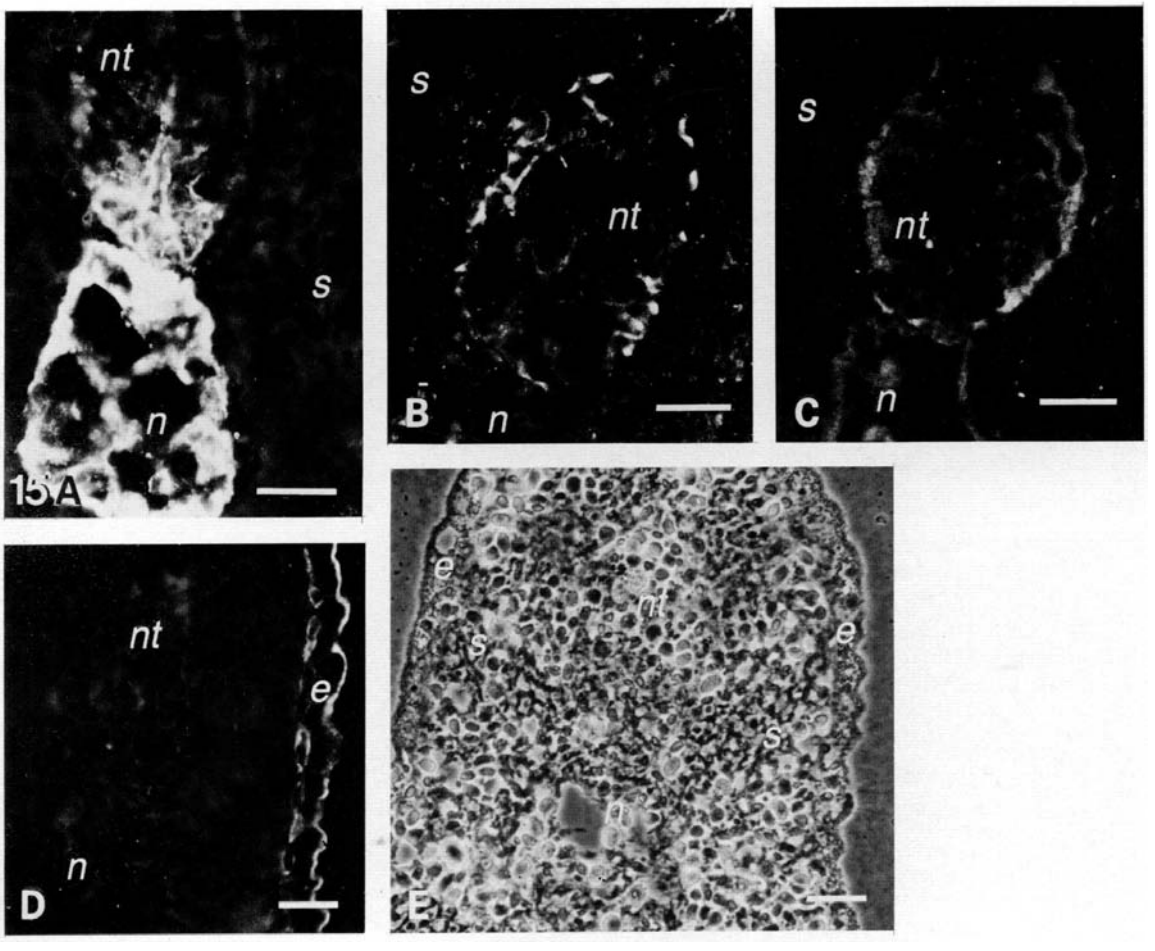

Fig. 15. Indirect immunofluorescence labelling of the stage 33/34 spinal cord and notochord by (A) RD35/2a; (B) rabbit anti-vimentin IgG; (C) rabbit anti-GFAP serum; (D) D3/3a. (E) Phase contrast micrograph of stage 33/34 spinal cord and notochord. Abbreviations: *nt*, neural tube; *n*, notochord; *s*, somites; *e*, epidermis. Bars, 20 μm.

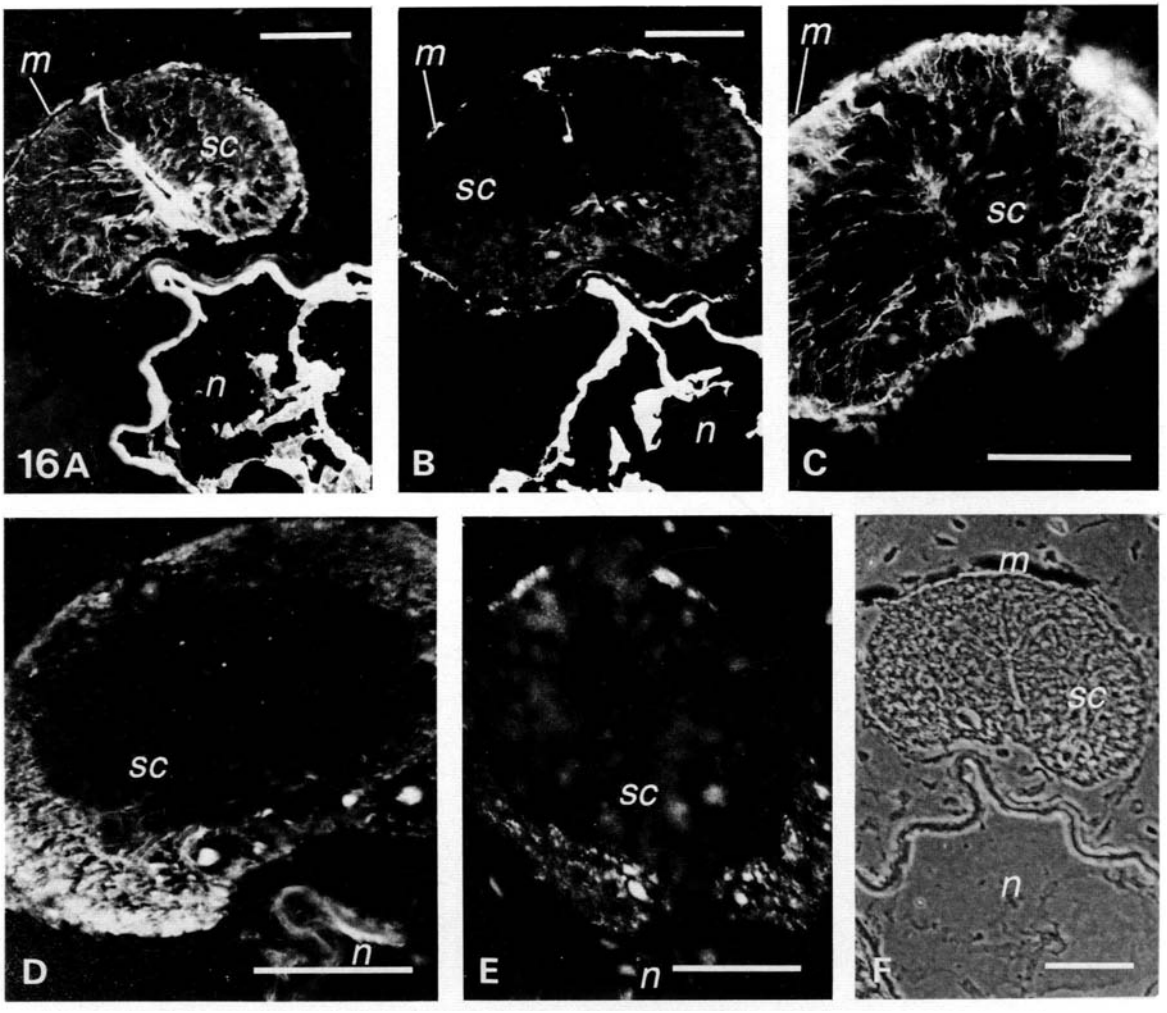

Fig. 16. Indirect immunofluorescence labelling of the spinal cord and notochord of stage 48 embryos with (A) RD35/2a; (B) LP3K; (C) rabbit anti-vimentin serum; (D) rabbit anti-GFAP; (E) monoclonal antibody to neurofilament 200 K protein (147). Abbreviations: *n*, notochord; *sc*, spinal cord; *m*, meninges. Bars, 50 μm.

Vimentin. Anti-vimentin stains radial cells between neurones (unstained) as well as a dense network of filaments in the grey matter (Fig. 17E). The ependymal cells appear to stain with vimentin as well as with the anti-cytokeratins (Fig. 17D).

GFAP. Anti-GFAP stains non-neuronal cells strongly (Fig. 17F). Many long radial glia are stained.

Neurofilaments. RT97 and 147 stain the neuronal processes in the white matter strongly (Fig. 17G).

(B) *The notochord*

The only intermediate filament antibodies to stain notochord were the anti-cytokeratins. Both LE65 and RD35/2a stained notochord right from the neural fold stage (not shown) and by the neural tube stage (25/26) when the cells are becoming vacuolated, LE65 and RD35/2a stained it distinctly (Fig. 14D). By the

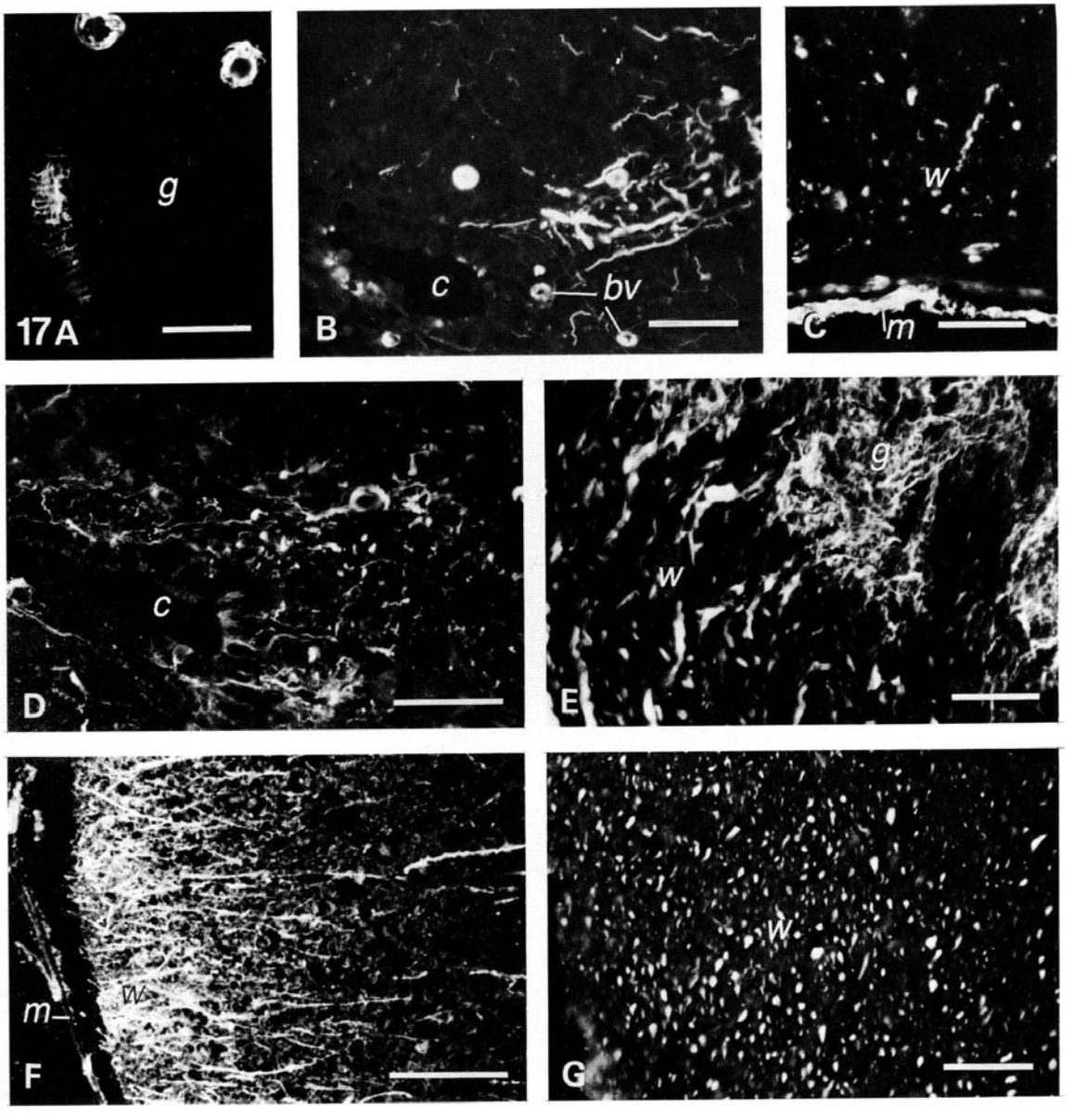

Fig. 17. Indirect immunofluorescence labelling of adult *Xenopus* spinal cord with (A) RD35/2a; (B) LE65; (C) LP3K; (D,E) rabbit anti-vimentin serum; (F) rabbit anti-GFAP serum; (G) 147, anti-neurofilament 200K protein monoclonal antibody. Abbreviations: *w*, white matter; *g*, grey matter; *m*, meninges; *bv*, blood vessels; *c*, central canal. Bars, 50 μm.

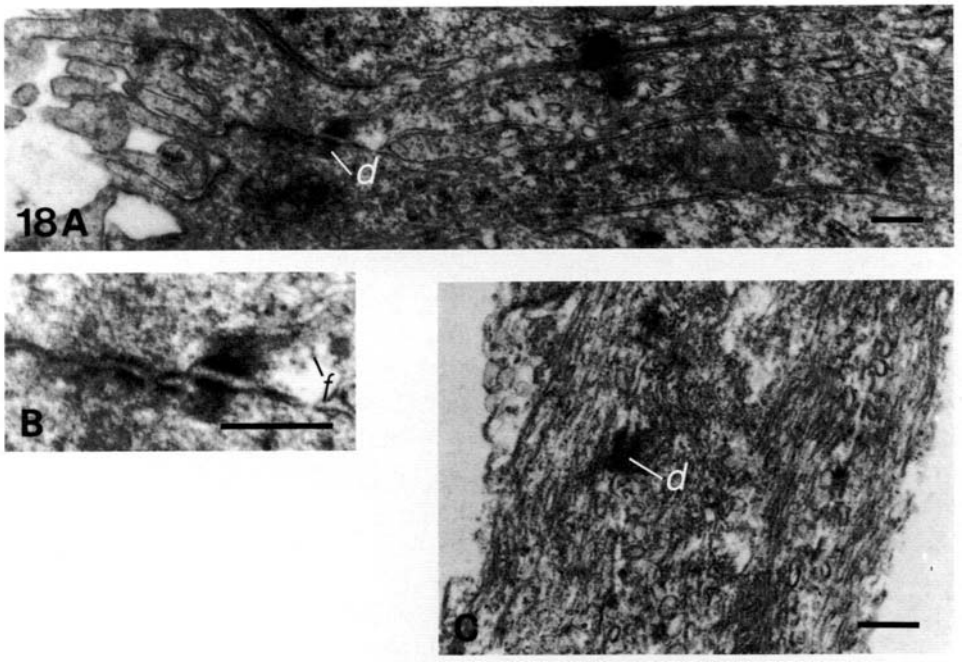

Fig. 18. Electron micrographs showing (A) desmosomes in ependymal cells in the spinal cord of a swimming tadpole; (B) high power view of desmosomes shown in A. Intermediate-sized filaments can be seen in association with the desmosome. (C) Desmosomes in cells of the notochord. Abbreviations: *d*, desmosome; *f*, intermediate-sized filaments. Bars, 1 μm.

swimming tadpole stage (stage 48) all five anti-cytokeratins tested reacted with it (e.g. Fig. 16A,B).

(C) *The epidermis*

Cytokeratins are present in the cortex of the oocyte and egg, and near the surface of the early embryo as previously shown (Godsave *et al.* 1984*a*; Gall *et al.* 1983). In this study, the earliest stage examined was neural fold stage (17/18) when three of the anti-cytokeratins stained both ectodermal layers (LP3K, RD35/3a and D3/3a) (Fig. 14). These three antibodies continue to stain epidermis throughout early development (Figs 14G, 15D).

Electron microscopy

Cytokeratins have not previously been reported in either the developing nervous system or notochord. Transmission electron microscopy was therefore carried out to look for the presence of desmosomes since they normally have cytokeratin filaments associated with them. Fig. 18A,B shows the inner (ependymal) layer of the swimming tadpole spinal cord, which stains strongly with anti-cytokeratins LE65 and RD35/2a (Fig. 16A). Abundant desmosomes and associated intermediate filament bundles are seen. The ependymal cells were seen to possess

radial processes which pass between the neuroblasts of the mantle layer (not shown). The notochord, too, is seen to contain occasional desmosomes (Fig. 18C) at the same stage.

DISCUSSION

(A) *The specificity of antibodies used*

The three new monoclonal antibodies described in this study, RD35/2a, RD35/3a and D3/3a, were all raised against Triton-insoluble preparations from whole swimming tadpoles. When these preparations were immunoblotted with anti-IFA, which reacts with all intermediate filament types, there was found to be a number of bands between approximately 43 K and 66 K M_r. All of the new monoclonal antibodies stained several of these Triton-insoluble tadpole proteins. No other bands were stained when whole tadpole preparations were used, therefore it is likely that the antibodies only recognize intermediate filament proteins. All of the new antibodies also bound to one or more of the cytokeratin polypeptides from the human epithelial HT29 cells. This fact, as well as their epithelial staining in immunocytochemical studies, led us to believe that they were specific for cytokeratins. RD35/2a stained some cell types that have not previously been reported to contain cytokeratins. However, in most cases these cells were also stained by LE65, a well-characterized anti-cytokeratin (e.g. Lane, 1982; Lane *et al.* 1983; Godsave *et al.* 1984*a*). The presence of cytokeratin intermediate filaments in notochord and neuroependymal cells was further supported by electron microscopic evidence of desmosomes and desmosome-associated filaments in these cells.

The other antibodies used have been characterized in other species (see references in Materials and Methods). They apparently maintain their tissue-specificity in *Xenopus*. It was interesting that the protein blotted by anti-GFAP in *Xenopus* spinal cord was of significantly higher M_r than mammalian and chicken GFAP (both approximately 50 K M_r proteins). Further characterization of intermediate filament proteins in *Xenopus* and other species is clearly necessary in order to explain species differences in intermediate filaments.

(B) *Intermediate filament proteins in the developing nervous system*

Several changes in type and distribution of intermediate filament proteins are seen during histodifferentiation of the *Xenopus* neural tube, which is derived from the surface ectoderm. This ectoderm contains cytokeratins that are stained by several antibodies (LP3K, RD35/3a and D3/3a) but some of these are lost in the presumptive neural tissue (see Fig. 15). Once the neural tube is formed, however, new cytokeratins can be detected (with LE65 and RD35/2a) and proteins recognized by one of the anti-cytokeratins (LP3K) are present both in surface ectoderm and at the luminal surface of the early neural tube. Cytokeratins are present throughout development in the neuroependyma which, at the swimming tadpole stage, includes some radially arranged cells extending to the outer surface of the spinal cord. Cytokeratins are also present in the adult spinal cord.

Anti-vimentin, like some of the anti-cytokeratins, strongly stains some radial cells in the CNS although these are probably radial glia, in which vimentin has been found in other species (Tapscott *et al.* 1981; Bovolenta *et al.* 1984). The staining patterns of vimentin and cytokeratin are similar at this stage but are never identical, though it is possible that there is coexpression of the two intermediate filament types in some cells. Immuno-electron microscopy would be needed to confirm this.

Neurofilaments and glial filaments appear in the spinal cord at later stages with the development of functional neurones and glia.

Changes in intermediate filament type and distribution such as those we have described in the developing *Xenopus* central nervous system have been reported in other species although there are some differences in the timing of appearance of specific intermediate filament proteins and in the complements of proteins found in particular cell types. In the mouse (Bovolenta *et al.* 1984) and chick (Tapscott *et al.* 1981), vimentin is the first intermediate filament protein to appear in the nervous system. Replicating neuroblasts contain vimentin and start to express neurofilament proteins when process formation is detectable. At later stages, vimentin expression ceases in neurones (Tapscott *et al.* 1981). In rat embryos, the two smaller neurofilament proteins and vimentin appear at the same time at the neural tube stage (Raju *et al.* 1982). Once again vimentin and neurofilament protein are coexpressed before neurones eventually express the latter exclusively. The largest (200 K) neurofilament protein appears later than the 70 K and 155 K proteins (Shaw & Weber, 1982).

In developing glial cells, the pattern is essentially the same, with the early coexpression of vimentin and GFAP in chick (Bignami & Dahl, 1975; Tapscott *et al.* 1981) and mouse (Bovolenta *et al.* 1984) embryos.

The major difference between the studies described here and previous work, is the demonstration that ependymal cells in *Xenopus* embryonic and adult spinal cord contain cytokeratin (possibly coexpressed with vimentin) whereas in other species only vimentin is detected (Schnitzer *et al.* 1981). Our finding is supported by electron microscopy, when desmosomes and desmosome-associated filaments are seen, although it has recently been reported that other intermediate filament types may in rare cases be associated with desmosome-like structures (Kartenbeck, Schwechheimer, Moll & Franke, 1984). It is possible that these interspecies differences are explained by the unusual pattern of development of the CNS in *Xenopus* in which the neural tube forms from two layers of ectoderm, one of which probably gives rise to the ependyma and the other to the nervous tissue (Nieuwkoop & Faber, 1967; Smith & Malacinski, 1983).

(C) *The notochord*

The notochord differentiates very early in *Xenopus* development and the large vacuolated cells are very distinctive. However, its function in *Xenopus* is still unclear. Fertilized *Xenopus* eggs that have been treated with low doses of ultraviolet light can give rise to notochordless embryos. However, the embryos are still

able to grow in length and the neural tube can apparently form normally although it is not known what effects the lack of a notochord may have after hatching (Malacinski & Youn, 1981, 1982; Youn & Malacinski, 1983). The unusual morphology of the cells must be controlled by the cytoskeletal network and perhaps desmosome adhesions and their associated filament networks are important for this. The notochord has not previously been reported to contain cytokeratins, although it has been described as an epithelial structure and a basement membrane has been described around its periphery (Nakao, 1974). It will be interesting to see if cytokeratins are present in the notochords of other vertebrates.

(D) *The skin*

The *Xenopus* embryonic epidermis remains as two layers of flattened cells until around stage 50 when further differentiation starts to occur. The embryonic epidermis is known to contain cytokeratins, some of which are embryo specific (Jonas *et al.* 1985; Dale, Smith & Slack, 1985; Ellison, Mathieson & Miller, 1985) and we find that three of our anti-cytokeratin antibodies stain *Xenopus* epidermis at all embryonic stages examined. However, two of these, RD35/3a and D3/3a, stain only the basal layer of the adult epidermis and a few perpendicularly arranged cells. The other antibody that stains embryonic epidermis (LP3K) fails to stain adult epidermis, but it does stain glands and blood vessels in the dermis. The complement of keratins in adult *Xenopus* epidermis has previously been examined (Hoffman, Franz & Franke, 1985) and a number of cytokeratins appear to be present.

One of the most interesting aspects of this work is that it provides a new range of differentiation markers to study the activities of embryonic cells. Since its inception as a science, one of the major problems of developmental biology has been to pinpoint the time of cell commitment to certain lineages, and the control of this process. However, experiments on this process have been bedevilled by the lack of reliable markers at early larval stages to demonstrate the type of differentiation that cells are undergoing (see Slack, 1984), although several markers for epidermis (Slack, 1985; Dale *et al.* 1985; Jones & Woodland, 1986) and muscle (Dale *et al.* 1985; Gurdon, Fairman, Mohun & Brennan, 1985) have now been described. In this paper, we show that the use of antibodies against different intermediate filament proteins can at least distinguish between epidermal ectoderm and ectoderm that is determined to become neural tube, and between cells of the inner and outer layers of the neural tube. At an early stage it is also possible to distinguish notochord cells from their neighbours. LE65 already stains notochord at the neural fold stage (authors unpublished observations). Also, at later stages glial cells can be distinguished from neuronal and ependymal cells.

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