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

II. Identification and molecular characterization of desmin

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Summary

During embryogenesis of avian and mammalian species the formation of intermediate filaments (IFs) containing desmin is characteristic for myogenesis. In view of important differences of patterns of IF protein expression in embryogenic pathways of amphibia on the one hand and birds and mammals on the other, we have decided to study the expression of desmin during early embryogenesis of *Xenopus laevis* by cDNA hybridization and antibody reactions. Here we describe the isolation of a cDNA clone encoding *Xenopus* desmin and the deduced amino acid sequence (458 residues; M_r 52 800) which displays a very high degree of conservation during vertebrate evolution from *Xenopus* to chicken and hamster, with a similar degree of sequence divergence between all three species compared. In addition, we have noted, by both cDNA-hybrid-selection–translation and immunoblotting of cytoskeletal proteins a second desmin-related polypeptide of $M_r \sim 49\,000$. RNA (Northern) blot analyses show the occurrence of three different desmin mRNAs (1·9, 2·6 and 3·0 kb) which seem to represent different polyadenylation sites, displaying quantitative differences in different kinds of muscle tissues. During embryogenesis, desmin mRNA has first been detected in stage-14 embryos and then increases drastically to high levels at stage 18 and thereafter. Immunofluorescence microscopy using desmin-specific antibodies shows that this synthesis of desmin is restricted to somite tissue. The embryonic time course of synthesis of desmin and desmin mRNA is discussed in relation to those of other muscle proteins.

Key words: *Xenopus laevis*, oocytes, cytoskeleton, intermediate filaments, desmin, somites.

Introduction

Of the various IF proteins that are differentially synthesized during embryogenesis, desmin has been shown to be typical for myogenesis, i.e. the formation of the cross-striated muscle tissue of myocardium and skeletal muscles and of the various kinds of smooth muscles (Cooke, 1976; Lazarides & Hubbard, 1976; Small & Sobieszek, 1977; Bennett *et al.* 1978, 1979; Lazarides *et al.* 1982). Desmin has also been found in the dispersed 'myoid cells' of the thymus (Henry, 1968; Hayward, 1972; Wekerle *et al.* 1975; Engel *et al.* 1977; Kao & Drachman, 1977; Drenckhahn *et al.* 1979; Dardenne *et al.* 1987; Kirchner *et al.* 1988) and the testis (Virtanen *et al.* 1986), in certain endothelial cells and pericytes (Fujimoto & Singer, 1986; Stamenkovic *et al.* 1986), stellate cells of liver of some species (Burt *et al.* 1986; Mijazaki *et al.* 1986), in cells of the interfollicular reticulum of lymph nodes and other lymphoid organs (Franke & Moll, 1987; Toccanier-Pelte *et al.* 1987) and in certain tumours, including myosarcomas as well as tumours not commonly regarded as myogenic (Osborn & Weber, 1983; Denk *et al.* 1983; Altmannsberger *et al.* 1985; Ellis *et al.* 1985; Gatter *et al.* 1986; Leader *et al.* 1987; Brown *et al.* 1987; Norton *et al.* 1987).

During myogenesis, the proportion of vimentin originally predominant in the muscle precursor cells appears to decrease gradually, whereas desmin increases, in some kinds of muscle to the extent that vimentin is no longer detectable (Bennett *et al.* 1979; Granger & Lazarides, 1979; Lazarides *et al.* 1982; Osborn *et al.* 1982). Clearly, vimentin and desmin can copolymerize and form heterotypic IFs in certain muscle tissues and cell culture lines (Steinert *et al.* 1981; Quinlan & Franke, 1982). However, desmin can also be coexpressed with cytokeratins such as in fetal myocardium of human and chicken, in myoid cells of bovine testis, and in some smooth muscle cells, notably in fetal tissues (Longo *et al.* 1987; Huitfeldt & Brandtzaeg, 1985; Brown *et al.* 1987; Jahn *et al.* 1987; van Muijen *et al.* 1987; Kasper *et al.* 1988; Kuruc & Franke, 1988).

During early mammalian and avian embryogenesis,

IFs containing desmin have not been detected (e.g. Jackson *et al.* 1980, 1981; Tapscott *et al.* 1981; Bignami & Dahl, 1984; Erickson *et al.* 1987), and its advent appears to be correlated with the synthesis of other myogenic marker proteins (e.g. Fuseler *et al.* 1981; Lazarides *et al.* 1982; Whalen *et al.* 1982; Danto & Fishman, 1984; Fishman & Danto, 1984; Hayward & Schwartz, 1986; Mahdavi *et al.* 1986; Robert *et al.* 1986). For early amphibian embryogenesis, in which the expression pattern of IF proteins is different in that most, if not all, cells contain cytokeratins (Franz *et al.* 1983; Godsave *et al.* 1984, 1986; Wylie *et al.* 1986; Herrmann *et al.* 1989), it has been reported that several typical muscle proteins, including α-actins, are first synthesized after gastrulation and are specifically induced in the forming muscle tissue (Sturgess *et al.* 1980; Gurdon *et al.* 1984; Mohun *et al.* 1984; Wilson *et al.* 1986; Mohun & Garrett, 1987; for a recent review, see Gurdon, 1987). However, the myogenic IF protein, desmin, has not yet been included in studies of amphibian embryogenesis. Therefore, we have isolated and characterized a desmin cDNA clone from an embryonic library and have used it, in combination with desmin-specific monoclonal antibodies, to identify the time and location of desmin synthesis in amphibian development.

Materials and methods

Maintenance of animals, cloning and sequencing procedures as well as RNA preparations and RNA blot experiments were performed as described in Herrmann *et al.* (1989). The radioactive probe for Northern blot analyses was constructed from a *Bam*HI/*Hin*dIII-restricted, 670 nucleotides long 5′end-fragment of pXenDes1 (containing 25 nucleotides of polylinker), using the random prime labelling kit from Boehringer (Mannheim, FRG). It was applied at $1{\cdot}2\times10^6$ cts min ml^{-1} hybridization solution.

For preparation of cytoskeletal proteins from the muscular layer of *Xenopus* oesophagus, 20 μm thick cryostat sections of oesophagus from adult animals were microdissected and extracted with buffers containing 1·5 M-salt and 1% Triton X-100 (Herrmann *et al.* 1989). Stage-18 embryos were extracted accordingly, and aliquots of high-salt-resistant fractions ('cytoskeletal proteins') equivalent to 50 embryos were loaded in each isoelectric focusing separation in two-dimensional gel electrophoresis.

Immunoblotting and immunofluorescence microscopy were performed as described (Herrmann *et al.* 1989), using a monoclonal antibody specific for desmin (Debus *et al.* 1983; from Boehringer, Mannheim, FRG).

Results

(A) *Characterization of desmin cDNAs*

In the course of screening an embryonic cDNA library for *Xenopus* vimentin we isolated two clones with insert sizes of about 3 kb that gave identical restriction maps which, on the other hand, were completely different from those obtained with the *Xenopus* vimentin clones. When one of these clones was used in hybrid-selection experiments with total RNA from *Xenopus* stage-18 embryos, mRNA was isolated that gave rise, after translation *in vitro* and two-dimensional gel electrophoresis, to two polypeptides of $M_r\sim53\,000$ and $\sim49\,000$, respectively, the larger of which was more basic than the smaller one (Fig. 1A,B). Both radioactive polypeptides comigrated with the most basic component of two prominent series of spots of cytoskeletal proteins from the smooth muscle layer of *Xenopus* oesophagus (Fig. 1A,B) identified as desmin-related by their specific reaction with a monoclonal desmin antibody (Fig. 1C). A cytoskeletal fraction from stage-18 embryos (Fig. 1D) revealed upon immunoblotting with a monoclonal desmin antibody two spots which corresponded to the two products of the hybrid-selection experiments (arrows in Fig. 1E). These results indicated that the isolated clone encoded desmin, and therefore we designated the clone pXenDes1. The $M_r\sim49\,000$ component is clearly a desmin-related polypeptide, but its chemical relationship to the M_r 53 000 polypeptide requires further clarification.

In Northern blot analyses of heart muscle RNA this clone reacted with two mRNA bands of ~3·0 and 1·9 kb (Fig. 2, lane 1). With skeletal muscle RNA we noticed the same two bands but, in addition, another band of ~2·6 kb (Fig. 2, lane 2). In RNA samples from ovary (lane 3) and cultured XLKE-A6 cells (lane 4) no signals were detected.

(B) *Nucleotide sequence and deduced amino acid sequence of desmin*

The sequence of the desmin clone, pXenDes1, is presented in Fig. 3. It contains 2995 bp and the second ATG is found within the consensus sequence ACCATGPu (Kozak, 1986). The 116 bp 5′-untranslated region is followed by an open reading frame of 1374 bp coding for 458 amino acids which corresponds to a calculated molecular weight of 52 799, including the initial methionine that is probably lost post-translationally. This value is slightly lower than that given for hamster desmin (cf. Quax *et al.* 1985) which contains 468 amino acids, corresponding to a molecular weight of 53 308. The TAA stop codon introduces a 1505 bp 3′-untranslated region that contains three possible poly-adenylation sites at positions 1754, 2114 and 2905. Whether these poly(A) addition sites correspond to the mRNA bands of 1·9, 2·6 and 3·0 kb in RNA blot experiments of cardiac and skeletal muscle (Fig. 2) or whether there are further downstream poly-adenylation sites remains to be examined.

Comparison of the amino acid sequence deduced from pXenDes1 with those of chicken and hamster desmin showed a close relationship (Fig. 4). The amino acids in the α-helical rod show 83% identical positions (270 out of 326) in all three species, most of the differing positions being conservative exchanges. Remarkably, this homology extends to the tail region, which displays 77% (*Xenopus*/chick) and 85% (*Xenopus*/hamster) identical amino acids. In direct contrast, the head region is, apart from the highly conserved first 20 amino acids, more variable in size and in sequence (58 and 65% identical amino acids with respect to hamster and

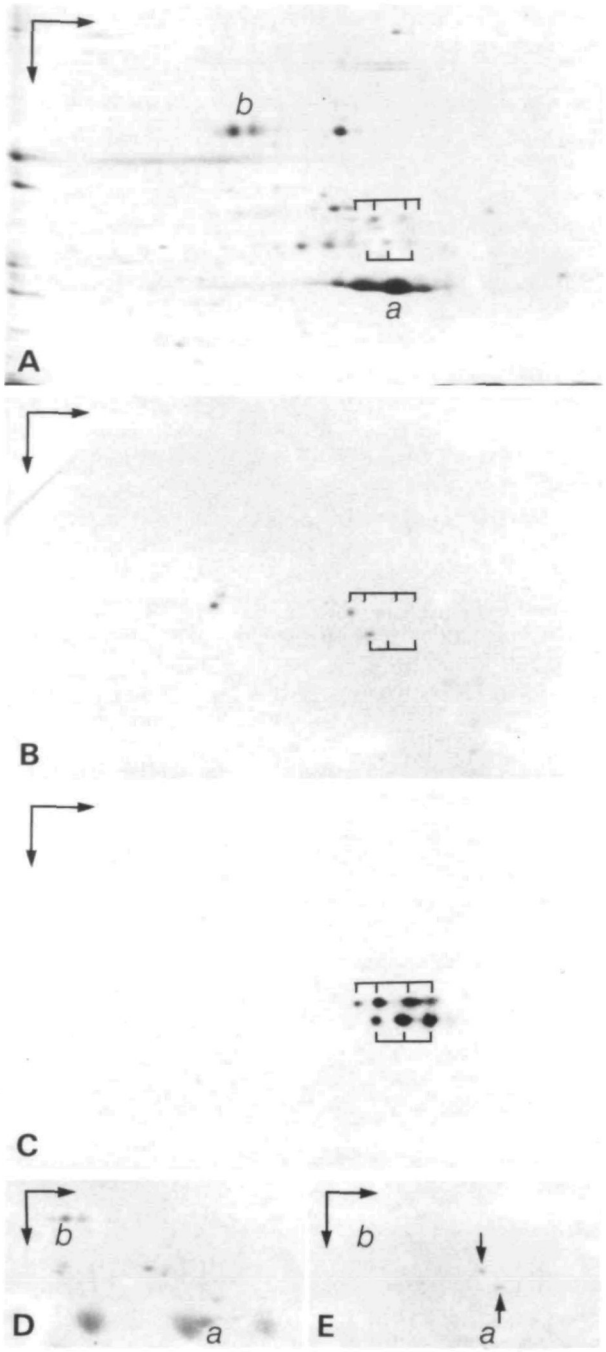

Fig. 1. Identification of the polypeptide encoded by the cDNA clone pXenDes1, using *in vitro* translation of mRNA selected from stage-18 embryo RNA and two-dimensional coelectrophoresis of the radioactively labelled translation products with cytoskeletal proteins from the muscular layer of *Xenopus* oesophagus (horizontal arrow, IEF; vertical arrow; SDS–PAGE). (A) Coomassie-brilliant-blue-stained gel loaded with cytoskeletal proteins from the muscular layer of oesophagus. (B) Autoradiograph of the gel shown in A, revealing [^{35}S]methionine-labelled products of *in vitro* translation of mRNA selected by hybridization with clone pXenDes1. (C) Immunoblot detection of desmin in a parallel gel electrophoresis, using monoclonal antibody to desmin. (D) Ponceau-S-stained nitrocellulose replica from gels loaded with cytoskeletal proteins from stage-18 embryos. (E) Immunoblot detection of desmin using monoclonal antibody to desmin. Arrows indicate the reactive polypeptides. Bars in A and B designate positions of immunoreactive desmin polypeptides as identified from the immunoblot in C. The second series of spots of lower M_r in B are either the corresponding proteolytic breakdown products or represent a genuine desmin-related polypeptide; in A and C additional, more acidic desmin variants are evident, compared to the components seen in the translational assay (B) and in the immunoblot of the cytoskeletal fraction of stage-18 embryos (E). *a*, Actin; *b*, bovine serum albumin.

chicken). Interestingly, however, the three possible phosphorylation sites of the protein kinase A type RXS reported for chicken desmin (cf. Geisler & Weber, 1988) are also present in *Xenopus* desmin (positions 40, 47 and 62; the second site is modified in the *Xenopus* sequence to KGAS, and to KGS in hamster desmin).

Sequence comparison of vimentin and desmin in the three species shows that the α-helical rod and the tail are very similar in the two IF proteins, whereas the head region seems to tolerate a higher degree of variability. Therefore, it is especially notable that in both vimentin and desmin the head contains shortly after the initial methionine, a highly conserved nonapeptide SSYRRXFGG (X being isoleucine or methionine in vimentin, and threonine in desmin; Fig. 5). Following this consensus sequence, a PSFS sequence is conserved in desmin of *Xenopus* and hamster, and a similar motif is seen in the corresponding position of chicken desmin. In desmin, the number and the se-

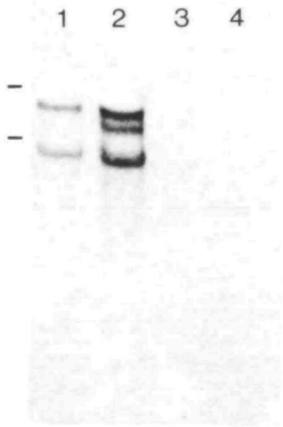

Fig. 2. RNA blot analysis of gel electrophoretically separated desmin mRNAs from various *Xenopus* tissues. Lane 1, cardiac muscle; lane 2, skeletal muscle; lane 3, ovarian tissue; lane 4, XLKE-A6 cells. 10 μg of total RNA were loaded and hybridized with a random-primed ^{32}P-labelled probe of pXenDes1. Bars indicate positions of 28S and 18S *Xenopus* rDNAs. Note the presence of two (in lane 1) and three (in lane 2) major mRNAs of different sizes.

quence of amino acids preceding this consensus sequence is also well conserved. Moreover, at position 10 of desmin, an arginine is found which introduces a possible phosphorylation site (RXS) for protein kinase A that is not present in vimentin. This conserved sequence motif is also seen as TSYRRTFGP, in the 'c73 IF protein' (Leonard *et al.* 1988), a recently detected rat IF protein expressed in a particular subset of mammalian neuronal cells ('peripherin'; Portier *et al.* 1984) that is highly homologous to desmin in the α-helical rod domain but shows little relationship to desmin in the head domain.

(C) *Expression of desmin during embryogenesis*

Using RNA blot analysis (Northern blots) we detected desmin mRNAs first in stage-14 embryos (Fig. 6, lane 5) whereas in stage 11 no signal was observed (Fig. 6, lane 4). Remarkably, all three mRNA species observed in skeletal muscle were identified in all these embryonic stages (Fig. 6, lanes 5–9) although their relative amounts varied considerably: in stage 28 the signal at ~1·9 kb was highest, compared to the ~2·6 and ~3·0 kb bands (Fig. 6, lane 7). The intensity of the reaction at ~3·0 kb increased over that at ~2·6 kb in swimming tadpoles (stages 42; Fig. 6, lane 9).

(D) *Localization of desmin in adult tissues and early embryonal stages*

By immunofluorescence microscopy on frozen sections of several tissues of adult *Xenopus laevis* with a monoclonal desmin-specific antibody, we observed positive reactions in smooth muscle tissue of the intestine and the oesophagus, in cardiac and skeletal muscle (where the fluorescence was concentrated in the Z-bands) as well as in cells of the endothelium (data not shown). When embryonic stages were examined by immunofluorescence microscopy on cryostat sections of frozen embryos, intense immunostaining was seen in early somite tissue of stages 14 (data not shown) and 18 (Fig. 7).

In parallel sections and double label experiments, the same somite regions were also positively stained with cytokeratin antibodies, including monoclonal antibody lu-5 (data not shown; see also figs 9 and 10 of Herrmann *et al.* 1989), indicative of the coexpression of cytokeratin and desmin IFs in the somite cells.

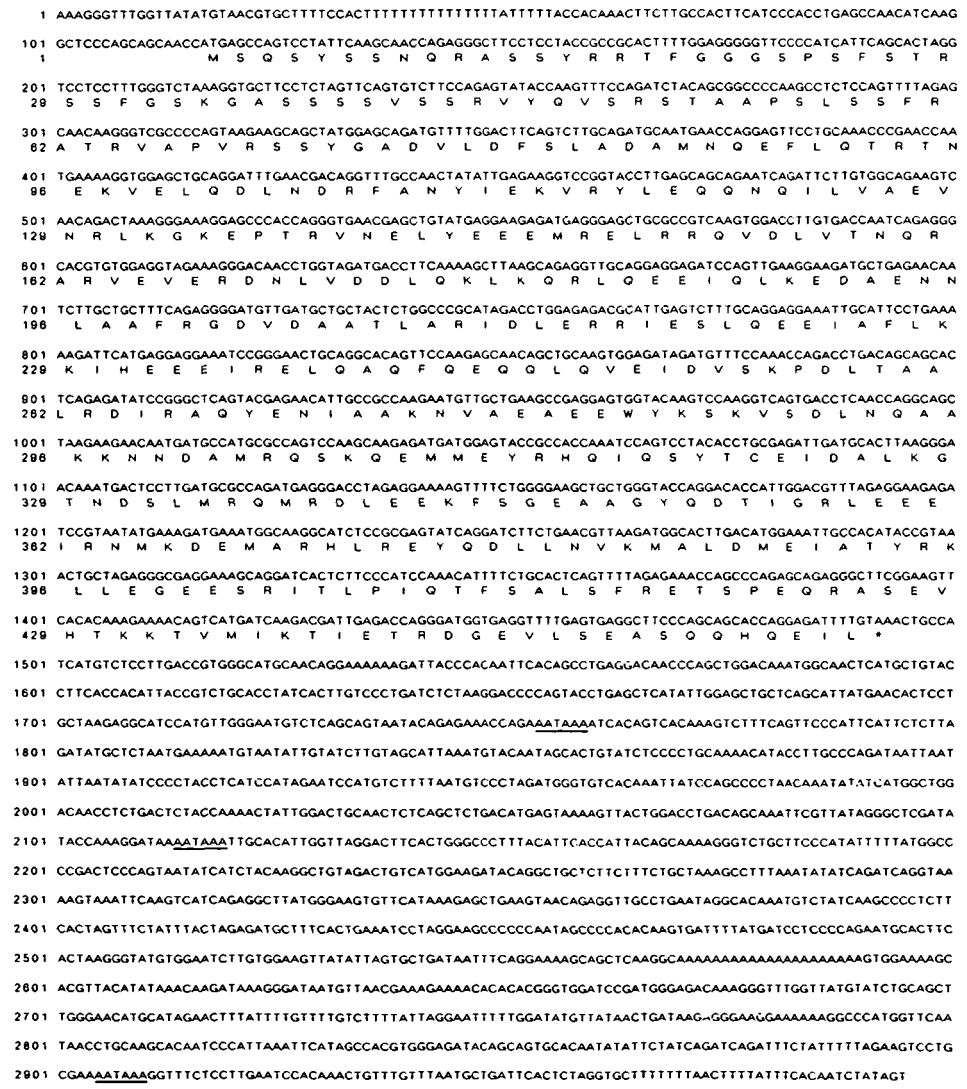

Fig. 3. Nucleotide sequence of clone pXenDes1 and deduced amino acid sequence of *Xenopus laevis* desmin. Asterisk denotes stop codon. Three putative polyadenylation signals are marked by bars.

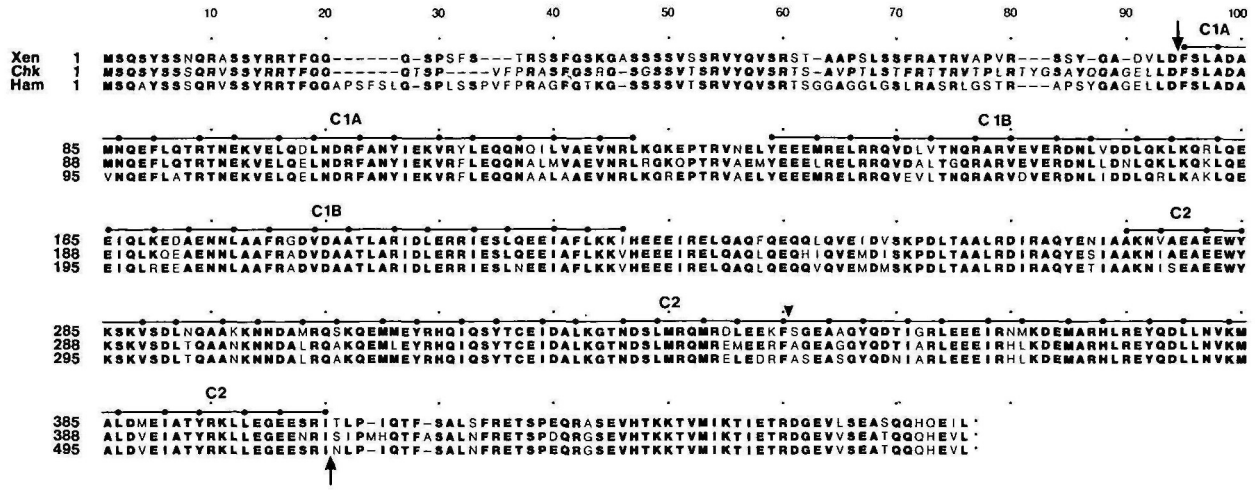

Fig. 4. Amino acid sequence comparison of *Xenopus* desmin (Xen), chicken desmin (Chk; taken from Geisler & Weber, 1984) and hamster desmin (Ham; taken from Quax *et al.* 1985). Bold-faced letters denote amino acids identical in *Xenopus* desmin and in at least one of the other two species. Amino acid 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 α-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-α-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 probaby results in a 'stutter' in coil 2.

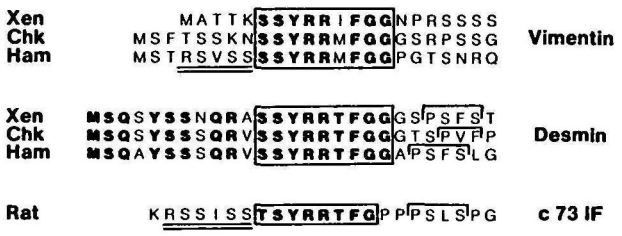

Fig. 5. Highly conserved sequence feature found near the *N*-terminus of the head domains of vimentin, desmin and neurone-specific IF protein c73IF. Vimentin and desmin from *Xenopus* (Xen), chicken (Chk) and hamster (Ham) and rat c73IF protein (taken from Leonard *et al.* 1988) are aligned with respect to the conserved nonapeptide consensus sequence (SSYRRXFGG). Bold-faced letters designate identical amino acids in all three proteins, including a conservative serine to threonine exchange in c73IF. In hamster vimentin and rat protein c73IF, a similar sequence precedes the nonapeptide (underlined). The motif PSFS present in *Xenopus* and hamster desmin appears in a modified form in protein c73IF as well as in chicken desmin (overlined). Note that in the desmins the sequence preceding the nonapeptide is also very similar.

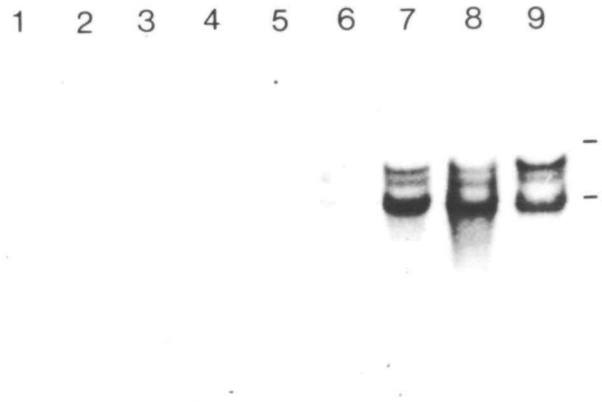

Fig. 6. RNA blot analysis of gel electrophoretically separated RNAs from various stages of *Xenopus laevis* development. Total RNA was prepared from embryos of the specific embryonal stage, and 20 μg each were loaded per slot for formaldehyde/agarose gel electrophoresis. Blotted RNA was hybridized with a random-primed, ^{32}P-labelled probe of pXenDes1. 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). Bars indicate positions of *Xenopus* 28S and 18S rRNA. Note that first reaction is seen in stage 14 (lane 5).

Discussion

The cDNA clone pXenDes1 encoding *Xenopus laevis* desmin extends our knowledge about this IF protein to amphibia and represents a valuable probe for studies of myogenesis and the regulation of expression of muscle-specific cytoskeletal proteins. Comparison of the deduced amino acid sequence with the sequences of avian (chicken) and mammalian (hamster, pig) desmin shows

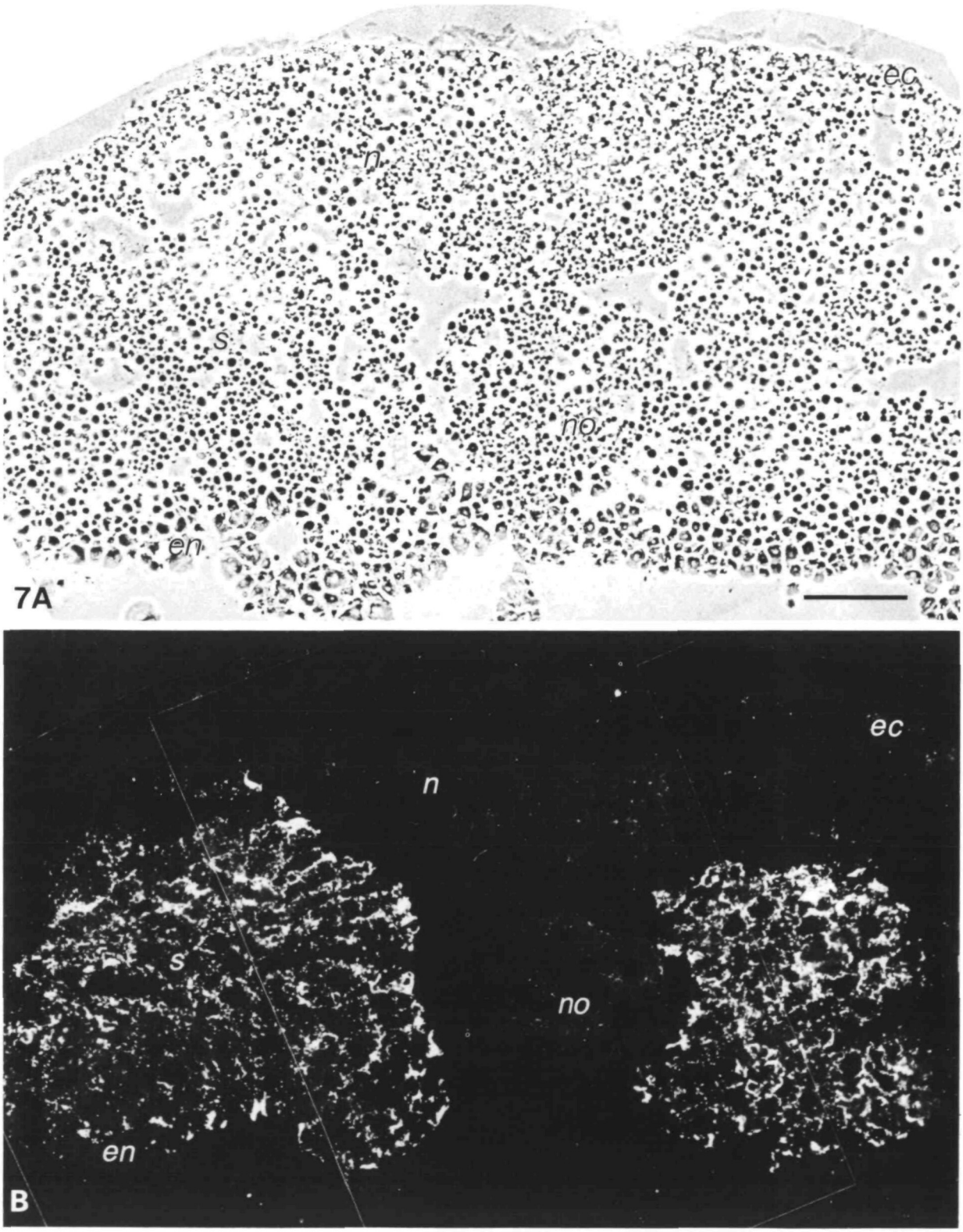

Fig. 7. Immunofluorescence microscopy performed on cryostat sections of snap-frozen stage-18 embryos of *Xenopus laevis* using a monoclonal desmin antibody. (A,B) The same field is shown in epifluorescence (A) and phase-contrast (B) optics. The somites are brightly stained, all other cell layers are negative. *ec*, ectoderm; *en*, endoderm; *n*, neural groove; *no*, notochord; *s*, somite. Bar, 50 μm.

that desmin has been well conserved during vertebrate evolution (more than 81% identical residues in *Xenopus* and hamster desmin). Despite the considerable general sequence homology between desmin and vimentin (for other species see Geisler & Weber, 1981; Weber & Geisler, 1984; Quax *et al.* 1985), which in *Xenopus* amounts to 71·3% identical amino acid residues in the rod portion, it has to be stated that the difference between desmin and vimentin is as great in the amphibian (182 amino acid exchanges) as in the avian (182 different residues) and mammalian (176 exchanges) species. This indicates that the separation of these two IF proteins, which probably are derived from a common ancestral gene (Franke *et al.* 1978; Quax *et al.* 1983, 1985), has occurred long before the evolution of the anuran amphibia, in agreement with previous data of Quax *et al.* (1984). Particularly noteworthy is the maintenance of certain common sequence features in the rod and tail domains of both proteins in otherwise more divergent regions, including a SSYRRXFGG motif near the aminoterminus that may represent important functional sites or signals.

In alignments of the vimentin and desmin sequences of *Xenopus*, chicken and hamster, we have noted several evolutionarily conserved features that provide distinguishing characteristics for vimentin and desmin, respectively. (*i*) The head domains of both proteins carry five or more possible kinase A type phosphorylation sites which, however, are arranged in patterns differing in the specific proteins. It will have to be seen whether these sites are indeed involved in regulations of the assembly of the specific IFs formed as it has been proposed by Geisler & Weber (1988; for vimentin see also Inagaki *et al.* 1987; 1988). (*ii*) Besides the nonapeptide common to the heads of both vimentin and desmin, the number and the kind of amino acids preceding this nonapeptide are also conserved in desmin but not in the two major vimentins. (*iii*) In the highly conserved α-helical rod, we have noticed a divergent site in coil 1B, which starts at position 196 of *Xenopus* vimentin Vim1 (LQSFFQDVDNA), which in desmin is replaced by LAAFFADVDAA, resulting in different surface domains on the α-helix (amides in vimentin, compared to hydrophobic residues in desmin). (*iv*) The carboxy-termini differ in that the vimentins show a very acidic tip, DDFE, compared to HEVL in the desmins.

Surprisingly, we have found two polypeptides reactive with both our desmin probes, i.e. by the monoclonal antibody and by cDNA-hybrid selection of mRNAs, followed by translation *in vitro*. While the larger ($M_r \sim 53\,000$) and slightly more basic component appears to correspond to the M_r 52 800 polypeptide encoded by clone pXenDes1, the component with higher electrophoretic mobility, corresponding to $M_r \sim 49\,000$, might represent a different, perhaps truncated, form of desmin. A proteolytic derivative of the latter component, although not fully ruled out at present, appears less likely in view of our *in vitro* translation results. More direct analytical work is needed to answer this question.

At both the protein and the mRNA level, we have not found desmin in appreciable amounts in oocytes, eggs or in early embryonic stages, including blastulae. With both the mRNA and protein probes, desmin has first been detected in a postgastrulation stage, i.e. stage 14, the neural plate stage. Probably, with greater sensitivity it might be possible to date the actual onset of synthesis of desmin mRNAs somewhat earlier. Obviously, these data and the demonstrated enriched, if not exclusive, occurrence of desmin in somite tissue correlate with the advent, in space and time, of several other muscle-specific proteins (e.g. Sturgess *et al.* 1980; Gurdon *et al.* 1984, 1985*a*,*b*; Mohun *et al.* 1984; Gurdon & Fairman, 1986; Wilson *et al.* 1986; Kay *et al.* 1987; Mohun & Garrett, 1987). It will be interesting to find the regulatory elements that are responsible for the concerted expression of this 'myogenic program'.

In mammalian and avian embryogenesis, vimentin appears in mesenchymal cells before, and in a wider range of cells than, desmin, and the latter IF protein has been detected only in myogenic cells that initially contain vimentin as their only IF protein (e.g. Lazarides *et al.* 1982; Bignami & Dahl, 1984; Bennett *et al.* 1979). In *Xenopus* embryogenesis, however, we have so far not detected vimentin in the somites or presumptive somite cells, although at the same time this IF protein is easily demonstrable in certain adjacent mesenchymal cell layers (Herrmann *et al.* 1989). Our finding that, in *Xenopus*, the somites and the presumptive somite cells contain cytokeratin IFs, like practically all early embryonic cells, suggests that the expression of desmin and the formation of desmin filaments can begin in cytokeratin-synthesizing cells and is compatible with ongoing cytokeratin synthesis. This observation may seem surprising on first sight, but is in agreement with recent reports of the coexistence of cytokeratin and desmin IFs in certain adult smooth muscle tissues of *Xenopus* (Jahn *et al.* 1987) and human (Brown *et al.* 1987; Jahn *et al.* 1987; Norton *et al.* 1987; Kasper *et al.* 1988) as well as in chicken and human embryonic myocardium (Van Muijen *et al.* 1987; Kuruc & Franke, 1988). Apparently, there exist regulatory factors that permit the simultaneous synthesis of certain IF proteins in one kind of tissue but lead to a mutually exclusive synthesis of the same IF proteins in other tissues.

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