Developmental expression of a neurofilament-M and two vimentin-like genes in *Xenopus laevis*

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**Summary**

A hamster vimentin cDNA probe has been used to isolate and characterize three *Xenopus laevis* intermediate filament genes, named XIF1, XIF3 and XIF6. Of these, XIF6 shows 89% homology at the amino acid level to a portion of porcine neurofilament-M. XIF6 is transcribed solely in nervous tissue of embryos, commencing at the late neural tube stage. Expression is totally dependent on an interaction between mesoderm and ectoderm during gastrulation and can be used as a marker of neural induction.

XIF1 shows 94% homology and XIF3 83% homology to hamster vimentin at the amino acid level over a region of the protein. Although XIF1 and XIF3 show more homology to vimentin than to any other intermediate filament gene, they have distinct temporal and spatial patterns of expression. XIF1 expression most resembles that of vimentin in higher vertebrates, being expressed in embryonic myotome and nerve cord, whilst XIF3 is unusual in that its expression is restricted predominantly to the head in tailbud embryos.

Key words: *Xenopus*, vimentin, expression, neural induction, intermediate filaments.

**Introduction**

Neural induction is one of the earliest interactions between the embryonic cell layers and results in the diversion of ectoderm from an epidermal to a neural pathway of differentiation. Recently, the study of neural induction in amphibia has benefitted from the availability of molecular markers that enable the early identification of the induced neural state in responding tissue without recourse to histology or morphology.

Two neural markers have recently been described in *Xenopus*. Neural cell adhesion molecule (N-CAM) is a cell surface protein expressed throughout the embryonic nervous system (Jacobson & Rutishauser, 1986; Kintner & Melton, 1987), whilst the homeobox-containing gene XLHbox6 is expressed only in neural tissue located posterior to the head (Sharpe et al. 1987).

In light of the proven success of molecular markers for the analysis of embryonic induction and the limited range of markers that recognize early neural tissue, I have attempted to isolate genes, the products of which may be useful for future studies on the development of the embryonic nervous system. The approach has been to identify those genes that are candidate neural markers in other organisms in the hope that these might then be applied to amphibian development. In many species, expression of members of the intermediate filament (IF) family of proteins is restricted to certain cell types (Lazarides, 1982). Indeed, one group, the neurofilaments, are expressed solely in neurones. These observations have for some time indicated that the IF proteins may prove to be a useful source of molecular markers (Osborn & Weber, 1982).

The intermediate filaments are a component of the structural network of the cell. They consist of a set of related proteins that can be divided into two main groups: the cytokeratins and the noncytokeratins. The latter include vimentin, desmin, glial fibrillary acidic proteins (GFAP) and three neurofilament (NF) proteins (reviewed in Steinert et al. 1985; Franke, 1987).

In this paper, I report the isolation and characterization of a *Xenopus* neurofilament-M gene and show that it can be used as an early molecular marker for neural induction. I also describe the temporal and
spatial expression of two vimentin-like genes that were isolated during the screen for IF sequences. One of these, XIF3, has an unusual pattern of expression in the tailbud embryo.

Materials and methods

(A) Screening the genomic library
Southern blots were performed under standard conditions (Maniatis et al. 1982) using as probe the PstI fragment of hamster vimentin (55–1040 bp) (Quax-Jeuker et al. 1983) subcloned into M13 (Messing, 1983). Hybridization followed standard conditions, low-stringency washing was performed at 42°C in 1×SSC (20×SSC is 3 M NaCl, 0.2 M sodium citrate) for 60 min.

5×10^6 plaques of a genomic library in AEMBL4 (Krieg & Melton, 1987b) were screened by the Benton & Davis (1977) technique and washed at low stringency as described above.

(B) Northern blots
10 μg of total RNA isolated from stage-25 to -30 embryos as previously described (Gurdon et al. 1985) was fractionated on a 1.5% agarose–formaldehyde gel and capillary blotted to GeneScreen (New England Nuclear). Hybridization followed the same regime as for the Southern blot, but the filters washed to a higher stringency (1×SSC, 65°C, 30 min).

(C) Isolation and characterization of gene-specific probes

(1) XIF1
A 4.3-kbp EcoRI–HindIII fragment of XIF1, that hybridizes to the hamster vimentin CDNA was isolated and ligated into pUC18 vector using standard techniques (Maniatis et al. 1982). The 4.3-kbp fragment was digested with AluI and sequences showing homology to hamster vimentin identified by dideoxy sequencing (Sanger et al. 1977) after transfer to M13 vectors (Messing, 1983). A partial AluI fragment of 630 bp that contained at 222 bp of the terminal exon of the central helical rod domain was transferred to the transcription vector, Bluescribe (Stratagene). Transcription from the T7 promoter of the resulting clone, pBSXIF1, produces an antisense RNA probe.

(2) XIF3
Similar techniques were used to identify and subclone fragments of XIF3. A 360-bp fragment containing the whole of the 222 bp terminal exon of the central helical rod region was subcloned into Bluescribe vector. Transcription from the T3 promoter of the resulting clone, pBSXIF3, produces an antisense RNA probe.

(3) XIF6
A 600-bp fragment that strongly cross-hybridizes to M13XIF1 was subcloned into M13 vectors for sequencing and into the transcription vector pSP64 (Krieg & Melton, 1987a) to give pSPXIF6 for the production of an RNA probe. The fragment contains the whole of the terminal exon of the central helical rod region, but no other coding sequences. This exon encodes 41–42 amino acids in 122 bp and is identical in length to the corresponding exon in human NF-M (Myers et al. 1987). The additional amino acid sequence shown in Fig. 2 is from a separate genomic fragment.

(D) Analysis of embryonic expression
Xenopus embryos were dejellied in cysteine HCl and grown at 18–23°C in 0.1×MBS (Gurdon, 1977). Embryos were individually staged according to Nieuwkoop & Faber (1967).

Neural conjugates were constructed from two entire animals caps of stage-10-25 embryos and the dorsal mesoderm of a stage-11 embryo, as described in Sharpe et al. 1987.

Dissected explants or conjugates were cultured in 1×MBS at 23°C until control embryos grown in parallel had reached the appropriate stage. Samples were normally stored at −80°C before standard RNA extraction method (Mohun et al. 1984).

RNase protection assays were performed according to Krieg & Melton (1987a), except that the probe pSPXIF6 was digested with RNase T1 alone. With the other probes both RNase T1 and RNase A were used. Protected probe was fractionated on 6% polyacrylamide, 7 M-urea gels which were then fixed and dried prior to autoradiography.

Results

(A) Isolation of Xenopus laevis intermediate filament genes
The proteins that assemble to form the intermediate filaments vary between different cell types (Lazarides, 1982), but constitute a family of proteins with conserved amino acid sequence and tertiary structure. The intermediate filament proteins generally consist of three domains, the central one forming a helical rod that is vital for its aggregation into filaments and it is within this domain that the greatest similarity in protein sequence exists between the family members (Steinert et al. 1985; Weber & Geisler, 1984; Bader et al. 1986).

The cytokeratins are a large subgroup of proteins within the intermediate filament family, they are expressed only in epidermal tissues (Lazarides, 1982). One of these, the Xenopus embryonic cytokeratin XK81 (Jonas et al. 1985), is expressed following gastrulation and has already been used as a marker for ectoderm and epidermis (Jamrich et al. 1987). It is also a negative marker for neural tissue, expression ceasing in ectoderm that has undergone neural induction.

The remaining IF proteins, the noncytokeratins, share approximately 70% homology across the central rod domain. However, they show only 30%
homology with the cytokeratins in this region (Bader et al. 1986). The members of this subgroup are also restricted in their expression to defined tissue types (Lazarides, 1982). These IF proteins include vimentin, expressed predominantly in cells of mesodermal origin, desmin in muscle cells, glial fibrillary acidic proteins in glia and the neurofilament proteins that are expressed exclusively in neurones.

The neurofilament proteins can be further subdivided on the basis of electrophoretic mobility into NF-L \((68\times10^3)\), NF-M \((145\times10^3)\), and NF-H \((170\times10^3)\) each encoded by a separate gene (Geisler et al. 1985; Lewis & Cowan, 1985; Myers et al. 1987; Robinson et al. 1986). The particular function of the neurofilament proteins remains unclear, though reported variations in the phosphorylation state of the protein might suggest they play more than a simple structural role (Myers et al. 1987).

To test the feasibility of isolating a range of noncytokeratin IF genes including one for a neurofilament, a radiolabelled cDNA probe derived from the region encoding the conserved central helical rod domain of a hamster vimentin clone was used (Quax-Jeuken et al. 1983) to screen a Xenopus genomic DNA Southern blot. This approach successfully showed that the hamster probe could hybridize to Xenopus sequences and therefore be used to isolate a group of Xenopus intermediate filament genes. At reduced stringency several discrete bands were observed in lanes containing either EcoRI or HindIII digested DNA, (Fig. 1). As Xenopus laevis is effectively tetraploid (Kobel & Du Pasquier, 1986), it is difficult to estimate the number of cross-hybridizing IF genes in the genome, from these observations, but it is clear that the hamster vimentin probe is capable of detecting several related Xenopus genes.

Having established that the hamster vimentin probe could productively cross-hybridize to sequences within the Xenopus genome, the actual isolation of genomic clones proceeded as follows. A Xenopus genomic library (Krieg & Melton, 1985) was screened with the hamster vimentin cDNA probe and one positive clone \(\lambda\)XIF1 isolated. Probes subcloned from \(\lambda\)XIF1 were then used to rescreen the library and the subsequent positive clones placed into three groups based on nucleotide sequence. One member of each group, \(\lambda\)XIF1, \(\lambda\)XIF3 and \(\lambda\)XIF6, was selected for further analysis.

The amino acid sequence was derived from the nucleotide sequence over a portion corresponding to the central helical rod region of each clone and compared to the published sequences (Geisler et al. 1984) of other IF proteins (Table 1, Fig. 2). XIF1 shows 94 % homology to hamster vimentin whilst XIF3 shows 82 % homology; in both cases, the Xenopus proteins are more related to vimentin than to any other IF protein. In contrast, XIF6 shows only 59 % homology to hamster vimentin but 89 % homology to porcine neurofilament-M and suggests that XIF6 is a Xenopus neurofilament gene.

(B) Sizes of transcripts

The comparisons of amino acid sequences suggest that the XIF genes encode different IF proteins and that XIF1 and XIF3 are closely related. To see whether these proteins were translated from different transcripts, Northern blots of pooled tailbud stage RNA were screened with probes subcloned from each of the three XIF genes (Materials and methods). Distinct hybridizing bands of approximately 1800, 2500 and 4000 bases were found for XIF 1, 3 and 6, respectively (Fig. 3). Each probe shows a small degree of hybridization to transcripts of the size primarily recognized by the other probes. It is assumed that this reflects cross-hybridization of sequences within the probes to transcripts from each of the different genes.

These results further suggest that the XIF clones represent three individual Xenopus IF genes.

(C) Temporal expression

The most useful molecular markers for analysing
development need to be expressed soon after early events such as gastrulation or the embryonic inductions (Gurdon, 1987). To determine the timing of expression the from XIF genes, embryos from artificially fertilized eggs were collected at different stages during development and RNA extracted. The presence of transcripts in amounts of RNA equivalent to one embryo (5 μg) was determined by RNase protection assay (Krieg & Melton, 1987a) using probes specific to each gene and encompassing the carboxy end of the central rod domain (Materials and methods). The results are shown in Fig. 4. The same staged RNA samples were assayed with a *Xenopus* cardiac actin probe (Mohun et al. 1984) as a control and for approximate quantification.

Transcripts from XIFl are present in ovarian tissue and have since been shown to be present throughout oogenesis (Tang et al. 1988). The level is at its lowest during cleavage stages before steadily increasing from early gastrulation onwards. XIFl therefore shows a similar pattern of expression to the cytoskeletal actin gene (Mohun et al. 1984).

The probe specific to XIF3 protects two fragments that differ only slightly in size from the expected protected fragment length of 222 bp. The lower band can first be detected at stage 10 and then accumulates at a faster rate than the upper band. The upper band is present throughout the early cleavage stages and mirrors the temporal expression of XIFl although it is not known whether it is expressed in oocytes. The degree of mismatch at the nucleotide level between XIF3 and XIFl, however, combined with a high stringency protection assay using both RNase T1 and RNase A makes it unlikely that the upper band represents cross-hybridization of the XIF3 probe to the XIFl transcript. The possibilities remain that the two transcripts are derived from the two homologous genes in the duplicated genome of *Xenopus* (Kobel & Du Pasquier, 1986) or that they represent alternatively spliced transcripts from the same gene.

Transcripts from XIF6 are easily detected by stage 26 of development and a weak signal can be seen at stage 22 (Fig. 4). When a developmental series, using RNA extracted from four embryos at each stage, was assayed by RNase protection using the same probe, transcripts from XIF6 were first detected in embryos at stage 20/21 (data not shown).

The level of cardiac actin transcripts in a stage-26 embryo has been accurately determined as 2.5 × 10⁸ transcripts per embryo (T. J. Mohun, personal communication). Assuming equivalent hybridization conditions and a linear autoradiographic response, then densitometry indicates a level of 1.1 × 10⁸, 3 × 10⁷ and 2 × 10⁶, transcripts per embryo at stage 26 for XIFl, and for each transcript from XIF3 and XIF6, respectively.

(D) Regional expression

The above section has shown that the XIF genes are temporally regulated during development. In order to determine whether the transcripts are also produced in a tissue-specific manner, embryos at the tailbud stage were dissected into component tissues (Fig. 5A) which were pooled and the RNA extracted. The presence of each transcript in these samples was assayed by RNase protection as above and the results are shown in Fig. 5B. Each lane of dissected tissue contains an amount of RNA equivalent to that obtained from a dissected component from one embryo. A whole-embryo sample was included in each analysis to mark the position of the protected fragment, in the XIF3 analysis this lane contains RNA from less than one embryo. The integrity of the RNA from the dissected samples was demonstrated by RNase protection assay using a 5S RNA probe (Sharpe et al. 1987).

It can be seen from Fig. 5 that all three genes show a distinct profile of expression. Transcripts from the

### Table 1. Amino acid identities between the XIF proteins and other IF proteins in the region of the carboxy-terminus of the helical rod domain

<table>
<thead>
<tr>
<th>Clone</th>
<th>Hamster vimentin*</th>
<th>Chicken desmin†</th>
<th>Porcine GFAP†</th>
<th>Porcine NF-M†</th>
<th>Porcine NF-L†</th>
<th>Xenopus cytokeratin XK81†</th>
</tr>
</thead>
<tbody>
<tr>
<td>XIF1</td>
<td>94</td>
<td>78</td>
<td>73</td>
<td>67</td>
<td>62</td>
<td>32</td>
</tr>
<tr>
<td>XIF3</td>
<td>82</td>
<td>70</td>
<td>66</td>
<td>68</td>
<td>62</td>
<td>34</td>
</tr>
<tr>
<td>XIF6</td>
<td>59</td>
<td>55</td>
<td>60</td>
<td>89</td>
<td>59</td>
<td>24</td>
</tr>
</tbody>
</table>

Comparisons for XIFl and XIF3 relate to the 74 amino acids in Fig. 2B, and for XIF6 relate to the 104 amino acids in Fig. 2C. The numbers represent the percentage of identical residues.

* Quax et al. (1983).
† Geisler et al. (1985).
‡ Jonas et al. (1985).
Fig. 2. *Xenopus laevis* possess genes with homology to hamster vimentin. (A) Domain structure of a typical IF protein. The hatched region marks the amino acid sequences compared in Fig. 2B and the stippled region the extra sequences compared in Fig. 2C. (B) Comparison of the deduced amino acid sequences of XIF1, XIF3, and hamster vimentin. (C) Comparison of the deduced amino acid sequences of XIF6 and porcine neurofilament-M. The arrowheads mark the position of exon–intron boundaries and demonstrates the presence of the characteristic small exon at the carboxy terminal of the rod region of neurofilament-M.

Fig. 3. Each XIF gene produces a transcript of a distinct size. Northern blots of total RNA from stage-32 embryos hybridized to the XIF probes (Materials and methods). Size markers are eukaryotic and bacterial ribosomal RNA.

Fig. 4. Temporal control of XIF gene expression. Transcripts from each gene were detected by RNase protection assays at different stages of development. Onset of transcription is marked by an arrowhead. XIF3 produces two transcripts under separate temporal control. An analysis of cardiac actin gene expression is included for comparison.
Fig. 5. Location of expression of the XIF genes. (A) Cross-section of a stage-25 embryo showing the dissected regions. (B) RNase protection assays to detect transcripts from each of the three XIF genes. The protected fragment is indicated by an arrowhead and expressing tissues denoted by a dot. Lanes contain RNA from the equivalent of one embryo for dissected samples. The whole embryo lane acts as a marker for the correct position of the protected fragment rather than as an indication of the amount of RNA in one embryo. The integrity of the RNA samples was confirmed using a 5S RNA probe. ect, ectoderm; myo, myotome; nv, nerve cord; noto, notochord; d en, dorsal endoderm; v en, ventral endoderm; hd, heads; wl, whole embryo; t, tRNA; ov, ovary.

XIF1 gene have a wide-ranging distribution but are found predominantly in myotomes and nerve cord. This is consistent with the detection of vimentin in embryonic Xenopus spinal cord by immunochemical techniques (Godsave et al. 1986).

Expression from XIF3 is markedly different from the other two genes. In the tailbud embryo, the gene is transcribed primarily in the head region though longer autoradiographic exposures reveal low levels of transcripts in ectoderm, notochord, nerve cord and dorsal endoderm.

Transcripts from the XIF6 gene are found predominantly in nerve cord and to a lesser extent in heads, supporting the proposition that XIF6 is indeed the Xenopus neurofilament-M gene.

Although the XIF proteins show many structural similarities and presumably have similar functions within the cell, I have shown that each possesses a distinct temporal and spatial pattern of expression.

(E) Explant analysis and embryo conjugates

The initial control of differential gene expression during development requires either the effects of localized factors or the influence of embryonic induction. The following experiments were designed to show to what extent transcription of the XIF genes is activated by cell interactions, and therefore whether they might be used as molecular markers of embryonic induction. In these experiments, embryos at stage 8 were dissected into animal cap, equatorial region and vegetal piece, cultured until control embryos reached stage 26 of development and then assayed for the presence of each gene-specific transcript. The results are shown in Fig. 6A.

Transcripts from XIF1 were found in explants of each blastula part though they were predominantly in the equatorial region. Expression in equatorial explants conforms with the pattern of expression in later embryos, where it is found mainly in myotomes and neural tissue. As XIF1 transcripts are found in both animal cap and vegetal piece explants, it is probable that embryonic induction is not required for the expression of this gene.

Transcripts from XIF3 were found in both animal cap explants and in explants from the equatorial region. It is likely therefore that in subsequent stages the transcripts will be found mainly in ectodermal derivatives. Further experiments are required to determine whether early embryonic inductions are required for the expression of XIF3.

XIF6 is believed to be a Xenopus neurofilament-M gene. Neural tissue in Xenopus is derived from animal cap ectoderm following neural induction by the underlying mesoderm. The equatorial regions of stage-8 blastula are the only explants to contain both mesodermal and ectodermal cells and it is only these explants that give rise to XIF6 expression (Fig. 6A). To test whether XIF6 can be used as a marker for neural induction, explants of stage-10i ectoderm and stage-11 mesoderm were placed in contact (as described in Materials and methods) and grown until control embryos had reached stage 26. Without interaction, these two cell types should remain negative for XIF6 expression. In contrast, expression from XIF6 would be expected in the 'sandwich' conjugates between isolated ectodermal and mesodermal pieces when grown to the equivalent of the tailbud stage (Asashima & Grunz, 1983; Sharpe et al. 1987), if this is indeed dependent on neural induction. As can be seen from Fig. 6B, expression from XIF6 is consistent with that expected for a neural marker.

Discussion

The aim of these studies is to provide molecular
Developmental expression of a neurofilament-M

In this report, I describe the isolation and characterization of a Xenopus neurofilament-M gene and illustrate its potential as a marker for neural induction. In the process of isolating the neurofilament gene, I have also characterized two other noncytokeratin IF genes and show that members of this closely related gene family are, nevertheless, expressed under independent temporal and spatial control during development.

What then are the proteins encoded by the XIF genes and do they possess a counterpart in higher vertebrates? The protein theoretically encoded by XIF1 displays 94% homology to hamster vimentin over regions of the central helical rod domain (Quax et al. 1983). XIF1 is likely to be a Xenopus vimentin gene. In stage-25 embryos, it is expressed predominantly in the myotomes, though in agreement with the studies of Godsave et al. (1986), it is also found in the embryonic nerve cord, while in other organisms it is transiently coexpressed with the neurofilaments during the early stages of neural differentiation (Tappett et al. 1981; Cochard & Paulin, 1984).

XIF3 encodes a noncytokeratin intermediate filament protein as can be seen from the amino acid homologies shown in Fig. 2B and Table 1. Like XIF1, the closest similarity is to hamster vimentin. However, the degree of identity is less than that between XIF1 and hamster vimentin. XIF3 also differs from XIF1 in that it encodes a message of about 2500 bases whereas XIF1 produces a transcript of around 1800 bases, a size more akin to that of the hamster vimentin message (1848 bases; Quax et al. 1983). The temporal and spatial patterns of expression of XIF3 and XIF1 are also different, leading to the conclusion that, in Xenopus laevis, there are at least two different vimentin-like genes. Experiments to determine the exact location of expression of the XIF3 gene in the head are in progress.

XIF6 displays greatest homology to the sequence of porcine NF-M (Geisler et al. 1984) and hybridizes to a band of approximately 4000 bases on a Northern blot. The relative molecular mass of NF-M estimated from SDS–PAGE is $145 \times 10^3$ though estimates from the sequence of the cloned gene in humans indicates a size nearer $102 \times 10^3$ (Myers et al. 1987). The size of the XIF6 message is more compatible with the smaller of the two molecular weights. XIF6 was detected despite its reduced homology at the amino acid level to hamster vimentin (59%) because at the
nucleotide level it is 76% homologous to the XIF1 probe over a region of 120 bp.

XIF6 transcripts are first detected around stage 20/21 (22 h postfertilization (p.f.)) corresponding to the time when the neuraxonic canal closes to form the neural tube (Nieuwkoop & Faber, 1967). This is somewhat earlier than in other species, where, by comparison, NF expression is first detected during initial axon elongation (Cochard & Paulin, 1984), a process that occurs at around stage 28 (32 h.p.f.) in Xenopus. Previous reports using antibodies against rat NF-H that cross-react with a Xenopus NF (presumably NF-H) have shown that this type of NF is first detected between stages 33 and 44 (44–92 h.p.f.) (Godsave et al. 1986). This has suggested that the NF proteins are products of late, terminal differentiation and as such would be of limited use as markers of neural induction. Apparently this is not the case for Xenopus NF-M. Neural induction probably commences as the mesoderm starts to invaginate beneath the ectoderm at the start of gastrulation, exerting its first influence at around stage 10. In this case, there is a lag of, at most, 11 h following neural induction to gene expression for NF-M compared to the 4 h required for the neural marker XLHbox6 (Sharpe et al. 1987). Muscle-specific actin gene expression, a commonly used marker for mesodermal induction, is first detectable 7 h following induction. These comparisons suggest that the NF-M gene is expressed sufficiently soon after the event to be a suitable marker of neural induction for future studies, and further emphasizes the rapidity of transcriptional activation of the homeobox gene XLHbox6 in response to an embryonic induction, compared to the expression of structural proteins such as the actins and neurofilaments.

XIF6 should prove to be a useful addition to the limited range of molecular markers for neural induction. The cell surface protein N-CAM can be detected using both monoclonal antibodies (Jacobson & Rutishauser, 1986) and nucleic acid probes (Kintner & Melton, 1987) and is expressed very soon following neural induction. Surprisingly, N-CAM can also be found at a low level in the mature oocyte (Kintner & Melton, 1987). The homeobox gene XLHbox6 is expressed only in neural tissue located posterior to the head and may prove of greatest value for investigating the regional variations in neural induction leading to the formation of forebrain, hindbrain and spinal cord (Sharpe et al. 1987).

XIF6 can be used as an additional molecular marker for pan-neural induction in Xenopus, whilst both XIF1 and XIF3 may provide some information on the processes that decide the fate of ectodermal cells. The analysis of factors involved in the distinct regulation of expression of each of these genes may uncover details of the molecular mechanisms that lead to the formation of neural tissue in Xenopus.

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References


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