Control of axis formation in *Xenopus* by the NF-κB-IκB system

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ABSTRACT We describe the isolation and analysis in *Xenopus* of Xrel2, a novel member of the NF-κB/Rel protein family that remains to be described in other vertebrates. We show that Xrel2 is expressed throughout development but with higher levels in pre-gastrula embryos. Like other NF-κB/Rel proteins, Xrel2 protein is able to bind DNA at a κB-Motif. Ectopic expression of Xrel2 disrupts normal morphogenesis at the early gastrula stages suggesting that the NF-κB/Rel family have developmental functions at stages earlier than previously thought. We also show that the Xrel2 over-expression phenotype can be rescued by co-expression of IκB-α and that ectopic expression of IκB-α or IκB-γ alone has no effect on development. Finally, we show that Xrel2 does not divert animal caps from an ectodermal to a mesodermal cell fate. Overall, these results suggest that the NF-κB/Rel family does have key functions in early vertebrate development, however, there is not a simple conservation of the *Drosophila* dorsal pathway.

KEYWORDS: *Xenopus*, embryology, gastrulation, NF-κB, IκB

Introduction

Many important decisions in growth and differentiation are controlled by the differential activity of transcription factors. For example during *Drosophila* development, the differential activation and repression of genes along the dorsal-ventral axis is controlled by the nuclear concentration of dorsal (Steward and Govind, 1993). Dorsal belongs to the NF-κB/Rel family of transcription factors whose members share a conserved region known as the Rel-homology domain that is responsible for DNA binding, nuclear localization and dimerization (for reviews see Liou and Baltimore, 1993; Baeuerle and Henkel, 1994; Siebenlist et al., 1994). The NF-κB/Rel proteins bind DNA either as homo or heterodimers at target sequences known as κB-motifs (Zabel et al., 1991; Baeuerle and Henkel, 1994; Siebenlist et al., 1994) and they are further regulated by the interaction of inhibitors that prevent their transport to the nucleus. These inhibitors form the IκB family and include cactus in *Drosophila* and the IκB-α, β and γ proteins in vertebrates (for reviews see Beg and Baldwin, 1993; Gilmore and Morin, 1993; Thanos and Maniatis, 1995).

It is intriguing that, in *Drosophila*, dorsal activates the mesodermal genes snail and twist, and that the *Xenopus* homologues of these genes are also expressed in mesoderm (Hopwood et al., 1989; Sargent and Bennett, 1990). This might suggest conservation of an important developmental pathway between invertebrates and vertebrates; however, the role of NF-κB/Rel proteins in early vertebrate development is less clear. In mammalian embryos, there is little evidence for an early developmental function of the NF-κB/Rel proteins. Expression of c-rel and relB is limited to a set of late hematopoietic and lymphoid lineages (Carrasco et al., 1993, 1994). Furthermore, relB or nfb1 (p50) germline mutations do not show any early developmental abnormalities (Sha et al., 1995; Wei et al., 1995). In *Xenopus*, a number of similar genes related to relA (p65) have been isolated (Kao and Hopwood, 1991; Richardson et al., 1994). These genes, termed Xrel1 or XrelA, are thought to be polymorphic variants of the same locus. XrelA is ubiquitously expressed throughout development with a peak during the late blastula and gastrula stages, however, no in situ hybridization data is available. XrelA protein has been shown to localize to embryo nuclei in over-expression experiments and using an anti-κB anti-serum, an endogenous *Xenopus* NF-κB/Rel protein has been detected in embryonic nuclei (Bearer, 1994; Richardson et al., 1994). Although XrelA can activate κB-motif-dependent transcription and there are κB-motif binding activities in early embryo extracts (Richardson et al., 1994), the function of XrelA in early *Xenopus* embryos remains to be addressed.

To further understand the role of NF-κB/Rel proteins in development, we have pursued the isolation of new members of the family in *Xenopus* as this provides a well characterized system for studying early developmental events. We present the sequence and expression analysis of Xrel2 which represents a novel NF-κB/Rel protein with DNA binding properties typical of...
this type of protein. We also show that Xrel2 is not sufficient for mesoderm formation and that over-expression disrupts development as early as the gastrula stages. Our results lead us to believe that the NF-κB/Rel proteins have important functions in early development, however, the Drosophila dorsal signalling pathway may not be simply conserved in vertebrates.

Results

Isolation of Xrel2, a novel member of the NF-κB/Rel family

Only one member of the NF-κB/Rel family has been described in Xenopus so it is likely that other members have yet to be isolated. Degenerate oligonucleotide primers were designed from conserved regions within the Rel-homology domain of the known NF-κB/Rel proteins and used to perform RT-PCR reactions using Xenopus embryonic RNA (see Materials and Methods). A PCR product of ~190bp was isolated and shown to be related but not identical to the known XrelA genes (data not shown). RNAase protection analysis with this fragment suggested that its mRNA should be present maternally (data not shown), hence this fragment was used to isolate a potential full length clone from a Xenopus oocyte cDNA library. This clone was named Xrel2 to designate it as the second NF-κB/Rel-related gene to be isolated in Xenopus. The Xrel2 cDNA comprises 2829bp and encodes a putative product of 583 amino acids. Figure 1 presents the sequence of Xrel2 and its conceptual translation.

The Xrel2 protein appears typical of the NF-κB/Rel family as it shows conservation to the family in the Rel-homology domain (Fig. 2). Beyond the Rel-homology domain, the similarity to the NF-κB/Rel family breaks down with the carboxyl-half showing no distinct homologies to any other proteins. This region has an overall net acidic charge of -7 with a high proportion of serine and proline residues suggesting that it might represent a transcrip-tional activation domain as found in other NF-κB/Rel proteins (Bull et al., 1990; Schmitz and Baeuerle, 1991; Dobrzenski et al., 1993). The Xrel2 Rel-homology domain shows a number of features shared between all NF-κB/Rel proteins (Fig. 1). A short sequence (RXR/RXXXC) essential for DNA binding is present (Kumar et al., 1992; Toledano et al., 1993) and there are conserved nuclear localization (KKQR) and protein kinase A phosphorylation sites (RRPS). Alignments of Xrel2 to other NF-κB/Rel proteins show that the closest relationship is to c-rel-type proteins at greater than 70% identity whereas the weakest relationship is to the NF-κB-type proteins at under 40% identity (Fig. 2, Table 1). It is unlikely that Xrel2 represents the Xenopus version of c-rel as there is little similarity between the Xrel2, mouse and avian c-rel proteins beyond the Rel-homology domain.

Developmental expression of the Xrel2 gene

If the NF-κB/Rel proteins have a role in early development then it would be expected that their mRNA should be present at the appropriate time. The temporal expression of Xrel2 was determined by RNAase protection analysis using a Xrel2-specific probe. Figure 3A shows that Xrel2 mRNA can be found con-tinuously from the egg to tailbud stages of development. However, by comparison to the internal Odc standard, it can be seen that the amount of Xrel2 mRNA drops from the egg to the gastrula stages by about 16 fold and that this lower level of Xrel2 is maintained throughout the rest of embryogenesis. Thus, Xrel2 mRNA is more abundant in pre-gastrula embryos which contrasts with the increasing expression of XrelA through the late blastula and gastrula stages (Kao and Hopwood, 1991; Richardson et al., 1994).

Whole-mount in situ hybridization has proven to be difficult for Xrel2, (D. Tannahill and J. Song, unpublished observations)
which is similar to the situation described for XrelA (Richardson et al., 1994) and is probably due to low mRNA abundance. RNAase protection analysis was therefore performed on dissected embryonic pieces to provide a general indication to the spatial expression of Xrel2 (Fig. 3B). During the blastula stages no differences in Xrel2 mRNA distribution were found along the animal-vegetal axis (data not shown), however, in the early gastrula ~3 times more Xrel2 mRNA is found ventrally than dorsally. Along the anteroposterior axis, the distribution of Xrel2 mRNA is roughly equivalent at all stages examined except that tailbud embryos show a small (~2 fold) increase in the middle piece. Thus, like XrelA, Xrel2 expression is essentially ubiquitous (Kao and Hopwood, 1991; Richardson et al., 1994) except for a small increase of Xrel2 expression on the ventral side of early gastrula.

Over-expression of Xrel2 interferes with gastrulation and results in a severe developmental phenotype

To begin to study the developmental role of Xrel2, we analyzed the consequences of supplying excess Xrel2 mRNA to eggs. Xrel2 mRNA levels in the egg are about 1-2% that of Odc, suggesting that Xrel2 is in the rare to moderately abundant class of mRNA. In our experiments greater than 100 fold excess of Xrel2 mRNA is being injected, however, the relative protein levels cannot be determined until an antibody to Xrel2 becomes available. Figure 4 shows the external phenotype of Xrel2-inject-
Xrel2-injected embryos show a clear defect at the onset of gastrulation with an absence of ventral lip formation and mesodermal involution. We therefore examined whether this was accompanied with a failure in primary mesoderm formation as assessed by the activation of early mesodermal markers (Fig. 6). It was found that the expression of \textit{wnt-6}, a ventral marker, and \textit{snail} and \textit{brachyury}, pan-mesodermal markers, was reduced in \textit{Xrel2}-injected embryos by ~2 fold as compared to control injected embryos. The effects of excess \textit{Xrel2} were not limited to ventral tissue as expression of \textit{goosecoid}, a dorsal marker, was also reduced ~3 fold.

\textit{IxB-\alpha} or \textit{IxB-\gamma} over-expression does not disrupt development but \textit{IxB-\alpha} rescues the \textit{Xrel2} phenotype

The NF-xB/Rel proteins interact with ixB inhibitors to retain them in the cytoplasm. By supplying excess ixB, it may be possible to interfere with the normal developmental NF-xB/Rel signalling. To test this, synthetic mRNA for avian \textit{IxB-\alpha} or human \textit{IxB-\gamma} was injected into eggs as the respective \textit{Xenopus} genes have not been isolated. It can be seen that the simple over-expression of \textit{Xrel2} RNA in embryos. The first observable abnormality is during gastrulation when the majority of \textit{Xrel2}-injected embryos fail to form the ventral blastopore lip (Fig. 4A and B, Table 2A). Histological sections show that mesoderm involution is not as extensive on dorsal side and that the archenteron has expanded less in \textit{Xrel2}-injected embryos (Fig. 5A and B). The \textit{Xrel2}-injected embryos continue to show this phenotype through gastrulation with many cases never forming a ventral blastopore lip.

The consequences of the gastrulation problems are clearly seen at the tailbud stages since virtually all the \textit{Xrel2}-injected embryos show a defect (Figs. 4C-F, 5C-F, Table 2B). The \textit{Xrel2} phenotype is somewhat variable and representative examples are shown in Fig. 4C-F. Variable phenotypes are often noted in \textit{Xenopus} over-expression experiments and are thought to arise, in part, from inadequate diffusion or differential stability of the injected mRNA. The mildest phenotype displays a distinct kink in the trunk that may result from a failure of the anteroposterior axis to extend properly during gastrulation. Sometimes, an accumulation of pigmented cells at the kink can be seen. The most severe phenotype is complex, often with severe reductions of the head and tail. The anteroposterior axis is often warped and can be split with yolk cells bulging through an open neural tube. Many somites are disorganized and in some cases, distinct protrusions are covered by a ruffled epidermis containing concentrations of pigment cells. A phenotype intermediate between the mild and severe cases can be seen in which the head and tail are relatively normal but the trunk is more severely affected.

Histological sections through \textit{Xrel2}-injected embryos highlight the disorganization of many embryonic tissues. Figure 5D shows an embryo with a large expansion of epidermal-like tissue, an accumulation of mesenchymal-like cells in the endoderm and an expansion of somitic tissue across the midline. The embryo in Fig. 5E shows a mass of neural-like tissue that appears adjacent to the neural tube. For the case in Fig. 5F, ectopic notochord can be found along with accumulations epidermal and mesenchymal-like cells.

\begin{table}
\centering
\caption{Sequence Relationship of the Rel-Homology Domain of the \textit{Xrel2} Protein to Other Members of the NF-xB/Rel Family}
\begin{tabular}{|l|c|c|c|c|c|c|c|c|c|}
\hline
\textit{Xrel2} & ddor & hnfkb1 & hnfkb2 & mrelb & mp65 & \textit{xrel1} & mcrel & \textit{tcrel} & \textit{tcrel} \\
\hline
\% Similarity & 54.6 & 57.5 & 56.7 & 49.3 & 20.4 & 65.1 & 67.3 & 65.6 & \\
\hline
\% Divergence & 45.4 & 42.5 & 43.3 & 50.7 & 79.6 & 34.9 & 32.7 & 34.4 & \\
\hline
\end{tabular}
\end{table}

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\textit{IxB-\alpha} or \textit{IxB-\gamma} over-expression does not disrupt development but \textit{IxB-\alpha} rescues the \textit{Xrel2} phenotype

The NF-xB/Rel proteins interact with ixB inhibitors to retain them in the cytoplasm. By supplying excess ixB, it may be possible to interfere with the normal developmental NF-xB/Rel signalling. To test this, synthetic mRNA for avian \textit{IxB-\alpha} or human \textit{IxB-\gamma} was injected into eggs as the respective \textit{Xenopus} genes have not been isolated. It can be seen that the simple over-expression of \textit{Xrel2} RNA in embryos. The first observable abnormality is during gastrulation when the majority of \textit{Xrel2}-injected embryos fail to form the ventral blastopore lip (Fig. 4A and B, Table 2A). Histological sections show that mesoderm involution is not as extensive on dorsal side and that the archenteron has expanded less in \textit{Xrel2}-injected embryos (Fig. 5A and B). The \textit{Xrel2}-injected embryos continue to show this phenotype through gastrulation with many cases never forming a ventral blastopore lip.

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\textit{IxB-\alpha} or \textit{IxB-\gamma} over-expression does not disrupt development but \textit{IxB-\alpha} rescues the \textit{Xrel2} phenotype

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Fig. 4. Representative whole embryo phenotype of embryos injected with Xrel2 or IκB synthetic mRNA. (A and B) Stage 10.5-11 mid-gastrula embryos; (C-J) stage 26-28 tailbud embryos. 1 ng of the following RNAs were injected into newly fertilized eggs: (A and C) β-galactosidase; (B,D,E and F) Xrel2; (G) IκB-γ; (H) IκB-γ-Xrel2; (I) IκB-α; (J) IκB-α-Xrel2. Arrowheads indicate the ventral blastopore lip which is missing in the Xrel2-injected embryo in (B). The mild, intermediate and severe phenotype of Xrel2 injection are shown in (D-F). The arrows in (E and F) indicate the accumulation of pigment that is found in Xrel2-injected embryos. Rescue of the Xrel2 phenotype by IκB-α is shown in (J). Scale bar: A, 150 μm and C, 450 μm.
TABLE 2

PHENOTYPES OF EMBRYOS INJECTED WITH Xrel2 AND/OR IκB INHIBITOR RNA

<table>
<thead>
<tr>
<th>RNA</th>
<th>Normal</th>
<th>Abnormal</th>
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<tbody>
<tr>
<td>β-gal</td>
<td>92</td>
<td>8</td>
</tr>
<tr>
<td>Xrel2</td>
<td>33</td>
<td>82</td>
</tr>
<tr>
<td>IκB-α</td>
<td>96</td>
<td>4</td>
</tr>
<tr>
<td>IκB-γ</td>
<td>93</td>
<td>7</td>
</tr>
<tr>
<td>Xrel2 + IκB-α</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>Xrel2 + IκB-γ</td>
<td>47</td>
<td>20</td>
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</tbody>
</table>

The percentage of embryos showing defects at the mid-gastrula stages (A) and at tailbud stages (B). The mid-gastrula phenotype is absence of ventral lip formation and the tailbud phenotype includes all defects displayed from mild to severe abnormalities as discussed in the text and illustrated in Fig. 4.

Xrel2 protein was found to bind specifically to a consensus κB-motif oligonucleotide as binding was competed by a 100-fold excess of cold target but not by an unrelated competitor oligonucleotide (Fig. 7, lanes 4-6). IκB-α and IκB-γ proteins synthesized in vitro were then used to test whether they could prevent Xrel2 binding to the κB-motif oligonucleotide. These IκBα were active as inhibitors as they prevented HeLa cell extract NF-κB/Rel complex from binding the κB-motif target (data not shown). The IκB proteins themselves do not bind the target oligonucleotide as only the endogenous background present in the translation system can be seen when these are used alone (Fig. 7, lanes 2 and 3). To test if these inhibitors could interact with Xrel2, IκB-α or IκB-γ protein was mixed with Xrel2 before adding the target oligonucleotide to allow time for protein interactions. Figure 7

![Fig. 5. Histological sections of Xrel2-injected embryos. (A and B) Roughly mid-sagittal sections through stage 10.5-11 mid-gastrula embryos injected β-galactosidase (A) or Xrel2 (B) mRNA. D and V indicate the dorsal and ventral lip of the blastopore respectively. B indicates the blastocoel. (C-F) Transverse sections through stage 26-28 tailbud embryos. (C) Section through the trunk of a control embryo injected with β-galactosidase. (D-F) Representative trunk sections through 3 different Xrel2-injected embryos. Nt, neural tube/tissue; S, somite; No, notochord; E, epidermis and M, mesenchyme. Scale bars in A and C are ~100 μm.]

expression of either IκB inhibitor has no effect on development (Fig. 5G and 1, Table 2). Possible explanations are considered in the discussion.

Even though the expression of the injected IκB has no effect on development, it is possible that they would interact with Xrel2 in the over-expression assays. The consequences of co-injection of equivalent amounts of Xrel2 and IκB-α or IκB-γ mRNA into eggs was therefore assessed (Fig. 4G-J, Table 2). It can be seen that IκB-α but not IκB-γ is able to completely rescue the Xrel2 phenotype. When IκB-α is co-expressed with Xrel2, the resulting embryos are indistinguishable from the control injections (Fig. 4J). Conversely, co-expression of IκB-γ and Xrel2 leads to embryos displaying the same spectrum and proportion of phenotypes as Xrel2-injected embryos alone (Fig. 4H, Table 2). The failure of IκB-γ to interact with Xrel2 is unlikely to be inefficient translation as all the mRNAs used in these experiments are efficiently translated in vitro (data not shown). These in vivo results are consistent with the in vitro studies presented below indicating that Xrel2 can physically interact with IκB-α but not IκB-γ. Furthermore, these results indicate that the Xrel2 phenotype does not arise non-specifically as it can be selectively rescued.

Xrel2 protein binds DNA at a consensus κB-motif

The in vivo data presented above suggests that IκB-α but not IκB-γ can interact with Xrel2 protein, therefore to examine this biochemically, a gel shift mobility assay was carried out using in vitro synthesized proteins (Fig. 7). To maximize the separation of specific complexes from the endogenous background present in the translation system (lane 1), the excess unbound probe was run off the end of the gel during the long electrophoresis run.
Fig. 6. Expression of mesodermal markers in Xrel2-injected embryos. 
RNAase protection analysis on 5 µg of total RNA from mid-gastrula (stage 10.5-11) embryos injected with 1 ng of β-galactosidase (Con) or Xrel2 mRNA (Xr2). The different panels show expression of goosecoid (Gcd), brachyury (Bra), wnt-8 (Wnt) or snail (Sna) markers. Ornithine decarboxylase (Odc) used as an internal control is shown in the bottom panel. Each panel is representative of results from 3 independent experiments. t indicates tRNA used as a negative control.

shows that IxB-γ cannot inhibit Xrel2 binding to the target kB-motif oligonucleotide (lane 8), however, DNA binding is prevented when IxB-α is mixed with Xrel2 (lane 7). The interaction between IxB-α and Xrel2 appears specific as there is no inhibition of DNA binding when β-galactosidase or IxB-γ is used in place of IxB-α (Fig. 7, lanes 8 and 9). Although DNA binding affinities have not been measured, these qualitative results are in keeping with the observations made in vivo, suggesting that IxB-α but not IxB-γ can interact with Xrel2.

Xrel2 is not sufficient for mesoderm induction

If mesoderm formation involves the redistribution of cytoplasmic NF-kB/Rel proteins to the nucleus in response to binding of mesoderm inducing factor then supplying excess NF-kB/Rel might bypass the requirement for inducing factor. To address this, we exploited the blastula animal cap which normally forms ectoderm but can be diverted to mesoderm in response to inducers and their downstream targets. Animal cap explants were made at the blastula stages from eggs previously injected with Xrel2 mRNA and assayed for the activation of mesodermal markers at gastrulation. Figure 8 shows that four mesodermal markers are not induced in Xrel2-injected animal caps which is similar to the control injection of β-globin mRNA. eFGF was used as a positive control and the strong expression of brachury, wnt-8 and snail is consistent with eFGF mimicking a ventral mesoderm inducer (Isaacs et al., 1992). Possibly, the failure of Xrel2 to induce mesoderm markers was due to the presence of carboxyl-terminal sequences regulating Xrel2 function, however, similar experiments with a truncated Xrel2 construct (XrT) lacking these sequences proved unable to activate mesodermal gene expression (Fig. 8). These results suggest that Xrel2 is not sufficient to direct mesoderm formation and therefore it does not have a simple role in mesoderm formation in vivo. Also, this suggests that the whole embryo Xrel2-phenotype is not simply due to a production of excess mesoderm.

Discussion

Xrel2 is a new member of the NF-kB/Rel family of transcription factors

We have described the isolation of the Xenopus Xrel2 gene which is a novel member of the NF-kB/Rel protein family. In Xenopus, there are often highly related polymorphic gene variants due to a presumed genome duplication giving rise to pseudo-tetraploidy (Bisbee et al., 1977) but the high divergence of the Xrel2 sequence makes it unlikely that it represents a polymorphic variant of XrelA. In the Rel-homology domain, Xrel2 is more closely related to c-Rel than other members of the family, however, the carboxyl-terminal sequences of Xrel2 bear little sequence relationship to other NF-kB/Rel proteins. The mouse and avian c-rel genes also show little similarity in the carboxyl-terminal domains therefore the c-rel genes may have evolved species-specific carboxyl-terminal domains. It is also possible that Xrel2 has no counterparts in other vertebrates and has evolved independently in Xenopus as a consequence of the presumed genome duplication. It is just as likely, however, that Xrel2-related genes remain to be isolated in other vertebrates.

We have shown that the Xrel2 protein binds DNA at a consensus kB-motif. It is likely that Xrel2 binds DNA as a homodimer in our experiments as there are no known NF-kB/Rel proteins in wheat germ lysates. In addition, it is known that the NF-kB/Rel proteins normally recognize DNA as dimers with each subunit interacting with a half-site of the recognition sequence (Zabel et al., 1991; Siebenlist et al., 1994). We have no information on the ability of Xrel2 to form heterodimers with any other NF-kB/Rel protein nor on the role of the Xrel2 carboxyl-terminal sequences; however, the Xrel2 sequence indicates that it may belong to the acidic class of transactivators. Our results also
suggest that Xrel2 can interact with IkB-α but not IkB-γ which may reflect the differing affinities that each IkB has for different NF-κB/Rel combinations (Liou et al., 1992; Dobrzanski et al., 1994).

**The role of NF-κB/Rel proteins in early Xenopus development**

We were struck by the similarity of the determination of the dorsal-ventral axis in *Drosophila* to the elements of the NF-κB/Rel pathway. So far, in vertebrates, there is little evidence for a role of NF-κB/Rel proteins in primary determinative events. Our work with Xrel2, together with that on XrelA in *Xenopus* (Kao and Hopwood, 1991; Richardson et al., 1994), has shown that there is early developmental expression of NF-κB/Rel proteins. The relevance of Xrel2 RNA distribution is unclear as the NF-κB/Rel proteins undergo regulated nuclear transport. Until an antibody to Xrel2 is available, we will be unable to confirm the distribution of ‘active Xrel2’ protein within the embryo. It is interesting to note that the overlapping expression patterns of Xrel2 and XrelA opens up the possibility of functioning heterodimers in the embryo.

Over-expression of Xrel2 does not induce mesoderm in isolated animal caps suggesting that Xrel2 is not involved in primary mesoderm induction. This result might be explained if Xrel2 does not act alone and normally functions as a heterodimer. Alternatively, there may be excess IkB inhibitors within the animal cap that can mop up the excess Xrel2 protein although this might be unlikely as free IkBs are known to be rapidly degraded (Rice and Ernst, 1993; Scott et al., 1993). Another possibility is that Xrel2 expression may induce the expression of IkB inhibitors as has been noted for other NF-κB/Rel proteins (Beg and Baldwin, 1993; Gilmore and Morin, 1993; Thanos and Maniatis, 1995), however, we have used a dose of Xrel2 that leads to clear whole embryo phenotypes. This might suggest that the animal cap is less sensitive than whole embryos because of such regulatory mechanisms.

The whole embryo phenotype displayed by Xrel2-injected embryos is complex. The earliest problems appear as an inhibition of gastrulation on the ventral side but we do not know if this is a consequence of Xrel2 function at the blastula stages. Similarly, we can not be sure that the tailbud phenotype arises from disturbed gastrulation or whether there are later effects of Xrel2. It is likely that Xrel2 has a specific over-expression defect as it can be rescued by the co-expression of IkB-α. This represents one of the few cases, in *Xenopus*, of an over-expression phenotype being specifically rescued by the co-expression of an interacting gene product. The Xrel2 phenotype probably arises, in part, through strong effects on mesoderm. This is supported by a reduction in expression of mesodermal markers and by an inhibition of convergent extension movements. We have also found that injection of IkB-α or IkB-γ alone into *Xenopus* eggs has no effect on development. This result does not rule out a role for Xrel2 or the NF-κB/Rel:IkB system in early development. IkBs are extremely unstable if they are not complexed to NF-κB/Rel dimers (Rice and Ernst, 1993; Scott et al., 1993; Thanos and Maniatis, 1995) therefore there may not be enough active inhibitor around the time of NF-κB/Rel signalling. Alternatively, a set of NF-κB/Rel dimers that does not interact with these particular IkBs might be employed during development.

Our work has suggested that NF-κB/Rel proteins might have important functions at the earliest stages of vertebrate development. Unlike the early *Drosophila* embryo, where there is only one NF-κB/Rel protein and one IkB inhibitor, the *Xenopus* embryo is likely to have multiple genes for each. We already know that there are at least two *Xenopus* NF-κB/Rel-related proteins expressed in early development. This extra complexity compared with the *Drosophila* situation makes it difficult to see a simple conservation of the *Drosophila* dorsal pathway in *Xenopus*. The further understanding of the role of NF-κB/Rel proteins in *Xenopus* development will require the isolation of *Xenopus* IkB homologues and more members of the *Xenopus* NF-κB/Rel family. In addition, the distribution of these proteins and their interactions with Xrel2 will need to be determined in order to assess the embryological function of the NF-κB/Rel:IkB system.
Recently after acceptance of this paper, the over-expression phenotype of XrelA has been reported, which appears to be comparable to that of Xrel2 (Richardson et al., 1995). This work suggests again that the Xrel proteins are not vertebrate counterparts of *Drosophila* dorsal and further suggests a role for Xrel in the patterning of embryonic terminal structures.

**Materials and Methods**

Embryological and histological procedures were carried out as described by Godsave et al. (1988). Embryo stages were according to Nieuwkoop and Faber (1967). Animal cap explants were made at stage 6-9 and cultured in NAM/2. RNA microinjections were carried out in 1xNAM + 5% Ficol 20-30 min before first cleavage furrow formation or in or both cells of the 2 cell-stage embryo. RNA injection was dissolved in water and 5-10 nl injected per embryo. Embryos were gradually transferred to NAM/10 before gastrulation.

Molecular methods were as described by Ausubel et al. (1992). Novel Xenopus NF-xb/Rel-related genes were isolated using RT-PCR with degenerate oligonucleotide primers as follows: equal quantities of total RNA were combined from a number of stages from gastrula to tailbud to increase the chances of amplifying cDNAs with limited temporal expression. 10 µg RNA template was heat denatured and reverse transcribed in a volume of 200 µl for 1 h at 42°C in 1xPCR buffer containing 200 U/jl MuLV reverse transcriptase (BRL), 0.5 µg/µl random primers (Boehringer), 1 mM dNTPs and 1 U jl RNasin (Promega). The reaction was terminated by heating to 95°C for 3 min and then 10 µl of this cDNA was added directly to 40 µl of 1xPCR buffer containing 0.2 mM dNTPs, 100 ng of forward and reverse primers and 1 U Tag DNA polymerase (Promega). Amplification was in a Techne PCH-2 thermocycler using an annealing temperature of 50°C for another 30 cycles. Degenerate PCR primers were selected from alignments of Rel-homology domains presented by Steward (1987). The forward primer had sequence 5'-TGC CAG GAG AAC TGC TTG-3' (16x degeneracy) and the reverse primer, 5'-GGI CAG CTG TGC ATC TT-3' (54x degeneracy).

PCR products were cloned and sequenced and the potential NF-xb/Rel-related clones used to screen a Xenopus oocyte lgt10 cDNA library. Positive phages were sub-cloned and DNA sequencing accomplished using Sequenase kits (USB). Sequencing primers were designed from sequence information produced from either end of the clone and presented by Steward (1987). The forward primer had sequence 5'.TGC CAG GAG AAC TGC TTG-3' (16x degeneracy) and the reverse primer, 5'-GGI CAG CTG TGC ATC TT-3' (54x degeneracy).

A forward primer with sequence 5'-TGC CAG GAG AAC TGC TTG-3' (16x degeneracy) and the reverse primer, 5'-GGI CAG CTG TGC ATC TT-3' (54x degeneracy). PCR products were cloned and sequenced using the potential NF-xb/Rel-related clones such as clone 13-2. RNase digestion of RNA was performed using the RNase inhibitor kit (Ambion). The coding regions of Xrel2, lxB-x (Davis et al., 1991), lxB-y (Inoue et al., 1992) and X-c, which contains the Rel-homology domain of Xrel2 (nucleotide 1 to 1240), were sub-cloned by standard techniques. The pspsAT-8-globin and pspsAT-eGFp plasmids have been described (Krieg and Melton, 1984; Isaacs et al., 1994).

Proteins for gel mobility shift assays were produced by in vitro transcription of synthetic mRNA in wheat germ lysates as described by the manufacturer (Promega). All translations products were confirmed to be the correct molecular weight by SDS-polyacrylamide gel electrophoresis and autoradiography. Gel shifts assays were carried out by a system purchased from Promega. 10 µl reactions were performed at 20°C with 1 µl of in vitro translation products and two stranded radiolabeled x-b-motif (5'-AGT TGA GGC GAC TTT CCC AGG C-3') or SP1 (5'-ATT CGA TCG GGG CGG GCC GAG C-3') oligonucleotides. For Xrel2 protein, the XrT construct containing the Rel-homology domain was used in the gel shift assay rather than full length Xrel2, as this gave a cleaner separation from the endogenous non-specific background found in the wheat germ lysate. Competitions were performed using 100 fold excess of unlabeled oligonucleotide. For inhibition experiments, 1 µl of Xrel2 in 1xgel shift buffer for 15 min at 20°C to allow protein interaction before adding oligonucleotide. Complexes were visualized by autoradiography of the reactions separated on 4% non-denaturing polyacrylamide gels.

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**References**


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