

Control of axis formation in *Xenopus* by the NF- κ B-I κ B system

DAVID TANNAHILL* and FIONA C. WARDLE¹

Department of Anatomy, University of Cambridge, Cambridge, United Kingdom

ABSTRACT We describe the isolation and analysis in *Xenopus* of *Xrel2*, a novel member of the NF- κ B/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- κ B/Rel proteins, *Xrel2* protein is able to bind DNA at a κ B-Motif. Ectopic expression of *Xrel2* disrupts normal morphogenesis at the early gastrula stages suggesting that the NF- κ B/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 I κ B- α and that ectopic expression of I κ B- α or I κ B- 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- κ B/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- κ B, I κ B

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- κ B/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- κ B/Rel proteins bind DNA either as homo or heterodimers at target sequences known as κ B-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 I κ B family and include cactus in *Drosophila* and the I κ B- 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- κ B/Rel pro-

teins in early vertebrate development is less clear. In mammalian embryos, there is little evidence for an early developmental function of the NF- κ B/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 *nfxb1* (p50) germline mutations do not show any early developmental abnormalities (Sha *et al.*, 1995; Weih *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-v-Rel antiserum, an endogenous *Xenopus* NF- κ B/Rel protein has been detected in embryonic nuclei (Bearer, 1994; Richardson *et al.*, 1994). Although *XrelA* can activate κ B-motif-dependent transcription and there are κ B-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- κ B/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- κ B/Rel protein with DNA binding properties typical of

*Address for reprints: Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 3DY, United Kingdom. FAX: 01223 333 786.

¹Present address: Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, United Kingdom.

CCCCAGCTATATGGTCAGTGTAGGGGTTTAGCTTGTTCAGCAGGAGGGCTGAAGTGGACAC 60
 GTTGAAGCTCAGAAAGACTGTGGATAGTCTGTTGGCTGGATATCTTTGGTGGAGTGGCG 120
 TCTCCTGAAAAATGTACCAGCTGTGAGTCTGAGCCGCTGCCAAGGATACCTGCCATTTA 180
 CTCGAGTGTATATCCGCGGCTCTTCACTGTCCCTGGTTCCTGTTGTCTACTCCGCTGCACT 240
 GAGCAACAATGGCTGGTAAATGCTCAACATTTCTCATCTGGTCTGCTCCAAACATCTTTT 300
M A G N A Q H S H H G R L Q T S F
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 G L N D P H I E I F E Q P R Q R G M R Y
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R Y K C E G R C A G S I H G E H S T E N
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 D L N V V R L C F Q V F L P D E H C S Y
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 I F L L C D K V Q K D D I E V R F P T D
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 N W E A K G T F R G Q A D V H R Q V A I V
 TTCAAAACGCCCCATTTCCAGTTCCTACTGTGTGTAACAGTAAATAATGCAAACTT 1080
 F K T P F H R S I T D V V T V K M Q L
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R R E V S E P M D F R Y L P D P
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 E D P H A G N K F K K Q R T S E V M Q K F
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 K F E M Q E R R E L L S P A K F N V N P I
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 C K R E H F T N S S G C G Q R S H A M Q P
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 P T A C R P N V T Y N D T T S V Q T R M P
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 N N S S S I Q P N L S Q L S V L N P A M
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 Q A N M H S T Y T S S I N N F M E T L R
 GCAGTTCCATCAGCTAAACACTACAACCTCCCATTCGCGAGCCCAAGTCCACATAGA 1560
 A V P S Q L N T H K K L P F A E P S H T R
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 P D V A P S N T T N M F R Q H I F N P N
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 V P N T S G Q V S S C P S V P R D I N L
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 Y T A H S N H M D I S E L G Q M T T S T
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 V N D S S I S S L S F N G V L P N P I F
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 S T P P S Y H P S I P E Q R L P D T S S I
 GCTGCTCCGACAGCACATCTATGGGTCTAGAAATCTTCCCGGCAATGTACAAAGCGAT 1920
 A A P H S T S M G H E I F P P G I V Q S D
 GACAATTAATCAGTGTGAAATCCGAACTTGATGATCAATGTCAGAACTTTGGAAATGCA 1980
 D N Y I T S V E S E L D S I L Q N F G S T
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 S E M L H D
 AGCCTTTTAGCACTTTCAACAGAAAGAGCTTACAGCCATCTCTCTGATATGGGTCCAACA 2100
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 CTGTGTCAGTATGTCATAAACCAGTATCCCAACAGTACTGATGAGCAACATGTTGT 2280
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 GTACTACGCTTTAAGACCTTTTCCAAATGTTGTGTGTGTGTATATATATATATATA 2640
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 TCTGATGAGAAATTAATCTGTAATCTAATGACTATGCTATTGATAAGAGGCTAGG 2760
 CTATAAAAATGTATCAAAATGTTGTAACTTAGAATTAGGCCAAAATAAATGTTTCTCAT 2820
 GTTTTACT

Fig. 1. DNA sequence and conceptual translation of *Xrel2*. Protein sequences indicated in bold are an essential DNA binding sequence (RXXRXRXXC), a protein kinase A phosphorylation site (RRPS) and a nuclear localization sequence (KKQR). The *Xrel2* sequence has been submitted to the EMBL database (accession number: Z49252).

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- κ B/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- κ B/Rel family

Only one member of the NF- κ B/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- κ B/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- κ B/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- κ B/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- κ B/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 transcriptional activation domain as found in other NF- κ B/Rel proteins (Bull *et al.*, 1990; Schmitz and Baeuerle, 1991; Dobrzanski *et al.*, 1993). The *Xrel2* Rel-homology domain shows a number of features shared between all NF- κ B/Rel proteins (Fig. 1). A short sequence (RXXRXRXXC) 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- κ B/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- κ B-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- κ B/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 continuously 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)

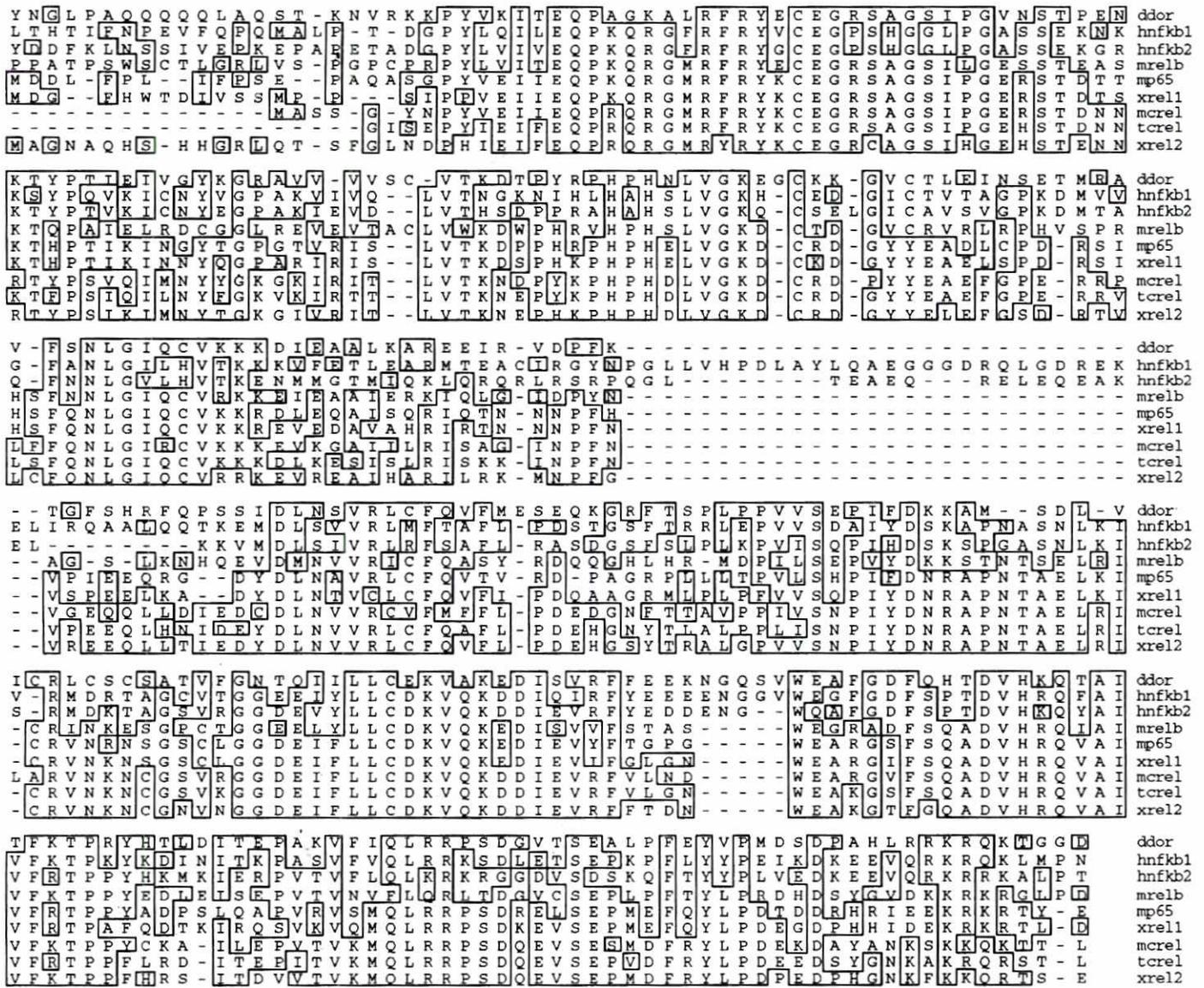


Fig. 2. Alignment of the Rel-homology domain of Xrel2 to other members of the NF-κB/Rel family. Boxes have been drawn round aligned residues with 3 or more matching amino acids. Data was produced using the Clustal method with a PAM250 residue weight. The representatives of each family member are as follows: ddor, *Drosophila dorsal*; hnfbk1, human p50; hnfbk2, human p52; mrelb, mouse RelB; mp65, mouse p65; xrel1, *Xenopus XrelA*; mcrel, mouse c-Rel and tcrel, avian c-Rel.

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 can not be determined until an antibody to *Xrel2* becomes available. Figure 4 shows the external phenotype of *Xrel2*-inject-

TABLE 1

SEQUENCE RELATIONSHIP OF THE Rel-HOMOLOGY DOMAIN OF THE *Xrel2* PROTEIN TO OTHER MEMBERS OF THE NF- κ B/Rel FAMILY

	% Similarity								
	ddor	hnfkb1	hnfkb2	mrelb	mp65	xrel1	mcrel	tcrel	xrel2
ddor		38.0	37.2	47.8	46.9	47.8	46.1	46.9	47.2
hnfkb1	55.5		56.7	38.5	37.2	38.3	41.1	40.8	39.4
hnfkb2	59.7	39.9		40.2	41.1	40.2	431.6	40.5	38.5
mrelb	54.8	56.1	57.7		53.6	53.6	50.6	53.1	52.0
mp65	54.8	59.2	55.8	47.7		77.7	62.0	65.9	63.4
xrel1	54.6	57.5	56.7	49.3	20.4		65.1	67.3	65.6
mcrel	53.7	52.6	55.1	49.8	36.1	34.7		80.7	72.9
tcrel	52.0	55.3	54.1	47.2	35.7	32.1	19.1		76.3
xrel2	53.6	57.7	59.6	51.5	40.3	36.4	23.5	22.5	

% Divergence

Figures above the diagonal represent the percentage similarity and figures below represent the percentage divergence between the respective comparisons. Data was produced using the Clustal method with a PAM250 residue weight table. The representatives of each family member are as follows: ddor, *Drosophila* dorsal; hnfkb1, human p50; hnfkb2, human p52; mrelb, mouse RelB; mp65, mouse p65; xrel1, *Xenopus* XrelA; mcrel, mouse c-Rel and tcrel, avian c-Rel.

ed embryos. The first observable abnormality is during gastrulation when the majority of *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 *Xrel2*-injected embryos (Fig. 5A and B). The *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 *Xrel2*-injected embryos show a defect (Figs. 4C-F, 5C-F, Table 2B). The phenotype is somewhat variable and representative examples are shown in Fig. 4C-F. Variable phenotypes are often noted in *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 *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.

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 *wnt-8*, a ventral marker, and *snail* and *brachyury*, pan-mesodermal markers, was reduced in *Xrel2*-injected embryos by ~2 fold as compared to control injected embryos. The effects of excess *Xrel2* were not limited to ventral tissue as expression of *gooseoid*, a dorsal marker, was also reduced ~3 fold.

***I κ B- α* or *I κ B- γ* over-expression does not disrupt development but *I κ B- α* rescues the *Xrel2* phenotype**

The NF- κ B/Rel proteins interact with I κ B inhibitors to retain them in the cytoplasm. By supplying excess I κ B, it may be possible to interfere with the normal developmental NF- κ B/Rel signalling. To test this, synthetic mRNA for avian *I κ B- α* or human *I κ B- γ* was injected into eggs as the respective *Xenopus* genes have not been isolated. It can be seen that the simple over-

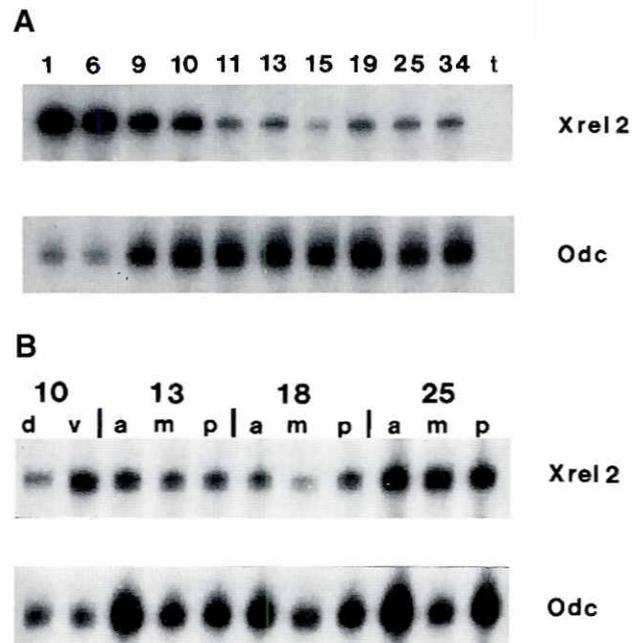


Fig. 3. Developmental expression of *Xrel2* RNA. (A) Temporal expression of *Xrel2*. RNAase protection analysis using 10 μ g of total RNA. The stage numbers are indicated above each lane and correspond to those given in the normal table (Nieuwkoop and Faber 1967). Briefly, stage 1 is the fertilized egg; blastula stages are up to stage 10; gastrula stages are 10-13; neurula stages are 13-19 and tailbud stages are from 19 onwards. t represents a tRNA negative control lane. Odc represents ornithine decarboxylase used as an internal control. Exposures were ~12 days for *Xrel2* and ~10 h for Odc. (B) Regional expression of *Xrel2*. RNAase protection analysis on 5 μ g of total RNA from dissected embryo pieces. Stages numbers are indicated above each dissection. For gastrula (stage 10), d represents the dorsal third segment and v represents the remaining ventral two thirds segment. For early and late neurula (stages 13 and 18) and tailbud (stage 25), a, m and p represent the anterior, middle and posterior thirds, respectively. Exposures were ~14 days for *Xrel2* and ~12 h for Odc.

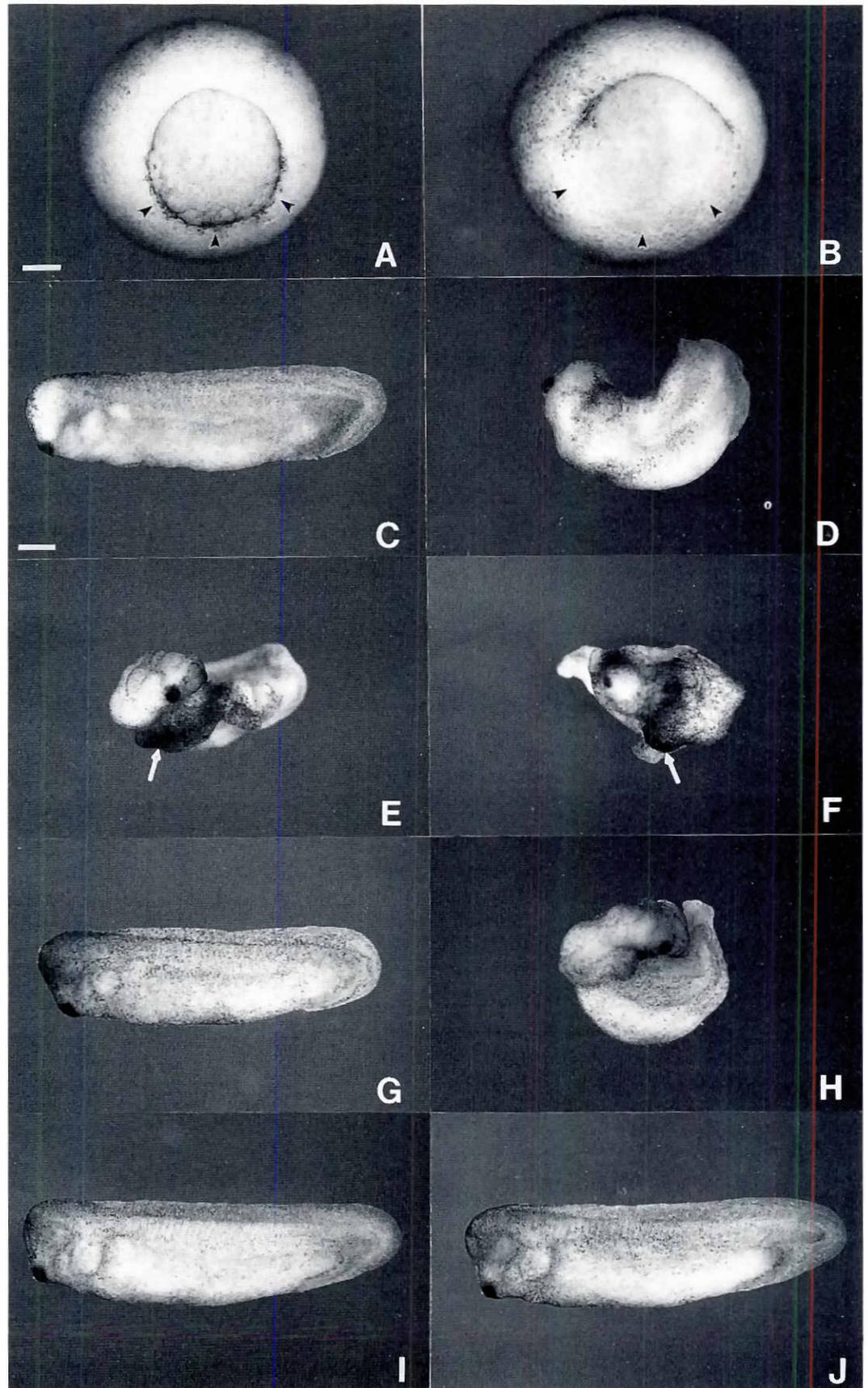


Fig. 4. Representative whole embryo phenotype of embryos injected with *Xrel2* or *IκB* synthetic mRNA. (A and B) Stage 10.5-11 mid-gastrula embryos; (C-J) stage 26-28 tailbud embryos. 1 ng of the following RNAs were injected into newly fertilized eggs: (A and C) β -galactosidase. (B,D,E and F) *Xrel2*. (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 μ m and C, 450 μ m.

TABLE 2

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

A. Phenotype at mid-gastrula stage 10.5-11

RNA	Normal		Abnormal	
	%	n	%	n
β-gal	92	86	8	8
<i>Xrel2</i>	33	41	67	82
<i>IκB-α</i>	96	49	4	2
<i>IκB-γ</i>	93	56	7	4
<i>Xrel2</i> + <i>IκB-α</i>	90	43	10	5
<i>Xrel2</i> + <i>IκB-γ</i>	47	18	53	20

B. Phenotype at tailbud stages 26-28

RNA	Normal		Abnormal	
	%	n	%	n
β-gal	88	65	12	9
<i>Xrel2</i>	1	1	99	96
<i>IκB-α</i>	90	44	10	5
<i>IκB-γ</i>	94	50	6	3
<i>Xrel2</i> + <i>IκB-α</i>	92	43	8	4
<i>Xrel2</i> + <i>IκB-γ</i>	6	2	94	31

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. 1 ng of each RNA was injected per embryo. n represents total number of cases. 2-4 independent experiments were performed with different RNA batches for each sample.

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

Even though the expression of the injected *IκB*s 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.

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 *IκB*s were active as inhibitors as they prevented HeLa cell extract NF- κB /Rel complexes 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

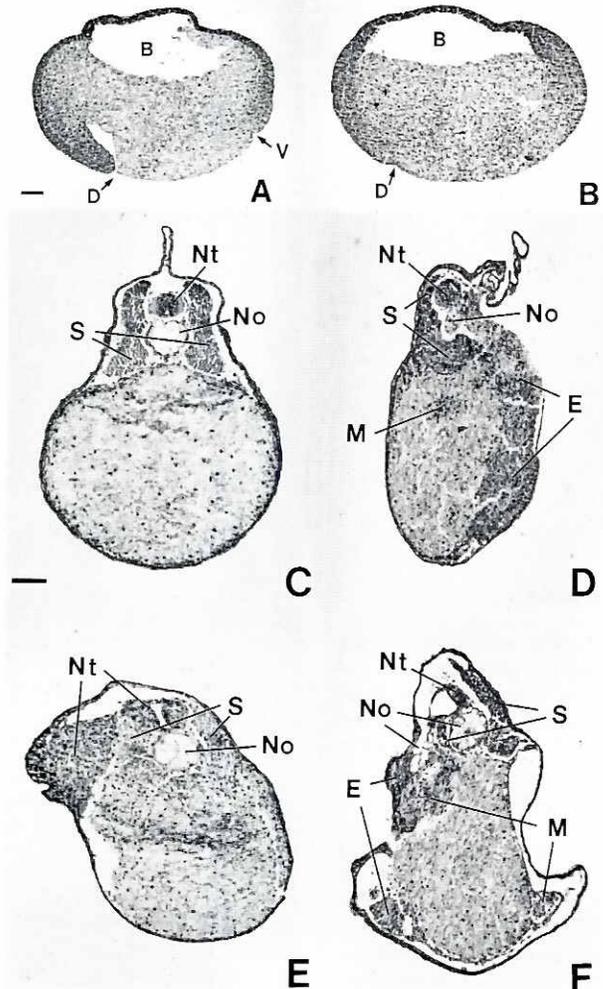


Fig. 5. Histological sections of *Xrel2*-injected embryos. (A and B) Roughly mid-sagittal sections through stage 10.5-11 mid-gastrula embryos injected β -galactosidase (A) or *Xrel2* (B) mRNA. D and V indicate the dorsal and ventral lip of the blastopore respectively. B indicates the blastocoel. (C-F) 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 $\sim 100 \mu\text{m}$.

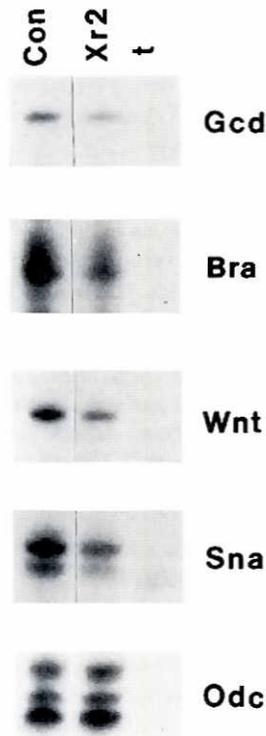


Fig. 6. Expression of mesodermal markers in *Xrel2*-injected embryos. RNAase protection analysis on 5 μ g of total RNA from mid-gastrula (stage 10.5-11) embryos injected with 1 ng of β -galactosidase (Con) or *Xrel2* mRNA (Xr2). The different panels show expression of goosecoid (Gcd), brachyury (Bra), *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 κ B- γ can not inhibit Xrel2 binding to the target κ B-motif oligonucleotide (lane 8), however, DNA binding is prevented when κ B- α is mixed with Xrel2 (lane 7). The interaction between κ B- α and Xrel2 appears specific as there is no inhibition of DNA binding when β -galactosidase or κ B- γ is used in place of κ B- 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 κ B- α but not κ B- γ can interact with Xrel2.

Xrel2 is not sufficient for mesoderm induction

If mesoderm formation involves the redistribution of cytoplasmic NF- κ B/Rel proteins to the nucleus in response to binding of mesoderm inducing factor then supplying excess NF- κ B/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 inducing factors 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 *brachyury*, *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- κ B/Rel family of transcription factors

We have described the isolation of the *Xenopus Xrel2* gene which is a novel member of the NF- κ B/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- κ B/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 κ B-motif. It is likely that Xrel2 binds DNA as a homodimer in our experiments as there are no known NF- κ B/Rel proteins in wheat germ lysates. In addition, it is known that the NF- κ B/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- κ B/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

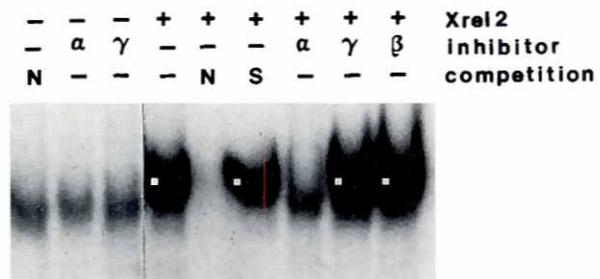


Fig. 7. DNA binding of Xrel2 protein to a consensus κ B-motif target oligonucleotide. Gel mobility shift assays were carried out as described in Materials and Methods. Specific complexes with Xrel2 and the κ B-motif target are indicated by dots (lane 4). Competition with an 100 fold excess of cold competitor κ B-motif (N) but not by an unrelated SP1 (S) oligonucleotide shows this binding to be specific (lanes 5 and 6). Note the general non-specific background endogenous to the wheat germ lysate in the absence of added protein even when competed with the κ B-motif target (lane 1). Xrel2 binding to the κ B-motif target is prevented by κ B- α but not κ B- γ or β -galactosidase (β) (lanes 7-9). Note the inhibitors κ B- α (α) and κ B- γ (γ) do not form complexes with the κ B-motif target (lanes 2 and 3).

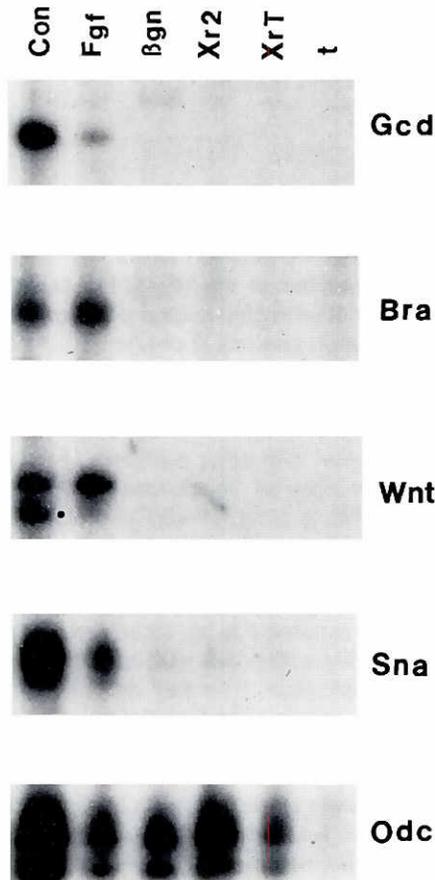


Fig. 8. *Xrel2* does not induce mesoderm in animal caps. RNAase protection analysis on 4 μ g of total RNA from mid-gastrula (stage 10.5-11) animal caps taken from eggs injected with either 10 pg eFGF (Fgf), or 1 ng β -globin (Gbn), *Xrel2* (Xr2) or the Rel-homology domain construct of *Xrel2* (XrT) synthetic mRNA. The resulting embryos were harvested at stage 10.5-11 and assayed for expression of goosecoid (Gcd), brachyury (Bra), wnt-8 (Wnt) or snail (Sna) markers. Orithine decarboxylase (Odc) was used as an internal control. Con is whole gastrula used as a control and t is a negative control using tRNA. For the Wnt panel, the dot represents a spurious non-specific band.

suggest that *Xrel2* can interact with $\text{I}\kappa\text{B-}\alpha$ but not $\text{I}\kappa\text{B-}\gamma$ which may reflect the differing affinities that each $\text{I}\kappa\text{B}$ 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 $\text{I}\kappa\text{B}$ inhibitors within the animal cap that can mop up the excess *Xrel2* protein although this might be unlikely as free $\text{I}\kappa\text{B}$ s 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 $\text{I}\kappa\text{B}$ 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 regulative 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 $\text{I}\kappa\text{B-}\alpha$. 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 $\text{I}\kappa\text{B-}\alpha$ or $\text{I}\kappa\text{B-}\gamma$ alone into *Xenopus* eggs has no effect on development. This result does not rule out a role for *Xrel2* or the NF- κB /Rel: $\text{I}\kappa\text{B}$ system in early development. $\text{I}\kappa\text{B}$ s 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 $\text{I}\kappa\text{B}$ s 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 $\text{I}\kappa\text{B}$ 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* $\text{I}\kappa\text{B}$ 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: $\text{I}\kappa\text{B}$ 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 8-9 and cultured in NAM/2. RNA microinjections were carried out in 1xNAM + 5% Ficoll 20-30 min before first cleavage furrow formation or into both cells of the 2 cell-stage embryo. RNA for 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- κ B/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 μ g RNA template was heat denatured and reverse transcribed in a volume of 200 μ l for 1 h at 42°C in 1xPCR buffer containing 200 U/ μ l MuLV reverse transcriptase (BRL), 0.5 μ g/ μ l random primers (Boehringer), 1 mM dNTPs and 1 U/ μ l RNasin (Promega). The reaction was terminated by heating to 95°C for 3 min and then 10 μ l of this cDNA was added directly to 40 μ l of 1xPCR buffer containing 0.2 mM dNTPS, 100 ng of forward and reverse primers and 1U Taq DNA polymerase (Promega). Amplification was in a Techne PCH-2 thermocycler using an annealing temperature of 40°C for the first 10 cycles, followed by 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 CGI GTI AAC AAG AAC TGC/T GG-3' (18x degeneracy) and the reverse primer, 5'-GGI CGI CGI AGC TGC ATC/T TT-3' (54x degeneracy).

PCR products were cloned and sequenced and the potential NF- κ B/Rel-related clones used to screen a *Xenopus* oocyte λ gt10 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 sequencing reiterated until the whole gene was sequenced on both strands at least twice. Sequences were analyzed by the UWGCG sequence software package version 7.3. Multiple sequence alignments were also produced using Megalign (Dnastar).

RNA isolation and RNAase protection analysis were carried out as in Isaacs *et al.* (1992). In all assays, the ubiquitously expressed *ornithine decarboxylase* gene (*Odc*) was used as an internal control. *Xrel2* protections were performed using a specific probe derived from the 3'UTR of the *Xrel2* cDNA giving a protected fragment of 0.26 kb. *Brachyury* and *snail* were used as pan-mesodermal markers (Sargent and Bennett, 1990; Smith *et al.*, 1991), *gooseoid* as a dorsal mesodermal marker (Cho *et al.*, 1991) and *wnt-8* as a ventral mesodermal marker (Christian and Moon, 1993) as described in Isaacs *et al.* (1994). Autoradiographs (Kodak X-OMAT) were exposed for 1-10 days and scanned using a Joyce-Loebl Chromoscan 3 densitometer.

Synthetic mRNA for embryo microinjection and *in vitro* translation was produced by *in vitro* transcription using a SP6 or T3 polymerase MEGAScript kit (Ambion) in the presence of the cap analogue m7G(5')ppp(5')G from cDNAs cloned into psp64T (Krieg and Melton, 1984) or into a modified psp64T containing a T3 promoter in place of the SP6 promoter (a gift from Patrick Lemmaire). The coding regions of *Xrel2*, *I κ B- α* (Davis *et al.*, 1991), *I κ B- γ* (Inoue *et al.*, 1992) and *XrT*, which contains the Rel-homology domain of Xrel2 (nucleotide 1 to 1240), were

sub-cloned by standard techniques. The psp64T- *β -globin* and psp64T-*eFGF* plasmids have been described (Krieg and Melton, 1984; Isaacs *et al.*, 1994).

Proteins for gel mobility shift assays were produced by *in vitro* translation of synthetic mRNA in wheat germ lysates as described by the manufacturer (Promega). All translation products were confirmed to be the correct molecular weight by SDS-polyacrylamide gel electrophoresis and autoradiography. Gel shift assays were carried out by a system purchased from Promega. 10 μ l reactions were performed at 20°C with 1 μ l of *in vitro* translation products and double stranded radiolabeled κ B-motif (5'-AGT TGA GGG GAC TTT CCC AGG C-3') or SP1 (5'-ATT CGA TCG GGG CGG GGC 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 μ l of I κ B inhibitor was incubated with 1 μ l 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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