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Xrel3 is required for head development in *Xenopus laevis*

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SUMMARY

The Rel/NF-κB gene family encodes a large group of transcriptional activators involved in myriad differentiation events, including embryonic development. We have shown previously that *Xrel3*, a *Xenopus* Rel/NF-κB-related gene, is expressed in the forebrain, dorsal aspect of the mid- and hindbrain, the otocysts and notochord of neurula and larval stage embryos. Overexpression of Xrel3 causes formation of embryonic tumours. We now show that Xrel3-induced tumours and animal caps from embryos injected with Xrel3 RNA express *Otx2, Shh and Gli1*. Heterodimerisation of a C-terminally deleted mutant of *Xrel3* with wild-type *Xrel3* inhibits in vitro binding of wild-type *Xrel3* to Rel/NF-κB consensus DNA sequences. This dominant interference mutant disrupts *Shh, Gli1* and *Otx2* mRNA patterning and inhibits anterior development when expressed in the dorsal side of zygotes, which is rescued by co-injecting wild-type *Xrel3* mRNA. In chick development, Rel activates Shh signalling, which is required for normal limb formation; *Shh*, *Gli1* and *Otx2* encode important neural patterning elements in vertebrates. The activation of these genes in tumours by Xrel3 overexpression and the inhibition of their expression and head development by a dominant interference mutant of Xrel3 indicates that Rel/NF-κB is required for activation of these genes and for anterior neural patterning in *Xenopus*.

Key words: Rel/NF-κB, Head development, Shh, Gli1, Otx2, Tumours, Oncogenes, *Xenopus*

INTRODUCTION

The Rel/NF-κB gene family encodes a large group of eukaryotic transcriptional factors that regulate a wide diversity of physiological cell differentiation events, including inflammation, immunity and apoptosis. These ubiquitous proteins are activated by a number of physiological stimuli and also activate a variety of cellular targets (for reviews, see Chen and Ghosh, 1999; Pahl, 1999; Israel, 2000).

Depending on the cell type, Rel/NF-κB dimeric complexes regulate transcription from target genes in either a constitutive or inducible manner. Inducible complexes are retained in the cytoplasm by a regulatory subunit related to the IκB family of inhibitors. Upon cellular activation, IκB is removed by the ubiquitin-proteosomal degradation pathway allowing the Rel/NF-κB complex to translocate to the nucleus, whereupon it binds to a consensus decameric sequence within target genes.

In addition to processes related to adult cell differentiation, Rel/NF-κB transcriptional regulators are required in embryonic and foetal development. The patterning of the dorsal-ventral axis of the *Drosophila* embryo, for instance, depends on the activity of the morphogen Dorsal. Dorsal is localised by the activity of a number of maternal genes, including *Toll*, *easter*, *spätzle*, *tube* and *pelle*, to nuclei of blastoderm cells in a dorsal-to-ventral gradient (Drier et al., 2000). Dorsal activates patterning genes in ventral and ventrolateral regions such as *twist* and *snail*, which specify mesodermal and neurogenic cell lineages (Drier and Steward, 1997; Govind, 1999). In *Xenopus laevis* embryos, the finding that members of the Toll/Spätzle signalling pathway could induce a secondary body axis suggests that a Dorsal-like morphogen might also exist in amphibians (Armstrong et al., 1998).

In mammalian development, the role of Rel/NF-κB proteins appears to be confined largely to immunity and organogenesis. Mice that are defective for *Nfkb1*, *Rel* (*c-rel*) or *Relb* develop deficiencies in specific cells of the immune system (Attar et al., 1997; Gerondakis et al., 1999). Rela-defective foetal mice die of liver failure resulting from massive apoptosis, illustrating the role of Rel/NF-κB as an anti-apoptotic factor (Foo and Nolan, 1999; Barkett and Gilmore, 1999; Chen et al., 2000). Thus, these specific Rel/NF-κB proteins are not required for body patterning, but there may be undiscovered Rel/NF-κB activities and/or embryonic genes, since mouse homologues of *twist* are required for mesoderm formation in mammals (Dixon, 1997).

The sonic hedgehog (Shh) ligand, its downstream target (the zinc-finger transcriptional activator Gli1), and related Gli proteins Gli2 and Gli3, form the basis of an important signalling pathway in organogenesis and tumourigenesis (Hahn et al., 1999; Ruiz i Altaba, 1999; Britto et al., 2000). There is evidence that Rel/NF-κB activates this pathway in vertebrates. In developing chick limbs, negative regulation of

Rel/NF-κB by constitutive expression of its natural inhibitor IκB, prevents the expression of *twist, Shh* and *Gli1*, all of which are required for normal limb development (Bushdid et al., 1998; Kanagae et al., 1998). In addition to limb morphogenesis, Shh/Gli signalling is required for normal patterning of the skeleton, nervous system and lungs (Matise and Joyner, 1999; Wickling et al., 1999; Park et al., 2000), but no relationship between these events and Rel/NF-κB has been found.

Rel/NF-κB genes are involved in embryonic development of the amphibian, *Xenopus laevis*. *XrelA* (Kao and Hopwood, 1991; Kao and Lockwood 1996; Richardson et al., 1995), *Xrel2* (Tannahill and Wardle, 1995), *XrelB* (Suzuki et al., 1995), *Xp100* (Suzuki et al., 1998) and *Xrel3* (Yang et al, 1998) are Rel/NF-κB genes expressed in embryos, some in a developmentally restricted pattern (Bearer, 1994; Suzuki et al., 1998; Yang et al., 1998). *Xrel3* mRNA, for example, accumulates in pre-gastrula embryos and in the late neurula stage embryo in the developing brain, inner ear primordium (otocyst) and notochord (Yang et al., 1998).

Interestingly, overexpression of *Xrel3* by injection of *Xrel3* mRNA into the animal pole of early embryos causes them to develop epidermal tumours that appear after gastrulation (Yang et al., 1998). Similar tumours are induced by overexpression of the *Gli1* oncogene in *Xenopus* (Dahmane et al, 1998) and in humans, abnormalities in Shh/Gli signalling that result in gain-of-function of Gli1, predispose individuals to a variety of cancers such as gliomas, medulloblastomas, meningiomas and fibromas (Hahn et al., 1999). In spite of the requirement for Rel in the activation of Shh/Gli1 during limb morphogenesis, no such link has been made in either Shh/Gli-dependent tumour formation or organogenesis.

In this paper, we show that Xrel3-induced tumours express *Gli1* in addition to the neural and anterior patterning genes *Shh* and *Otx2*. When a dominant negative mutant of Xrel3 is expressed in the dorsal side of the embryo, it reversibly causes defects in dorsal and head development and disrupts expression of dorsoanterior neural markers. Thus, ectopic expression of Xrel3 in ectoderm results in the formation of tumours that express neural-patterning oncogenes while interference of Xrel3 function results in defective anterior development. These findings are consistent with the hypothesis that Xrel3 is required for head development.

MATERIALS AND METHODS

Embryos

We previously reported the cloning and early embryonic expression analysis of Xrel3 (Yang et al., 1998). Albino and wild-type embryos, staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994) were obtained by in vitro fertilisation and microinjected as described (Kao and Lockwood, 1996; Yang et al., 1998) with capped, synthetic Xrel3 RNA using the Ribomax Kit (Promega) or as per Krieg and Melton (Krieg and Melton, 1987). The Xrel3 cDNA template encodes only the translated portion of the *Xrel3* gene to distinguish it from endogenous embryonic *Xrel3* mRNA, which naturally contains both coding and non-coding sequences. As a control, a mutant Xrel3 cDNA was constructed in which the protein kinase A site of the Rel homology domain was altered. This mutation disables the dimerisation potential of the protein and does not affect development (B. B. L., unpublished observations).

Embryos were obtained by in vitro fertilisation. Animal caps were dissected in agar-coated plastic or glass petri dishes using fine forceps in NAM/2 as described (Kao and Lockwood, 1996) and cultured at room temperature or 14°C. Embryos were fixed in Smith's fixative (Kao and Lockwood, 1996), embedded in Historesin (Leica), sectioned at 3 μm and stained with Haematoxylin and Eosin.

Experimental manipulation of zygotes was performed in 4% Ficoll in NAM/2. To ensure the position of the dorsal side, fertilised eggs were dejellied and tilted by 90° in agarose wells so that the sperm entry site faced towards gravity, as described (Kao and Lockwood, 1995). Zygotes were kept in this position until first cleavage, and microinjected on either side of the cleavage furrow directly in the middle of the depigmented 'grey crescent' region. Embryos that did not exhibit this stereotypical dorsal landmark were not used.

RT-PCR

RNA was extracted from embryos and explants and processed for RT-PCR (Kao and Lockwood 1996; Yang et al., 1998) using oligonucleotide primers complementary to *Xenopus* sequences as described previously for Xrel3 and histone (Yang et al., 1998) or from published sequences (Table 1). Primers were purchased from Gibco or Oligos, etc. Histone levels were used to normalise the amount of cDNA used in PCR amplification of the other primers. Our cycling parameters did not allow quantitative comparisons of amplification between different sets of primers but provided comparison of levels between samples for each primer set. Thus, we were able to determine very obvious differences in expression of individual genes between samples. All analyses were repeated three to five times to ensure reproducibility of results.

In situ hybridisation

Embryos were fixed in MEMFA and methanol and processed for

Table 1. Primers used in RT-PCR analysis

Marker	Upstream Primer (5′-3′)	Downstream Primer (5′-3′)	T_M (°C)	Product size (base pairs)	Reference
Cardiac actin	GCTGACAGAATGCAGAAG	TTGCTTGGAGGAGTGTGT	65	666	Mohun et al., 1984
Cephalic hedgehog	TTCTGGCTATCTGCTGCGGG	AACCTTTCTGAGCCCCGGTG	72	198	Ekker et al., 1995
Xenopus Otx2	CGGGATGGATTTGTTGCA	TTGAACCAGACCTGGACT	65	203	Pannese et al., 1995
Epidermal keratin	CTTATCGTACCAGTTACGGATC	CATCTAGCAAAGGGTGGGCTTTGG	72	318	Jamrich et al., 1987
XGli1	CACACTGCGAAATACAGGAGC	TAAGTGGGTGAGCTCTGCGCC	70	195	Lee et al., 1997
XGli3	ATGAACAATGAGCAAGCCCG	ACATTCCAAACATGCGCGGC	69	218	Marine et al., 1997
XAg1	GTATGATGTGGGGCAGTTCC	CGTCTTGCTCTTCCAGGTAG	70	203	Sive et al., 1989
Hoxb9	TACTTACGGGCTTGGCTGGA	AGCGTGTAACCAGTTGGCTG	72	217	Sharpe et al., 1987
Nrp1	GGGTTTCTTGGAACAAGC	ACTGTGCAGGAACACAAG	65	266	Good et al., 1993
Sonic hedgehog	CTTCGCTCGGACGAGATGCTGG	CCTTCGTATCTGCCGCTGGCC	68	211	Ekker et al., 1995
Twist	GTCCAGCTCGCCAGTCTC	CCGGTGCTGCTCGCCTTC	72	170	Hopwood et al., 1989
Endogenous Xrel3	CACCTGCAAATCCTATGG	GCTAGTCATGTGTAACATTTCTC	64	128	Yang et al., 1998

whole mount in situ hybridisation using maleic acid buffer, digoxigenin- and fluorescein-linked probes, stained with NBT/BCIP as described (Harland, 1991) and modified by Sagerström et al. (1996). To eliminate variability in staining between samples, we processed both control and experimental embryos in separate chambers but within the same reaction vials for each probe. Thus all embryos were treated exactly the same for each probe. Embryos were dehydrated in ethanol and cleared in benzyl-benzoate;benzyl alcohol (2:1). Templates for probes complementary to *Xenopus* Shh and Gli1 mRNA were obtained as gifts from Randy Moon and Ariel Ruiz i Altaba, respectively, and for *Xenopus* Otx2 and Nrp1 mRNA from Tamara Holowacz.

In vitro translation

The Xrel3-, Xrel3-RHD- and Xrel3Δ58-coding regions were subcloned into pCS2+ and/or pCS2+mt (gifts from Dave Turner). Wild-type and truncated Xrel3 proteins were synthesised in vitro using the SP6 transcription/translation-coupled rabbit reticulocyte lysate system (Promega). For protein used in immunoprecipitation, 17 μCi of [^{35}S]-methionine (1200Ci/mmol; Mandel) and either 2 μg of pCS2+mtXrel3 alone or 1 μg each of pCS2+mtXrel3 and pCS2+Xrel3 or pCS2+mtXrel3 and pCS2+Xrel3Δ58 to a total of 2 μg were added to the reticulocyte lysate (total volume of 50 μl) and incubated at 30°C for 90 minutes. For EMSAs involving cold κB competitive binding, 2 μg of pCS2+Xrel3RHD and pCS2+Xrel3Δ58 were used for translation as above. However, for EMSAs involving competitive inhibition by Xrel3Δ58 of Xrel3 DNA binding, 1 μg of pCS2+ Xrel3 or pCS2+Xrel3Δ58 was used alone and 1 μg of pCS2+Xrel3 was used in combination with either 0.5 μg, 1 μg or 2 μg of pCS2+Xrel3Δ58. These latter protein products were examined by SDS-PAGE and levels analysed by spot densitometry (ChemiImager^{tm}4000; Alpha Innotech Corporation). Xrel3 protein levels were shown to be consistent between individual and co-translated samples while Xrel3Δ58 levels were approximately 0.6×, 0.9× and 1.3× that of Xrel3 in the co-translated samples.

Electrophoretic mobility shift assays (EMSA)

Double stranded κB DNA elements (5 picomoles; 5′-GCAGGGGAATTCCCCT-3′) were forward labelled using T4 polynucleotide kinase (10 units; GibcoBRL) and 1 μCi/μl [γ^{32}P]-dATP (Mandel) in a total volume of 25 μl for 10-30 minutes at 37°C. Between 150,000 and 500,000 cpm of κB probe was incubated with 1 μl of in vitro translated protein for 20 minutes at room temperature, according to Mavrothalassitis et al. (Mavrothalassitis et al., 1990). Samples were then electrophoresed onto a 5% non-denaturing polyacrylamide mini-protein gel for 50 minutes (100V) at room temperature. Competition of protein binding to labelled κB sequences in testing specificity and affinity involved addition of labelled κB DNA as well as 2, 5, 10, 20, 100 or 1000 ng of unlabelled κB sequence to the protein/DNA-binding mixture and proceeding as described.

Immunoprecipitation

Xrel3 fused with the Myc epitope was either translated alone or co-translated with Xrel3 or Xrel3Δ58. Dimeric protein complexes were covalently linked by incubating 10 μl of the in vitro translated proteins with glutaraldehyde in 8 mM potassium phosphate buffer (pH 8) for 1 hour at room temperature. Crosslinked protein dimers were then incubated overnight at 4°C with 20 μl mouse monoclonal anti-Myc antibody 9E10 cell supernatant, purchased from DSHB (Iowa), in 1 ml 1× Triton Medium (10 mM Tris pH 7.5; 1% Triton X-100; 10 mM EDTA; 0.002% sodium azide) with 20 mM methionine and 5 μl of 0.2 M phenylmethylsulfonylfluoride. Antibody-antigen complexes were immunoprecipitated with Protein A-Sepharose beads (Pharmacia) then washed three times with 1× Triton medium/20 mM methionine then twice with 150 mM NaCl. Immunoprecipitate was boiled for 4 minutes in 0.125 M Tris-HCl, 2% SDS, 5% β-mercaptoethanol and 20% glycerol, and run on SDS-PAGE, along with pre-stained molecular weight standards (12.5 μg/lane; BioRad). All gels were visualised and recorded using a Cyclone (Canberra-Packard) phosphorimager.

RESULTS

Activation of neural patterning genes in Xrel3-induced tumours

Our previous results indicate that 0.5 to 1.0 ng of synthetic *Xrel3* mRNA injected into either the animal or vegetal pole of two-cell stage *Xenopus laevis* embryos causes them to develop tumours without affecting normal axial patterning (Yang et al., 1998). Tumours were not visible on the surface of the embryo until the neurula stage when they appeared as a patch of pigmented cells in the ventral or flanking epidermis (Fig. 1A). The pigmented regions then enlarged into easily removable growths (tumours) that persisted until as late as the early tadpole (Fig. 1B). Initially stage 20 tumours were chosen for analysis. To determine whether injected *Xrel3* mRNA was present in the tumours, RT-PCR was performed on total tumour RNA and from RNA of control-injected epidermis using Xrel3-specific primers. When assayed at stage 20, total Xrel3 RNA was present at much higher levels in tumours than in normal epidermis (Fig. 2A). The endogenous level of Xrel3 was determined using primers complementary to sequences within the 5′ untranslated region of Xrel3 that are not present in injected Xrel3 RNA. The level of endogenous Xrel3 mRNA was not affected by introduction of exogenous Xrel3 mRNA (Fig. 2A). Thus, Xrel3 did not activate itself in these cells.

We next performed RT-PCR on tumour-derived RNA using primers specific to genes expressed in terminally differentiated tissues (Fig. 2B). The expression of these markers was assayed

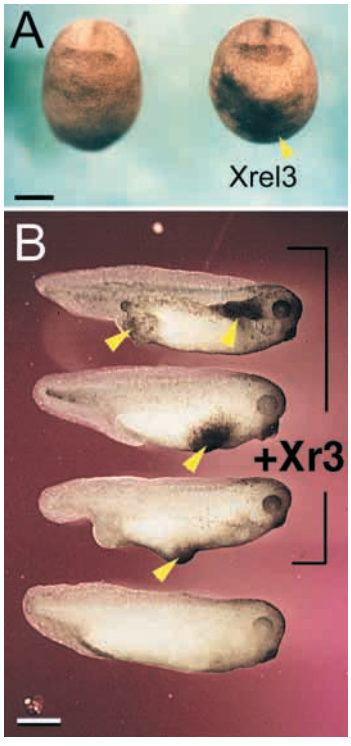

Fig. 1. Xrel3-induced tumours first appear at the late neurula stage. (A) Tumours appear as a pigmented patch (arrowhead) on the ventral epidermis of embryos injected with 0.5 ng of Xrel3 (Xr3) mRNA. Darkening on control embryo at left is due to shadow effect. (B) Xrel3-induced tumours (arrowheads) persist until at least the early tadpole stage. Scale bars: 0.5 mm.

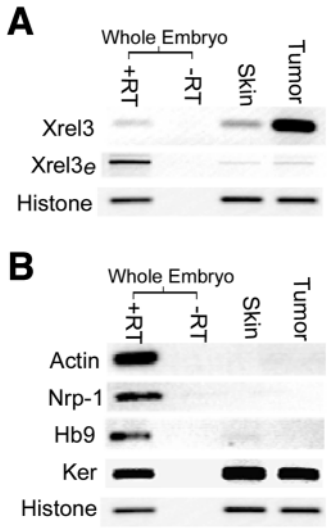

Fig. 2. RT-PCR analysis of Xrel3-induced tumours dissected from stage 20 embryos. (A) Endogenous Xrel3 is not activated by introduction of exogenous Xrel3 mRNA. Total Xrel3 RNA (Xrel3) levels remain higher in tumours than in skin but endogenous levels are not altered. (B) Xrel3-induced tumours express epidermal (epidermal keratin, Ker) but not mesodermal (cardiac actin) or neural (Nrp1 and Hoxb9 (Hb9)) differentiation markers. Histone levels in all RT-PCR assays were used to standardise loading amounts.

in order to determine the embryonic germ layer origin of the tumours. They included muscle mesoderm (cardiac actin), neural tissue (Nrp1 and Hoxb9) and epidermis (epidermal keratin). Analysis of Hoxb9, cardiac actin and Nrp1 mRNA indicated that these markers were not expressed or were expressed at very low basal levels in skin and tumour samples. Epidermal keratin, however, was expressed at very high levels in both skin and tumour samples. These observations indicate that the tumours were not induced to commit along a mesodermal or neural tissue lineage.

Both *Gli1* and *Shh* have been associated with cellular proliferation and certain types of cancer, particularly the skin malignancy nevoid basal cell carcinoma (Matise and Joyner, 1999). In normal *Xenopus* development, mRNAs encoded by these genes accumulate in the notochord, ventral floor plate of the neural tube and in the developing brain (Ekker et al, 1995; Lee et al, 1997; Marine et al., 1997). *Xrel3* mRNA accumulates in the notochord and brain and in otocysts of neurulae and tailbud embryos (Yang et al., 1998). Rel is also required to activate Shh and Gli1 signalling in chick limb development (Kanagae et al., 1998; Bushdid et al., 1998) and overexpression of *Gli1* in *Xenopus* embryos gave rise to epidermal growths resembling *Xrel3*-induced tumours (Dahmane et al., 1997). These lines of evidence suggested that Xrel3 might also activate *Shh* and *Gli1* in tumours. Indeed both *Gli1* and *Shh* mRNA levels were upregulated in tumours removed from embryos at Stage 20 (Fig. 3A).

We determined if other markers, present in tissues that normally express *Xrel3*, were also expressed in Xrel3-induced tumours. *twist* is a mesodermal marker that is normally expressed in neural crest and in the non-muscle mesoderm (including the notochord) (Hopwood et al., 1989), while *Otx2* is an anterior marker that is normally expressed in the otocysts and head region of *Xenopus* embryos (Pannese et al., 1995; Kablar et al., 1996). Expression of both of these genes was upregulated in stage 20 tumours (Fig. 3A). Other members of the Hedgehog and Gli gene families, including *cephalic hedgehog* (Ekker et al., 1995), *Gli3* (Marine et al., 1997) (Fig. 3A) and *banded hedgehog* (Ekker et al., 1995) (not shown), were not expressed or were expressed at very low levels in both skin and tumour samples.

At Stage 16/17, tumours are visible as pigmented patches on the embryo surface. Both *Otx2* and *Shh* mRNA levels in tumours were higher at this stage, while both *twist* and *Gli1* mRNA levels were low and relatively at the same level with that found in normal embryonic epidermis (Fig. 3B). At Stage 26/27, *Gli1*, *Otx2* and *twist* levels all were higher in tumours than in the skin (Fig. 3C). This observation indicates that the expression of *Shh* and *Otx2* is associated with tumours that are initiated early in development by Xrel3 overexpression, but that expression of *Gli1* and *twist* occurs later.

The activation of the above patterning markers was examined in isolated animal cells. Embryos were injected as usual at the two-cell stage. At Stage 8, the animal regions (caps) were dissected away from the rest of the embryos. At this stage there are low levels of Shh, Gli1 and Otx2 RNAs, which do not differ between injected and control cells (Fig. 4A). Twist was not expressed at this stage in either embryos or animal caps. These results indicate that Xrel3 (either endogenous or exogenous) is insufficient at the blastula stage to activate expression of *Shh*, *Gli1*, *Otx2* or *twist* above background levels.

The dissected caps were allowed to heal and assayed at a time when tumours are normally visible on the embryo (Stage 20). There was no difference in levels of endogenous *Xrel3* mRNA between the control and Xrel3-injected cells (Fig. 4B), but there was still a significant amount of total (injected plus endogenous) *Xrel3* mRNA in the Xrel3-injected caps. Thus, as in dissected tumours, the effects we observed in isolated animal cells at stage 20 were due to introduction of exogenous Xrel3

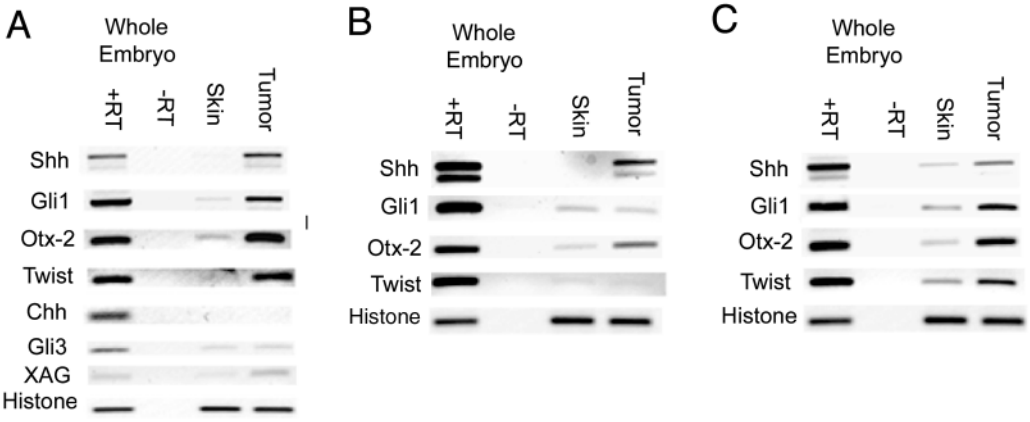

Fig. 3. Increased expression of *Shh*, *Gli1*, *Otx2* and *twist* mRNA in Xrel3-induced tumours as compared with normal epidermis. (A) Tumour RNA from stage 20 embryos analysed by RT-PCR using primers specific for *Xenopus Shh*, *Gli1*, *Otx2* , *twist*, cephalic hedgehog (*Chh*), *Gli3* and *Xag1* (*XAG*). (B) Stage 16 tumours have higher levels of *Shh* RNA only. (C) Analysis of RNA from stage 26 tumours shows continued higher expression of *Gli1*, *Otx2* and *twist* than in epidermis.

RNA. *Shh*, *Gli1* and *Otx2* expression levels at stage 20 were higher in animal caps isolated from Xrel3-injected embryos than in control caps (Fig. 4B).

twist RNA expression did not change as a result of Xrel3 overexpression, suggesting that the dissected tumours (as in Fig. 3) probably contained contaminating *twist*-expressing cells (Fig. 4B). In addition, we did not detect *cardiac actin* expression in the Xrel3-injected caps (Fig. 4B), indicating that mesoderm induction did not occur. To confirm the expression of Shh, Gli1 and Otx2 in tumours, we performed whole-mount in situ hybridisation on embryos that had been injected with *Xrel3* mRNA at the two-cell stage. These analyses directly illustrated that the tumours accumulated mRNA corresponding to these markers (Fig. 4C).

The above results indicated that inductive interactions from the underlying tissue were not required for *Shh*, *Gli1* and *Otx* gene activation by Xrel3. They show that activation of these genes by Xrel3 was cell autonomous but required an additional event that occurred after gastrulation.

Disruption of normal anterior development by a C-terminal deletion mutant of Xrel3

The Rel/NF-κB proteins consist of a highly conserved N-terminal domain, called the Rel homology domain (RHD), that is required for DNA binding, dimerisation and nuclear localisation (Chen and Ghosh, 1999). The C-terminal region is variable in length and structure, but in some members, particularly RelA and cRel, the C-terminal domain contains sequences required for transactivation (Schmitz et al., 1985; Martin and Fresno, 2000). In studies designed to determine the domains of the Xrel3 protein that are required for tumour formation (B. B. L., unpublished observations), we developed a mutant clone in which the 58 C-terminal residues were deleted. This region comprises the putative transactivation domain of Xrel3 and is required for tumour formation (data not shown).

The mutant protein, Xrel3Δ58, interfered with the normal function of synthetic Xrel3. To explain the biochemical mechanism of action of Xrel3Δ58, we determined its DNA binding and dimerisation properties by electrophoretic mobility shift assays using DNA probes with known Rel/NF-κB binding consensus sequences and by immunoprecipitation. The in vitro-translated RHD of Xrel3 (Fig. 5A) and wild-type Xrel3 (Fig. 5B) bound the κB perfect palindromic consensus sequence with high affinity. Xrel3Δ58 protein, however, bound the κB sequence very weakly or not at all (Fig. 5). Full-length Xrel3 protein, when co-translated with Xrel3Δ58 RNA, progressively lost its ability to bind DNA with increasing proportion of Xrel3Δ58 (Fig. 5B). This result indicates that Xrel3Δ58 competitively inhibits the ability of Xrel3 to bind DNA.

The reduced ability for Xrel3 to bind DNA when co-translated with Xrel3Δ58 might be due to its decreased in vitro translation efficiency or through interference by Xrel3Δ58. Labelling of co-translation products with [^{35}S]methionine indicated that Xrel3 and Xrel3Δ58 mRNAs were translated to roughly equivalent levels, ruling out the possibility of reduced efficiency (Fig. 6B). We also predetermined the relative proportions of Xrel3 and Xrel3Δ58 before subjecting them to EMSA analysis. Thus, the competition experiments shown in Fig. 5B are a true indication of the ability for Xrel3Δ58 to interfere with the function of wild-type Xrel3 protein. Finally, western blot analysis of protein extracts from embryos injected with Xrel3Δ58 and Xrel3 RNA showed that their translation products were proportionately stable at least until the late neurula stage, indicating efficient in vivo translation of the mRNA (B. B. L., R. F. and K. R. K., unpublished observations).

The hallmark of dominant negative interference by mutant proteins like Xrel3Δ58 is their ability to inhibit their wild-type counterparts when they oligomerise with them (e.g., Amaya et al., 1991). Xrel3Δ58 on its own does not bind the consensus κB DNA sequence, yet it also inhibits the DNA-binding ability of wild-type Xrel3, probably by dimerisation. To confirm the dimerisation potential of Xrel3, Xrel3 and Xrel3Δ58 cDNAs were subcloned into vectors encoding a peptide tag containing six copies of a human Myc epitope. Translation products made from these plasmids were fusion proteins that contained the Myc tag at the N terminus (Fig. 6A). Myc-Xrel3 RNA was also able to cause tumours when injected into embryos indicating that the epitope did not interfere with the activity of Xrel3. Using an anti-Myc monoclonal antibody, Xrel3Δ58 consistently co-precipitated with Myc-tagged Xrel3, and heterodimeric complexes containing both species could be covalently linked by glutaraldehyde fixation (Fig. 6B). These results indicate that Xrel3Δ58 dimerised with wild-type Xrel3, but in so doing, also reduced its ability to bind DNA. They also indicate that Xrel3Δ58 can act as a dominant negative interference mutant.

When Xrel3Δ58 RNA was injected into the animal poles of two-celled embryos, it was unable to cause tumours, but the embryos developed anterior patterning defects when examined later in development. Injection of Xrel3Δ58 mRNA resulted in

Table 2. Distribution of phenotypes caused by Xrel3Δ58 overexpression

Picograms of injected mRNA			Numbers (percentages) of embryos				
Xrel3Δ58	Xrel3	Number of experiments	I (normal)	II (Cyclopia)	III and IV	V (acephaly)	Total
0	0	10	426 (94)	20 (4)	2 (1)	1 (1)	452
250	0	4	69 (67)	28 (27)	3 (3)	3 (3)	103
500	0	6	77 (44)	70 (40)	16 (10)	11 (6)	174
1000	0	4	29 (25)	40 (35)	23 (20)	23 (20)	115
500	50	3	61 (55)	41 (37)	6 (5)	3 (3)	111
500	150	3	60 (61)	35 (35)	3 (3)	1 (1)	99
500	250	3	68 (69)	30 (30)	0 (0)	1 (1)	99
500 (dorsal)	0	4	4 (6)	21 (36)	21 (36)	12 (21)	58
500 (ventral)	0	4	31 (58)	13 (25)	7 (13)	2 (4)	53
500 (dorsal)	250 (dorsal)	3	28 (65)	9 (21)	4 (9)	2 (5)	43

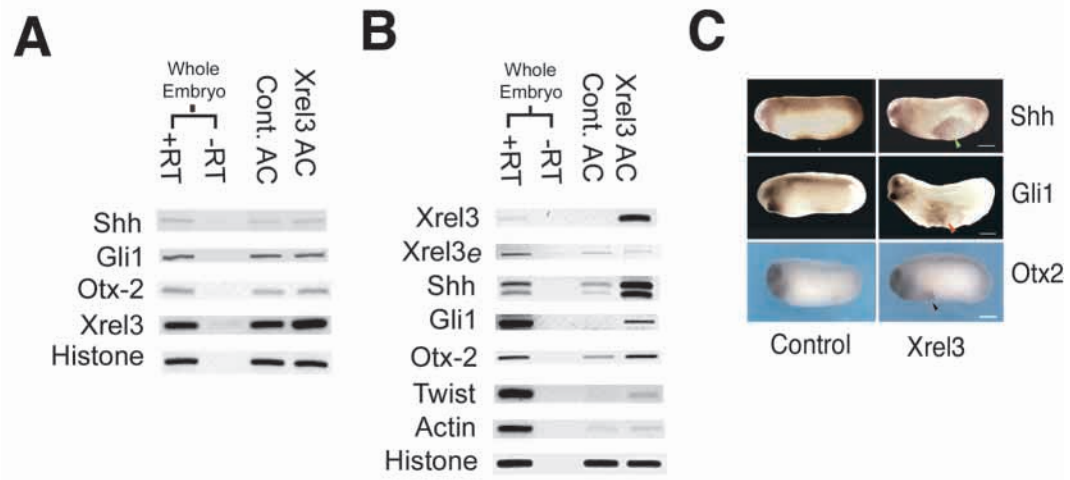

Fig. 4. Expression of *Shh*, *Gli1* and *Otx2* in dissected animal caps is cell-autonomous. (A) Animal caps, dissected from embryos injected with 0.5 ng *Xrel3* mRNA at stage 8 and assayed at stage 10 by RT-PCR show no differences in levels of *Shh*, *Gli1* and *Otx2*. (B) *Xrel3*-overexpressing animal caps at the equivalent stage 20 have higher levels of expression of *Shh*, *Gli1* and *Otx2*. (C) Analysis of expression of *Shh* (green arrowhead), *Gli1* (red arrowhead) and *Otx2* (black arrowhead) in tumours by whole mount in situ hybridisation. Albino embryos were injected with 0.5 ng Xrel3 and processed at the tailbud stage. Scale bars: 0.5 mm.

a graded increase in the proportion of anterior-defective embryos in a dose-dependent manner (Fig.7A). The defective embryos displayed a variety of phenotypes, including reduced eyes (grade II), anopthalmy or synopthlamy and microcephaly (no eyes or fused eyes and small head, grades III and IV) to acephalic embryos (no heads, grade V) (Table 2). Although these phenotypes superficially resembled those of the dorsoanterior index (DAI; Kao and Elinson, 1988), they differed substantially because they did not have apparent defects in dorsoanterior mesoderm (see below). We could not, therefore, apply the DAI scale to these embryos. Co-injection of 250 pg of wild-type Xrel3 with 500 pg of Xrel3Δ58 mRNA resulted in rescue of normal development, indicating that the effects were due specifically to Xrel3Δ58 mRNA (Fig. 7B).

We were concerned that the penetrance of the effect of animal pole injection of Xrel3Δ58 mRNA on anterior patterning was relatively low. At 0.5 ng of Xrel3Δ58 RNA, only 57% of the embryos developed head defects, while co-injection of wild type Xrel3 up to 0.25 ng resulted in rescue of normal embryos from 43% to 73% (Fig. 7B). The low percentage of affected embryos might be due to a variety of possibilities, but a likely scenario is that injection into the animal pole does not sufficiently target the mRNA to the appropriate region of the embryo. We therefore performed experiments to more precisely localise the injected RNA to the future head-forming region.

Dorsoanterior structures, including the head, arise from the side opposite the sperm entry site in fertilised embryos. To target injection of the mRNA to the future dorsal side, we tilted zygotes by 90° within 40 minutes of fertilisation to ensure the

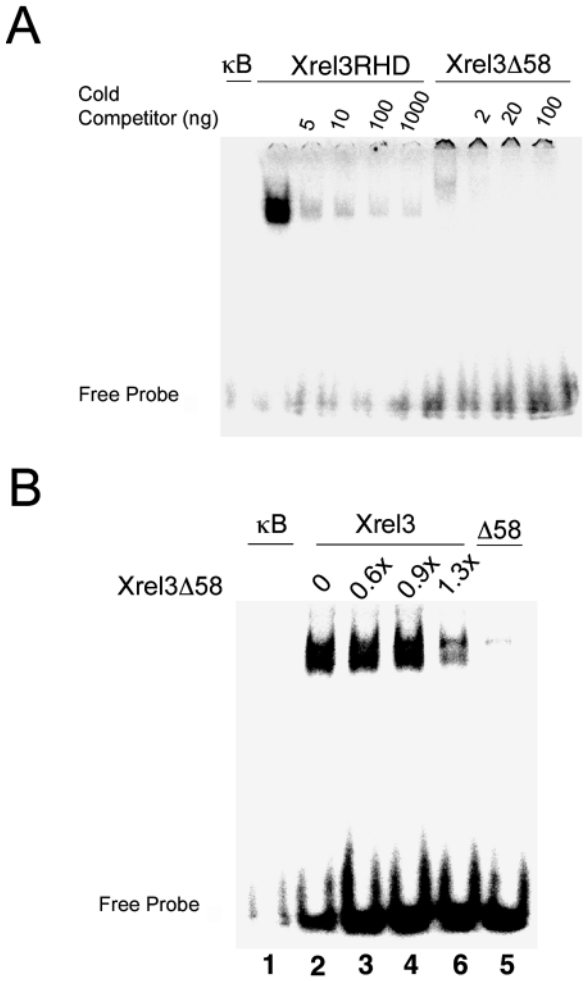

Fig. 5. Xrel3Δ58 competitively inhibits binding by Xrel3 to the κB enhancer sequence. (A) Xrel3Δ58 binds the perfect κB element very weakly in an EMSA reaction, when compared with the RHD of Xrel3 alone. Binding specificity is shown using increasing quantities of unlabeled κB sequence (cold competitor). Much less competitor is required to eliminate Xrel3Δ58 binding (2 ng) than Xrel3RHD binding (1000 ng). (B) Equal amounts of Xrel3 (lanes 2-4) were co-translated with increasing proportions of Xrel3Δ58 (0-1.3x) and used in an EMSA with the κB perfect palindrome as a probe. Free probe (lane 1) is not retarded in the gel, and Xrel3Δ58 alone (lane 6) binds the palindrome weakly.

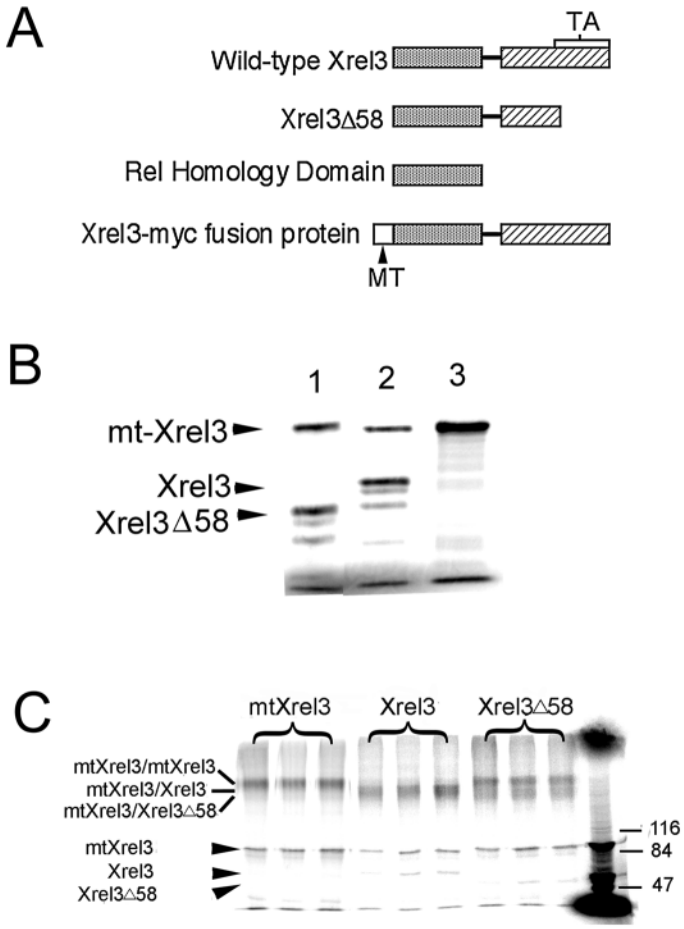

Fig. 6. Xrel3Δ58 competitive inhibition occurs through dimerisation with full-length Xrel3. (A) Maps of constructs. Wild-type Xrel3 consists of a Rel-homology domain (stippled box) and a C-terminal domain (hatched box) with transactivation motifs (TA) at the extreme C terminus. The 58 C-terminal residues are deleted in Xrel3Δ58, which includes the putative TA-region. Xrel3 was fused with a human Myc-epitope by subcloning the Xrel3-coding region into pCS2+mt, which encodes six copies of the epitope and is recognised by the 9E10 antibody. (B) Xrel3-Myc fusion protein (mt-Xrel3) was co-translated with Xrel3 (lane 2) or Xrel3Δ58 (lane 1) and resolved by SDS-PAGE. Arrowheads indicate full-length translation products. (C) mt-Xrel3, alone or covalently, crosslinked to co-translated Xrel3 or Xrel3Δ58 using 0.005%, 0.01% and 0.015% glutaraldehyde, was immunoprecipitated and resolved on SDS-PAGE. Sample in lane on extreme right is Myc-tagged Xrel3 co-translated *in vitro* with Xrel3Δ58. The top bands represent covalently linked Myc-tagged Xrel3 (mtXrel3/mtXrel3), Myc-tagged Xrel3 dimerised with wild-type Xrel3 (mtXrel3/Xrel3) or Myc-tagged Xrel3 dimerised with Xrel3Δ58 (mtXrel3/Xrel3Δ58). The lower bands represent unlinked monomeric subunits (mtXrel3, Xrel3 or Xrel3Δ58) that co-immunoprecipitated with mtXrel3.

position of the dorsal axis opposite the sperm entry site. Injection of 500pg Xrel3Δ58 mRNA in the dorsal side caused 94% of the embryos to develop anterior defects (Fig. 7C). Alternatively, injection of the same quantity of mRNA in the ventral side caused only 40% of the embryos to develop head defects (Fig. 7C) and most of these were scored as grade II embryos (Table 2). This number of defective embryos could have been due to improper placement of the injection needle exactly opposite the dorsal side, or to diffusion of mRNA to the target on the dorsal side. Co-injection of 500pg Xrel3Δ58 mRNA with 250pg wild-type Xrel3 mRNA into the dorsal side of tilted embryos reduced the number of defective embryos to 35% (Fig. 7C), with mostly minor (grade II) head defects (Table 2). These experiments indicate that injection of dominant negative *Xrel3* mRNA on the dorsal side specifically inhibits anterior development.

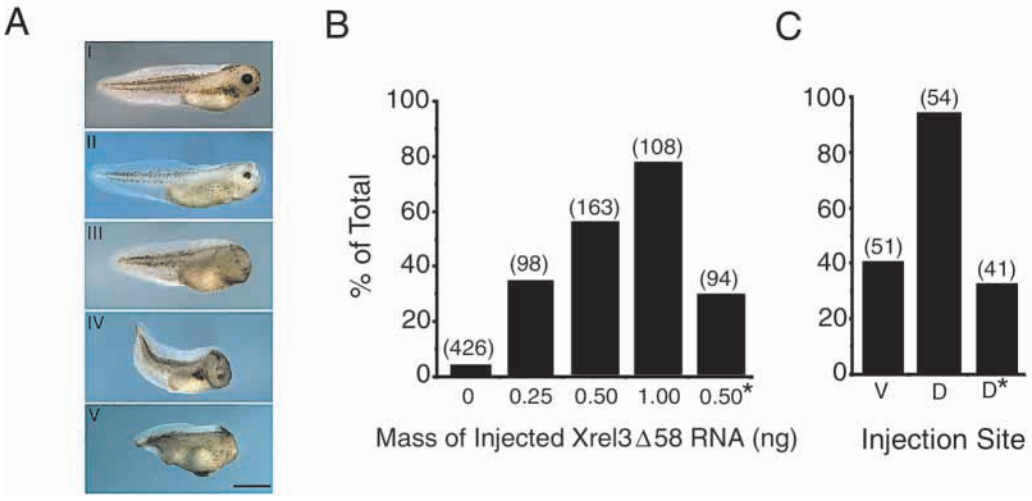

Fig. 7. Overexpression of a dominant negative Xrel3 mutant causes anterior-defective embryos. (A) Embryos injected at the two-cell stage with varying concentrations (0.25 to 1.0 ng) of Xrel3Δ58 mRNA showed varying degrees of phenotypic alterations when compared with normal embryos (top, I) when assessed at the tadpole stage. Defects ranged from mildly reduced eyes, forebrain and cement gland (II) to more significant reduction or loss of these structures (III), microencephaly, cyclopia (IV) and anencephaly (V). (B) The percentages of anterior defective embryos combined from several experiments is shown in the histogram. Dead embryos and embryos that failed to gastrulate were not included in these analyses. The bar showing reduction of defects in Xrel3Δ58 mRNA-injected embryos by co-injection of 0.25 ng wild-type Xrel3 mRNA is indicated by the asterisk. Numbers at tops of bars indicate total number of embryos scored. (C) Microinjection of 0.5 ng Xrel3Δ58 in the dorsal side of the embryo causes head defects. D, dorsal injection; D*, dorsal co-injection of 0.25 ng Xrel3 and 0.5 ng Xrel3Δ58; V, ventral injection. Scale bar: 0.5 mm.

The injection of Xrel3Δ58 mRNA caused a graded loss of anterior neural development (Fig. 8I-K). The embryos had normal looking notochords and spinal cords from the head (Fig. 8E) to the trunk region (Fig. 8F) but rhombencephalic (hindbrain) development was over represented. The ventral hindbrain structure was greatly exaggerated with an enlarged brain vesicle accounting for the large gap that was visible on the dorsal anterior aspect of the head (Fig. 8E,H). Depending on the severity of the phenotype, the mesencephala (midbrains) of these

embryos were reduced in size and lacked the typical diencephalic, eye and prosencephalic (forebrain) structure of control embryos (Fig. 8D). All embryos partially or completely lacked cement gland cells normally located ventrally. The apparent over-representation of hindbrain and lack of more anterior elements suggested that the former was expanded at the expense of the latter. These observations indicated that although neural tissue was present at the anterior end of the embryo, it was not patterned into normal head elements caudal to the hindbrain.

We performed in situ hybridisation on Xrel3Δ58-injected embryos at the neurula stage that had clear anterior defects using probes against Otx2, Shh, Gli1 and Nrp1. Injection of 0.5 ng of Xrel3Δ58 mRNA caused disruption of normal Otx2 and Nrp1 patterning at the anterior end of the embryo (Fig. 9C,D). At the early neurula stage, *Shh* mRNA was normally found in the anterior neural plate, and in the dorsal midline of the neural plate that develops into the ventral floor plate (Fig. 9E). In Xrel3Δ58-injected embryos, this pattern was lost or diminished greatly (Fig. 9F). Gli1 transcripts appeared to normally accumulate in the developing nervous system (Lee et al., 1997), with higher levels in the prospective brain region (Fig. 9G), while in Xrel3Δ58-injected embryos, there was a significant reduction in this pattern of accumulation (Fig. 9H).

These effects on anterior and neural patterning are not likely to be due to early effects of Xrel3Δ58 on dorsoventral specification before gastrulation, since injected embryos undergo gastrulation with complete blastopore closure. In addition, expression of mRNA encoding chordin, a secreted patterning morphogen required for the specification of dorsal and neural fates (Larrain et al., 2000) in the dorsal lip region of the gastrula (Fig. 9I,J), and that of the pan-mesodermal marker Xbra (Smith et al., 1991), were unaltered by overexpression of Xrel3Δ58 (Fig. 9K,L). These results suggest that Xrel3Δ58 specifically inhibits the expression of neural patterning genes, and imply that Xrel3 is required for anterior neural patterning.

DISCUSSION

The formation of tumours in embryos indicates that Xrel3 cannot itself direct the development of anterior embryonic structures, even though the tumours expressed neural and anterior patterning genes. One pathway by which this proliferative response by Xrel3 overexpression might occur is through Gli1 signalling. Several lines of evidence support this hypothesis. First, overexpression of Gli1 causes epidermal embryonic tumours in *Xenopus*, and Gli1 is overexpressed in human basal cell carcinoma (Dahmane et al, 1998; Matise and Joyner, 1999). Second, Rel is required in the proliferation zone of the developing chick limb bud for the expression of *Shh* and *Gli1* (Kanagae et al., 1998; Bushdid et al., 1998). Third, our results show that Xrel3-induced tumours appear coincidentally and express *Shh* and *Gli1* mRNA at the early neurula stage. Finally, although ectopic overexpression in mammals of *Shh* and *Gli* genes is oncogenic, Shh/Gli signalling has a well-conserved role in normally promoting epithelial stem cell proliferation, including cells of the developing nervous system (Hynes et al, 1997; Parisi and Lin, 1998; Fan and Khavari, 1999; Matise and Joyner, 1999; Weschler-Reya and Scott, 1999, Britto et al., 2000). We propose that Xrel3 in *Xenopus* is normally required for the activation of *Shh*/*Gli1*signalling, which leads to cell proliferation.

Our data also indicate that Xrel3 is required for the expression of *Xenopus Otx2*. The Otx family of proteins represent a class of homeodomain-containing transcriptional regulators related to the *Drosophila* anterior pole-patterning gene *orthodenticle* (*otd*), which are required for anterior development (Hirth and Reichert, 1999; Acampora et al., 2000). In *Xenopus* embryos, Otx2 is expressed in the cement gland, the anterior neural region and in the otocysts (Pannese et al., 1995), and ectopic expression of Otx2 results in cement gland formation (Gammill and Sive, 1997). In mice, targeted disruption of *Otx2* causes embryos to die during embryogenesis (Ang et al., 1996). The aborted embryos lack heads and have body axis defects that are analogous to Xrel3Δ58-injected embryos. In addition to head patterning, *Otx2* is required for the formation of the otocyst, or inner ear structures in mouse embryos (Morsli et al., 1999). Whole-mount in situ hybridisation in *Xenopus* indicates strong expression of Xrel3 in otocysts which are abnormal in Xrel3Δ58-injected embryos (Yang et al., 1998) (Fig. 8).

Neural development in frogs results from an inhibition of bone morphogenetic protein (BMP)-signalling in the dorsal side of the embryo by factors such as noggin and chordin (Hemmati-Brivanlou and Melton, 1997; Larrain et al., 2000). The cement gland develops at the most anterior extent of the head, resulting from an interaction between Bmp4 and Otx2 (Gammill and Sive, 2000). Higher levels of Bmp4 prevent cement gland and neural tissue differentiation and lower levels permit brain development (Gammill and Sive, 2000). Ironically, Xrel3-induced tumours did not express cement gland markers even though they inappropriately expressed Otx2. It is possible that the tumours expressed high enough levels of Bmp4 to inhibit cement gland formation, initiated by ectopic Otx2. We are presently determining the more precise role of Xrel3 and other Rel/NF-κB proteins in neural differentiation, by activating its ectopic expression after gastrulation.

At the cellular level, Xrel3 may be required to support cell proliferation to ensure that adequate numbers of cells are present in the structures in which it is expressed, accounting for the formation of tumours in either ectoderm- or endoderm-derived cells (Yang et al., 1998). Another possible function related to proliferation might be that Xrel3, like other Rel/NF-κB proteins (de Martin et al., 1999; Barkett and Gilmore, 1999) prevents programmed cell death in the developing embryo. In support of this latter idea, previous studies have shown that large numbers of apoptotic cells were detected in the developing *Xenopus* neurula (Hensey and Gautier, 1998).

Xenopus embryos have been used as an in vivo model system to study oncogenic activity (Wallingford, 1999). So far, in addition to Xrel3, overexpression of Gli1 (Dahmane et al., 1998) and a dominant negative form of the p53 tumour suppressor and its wild-type antagonist Mdm2 all cause similar epidermal tumours when injected into the early embryo (Wallingford et al., 1997). One possibility is that each of these proteins initiates the same responses leading to tumour formation, or it is possible that they represent steps along the same pathway(s) that lead to loss of cell-cycle control in early development.

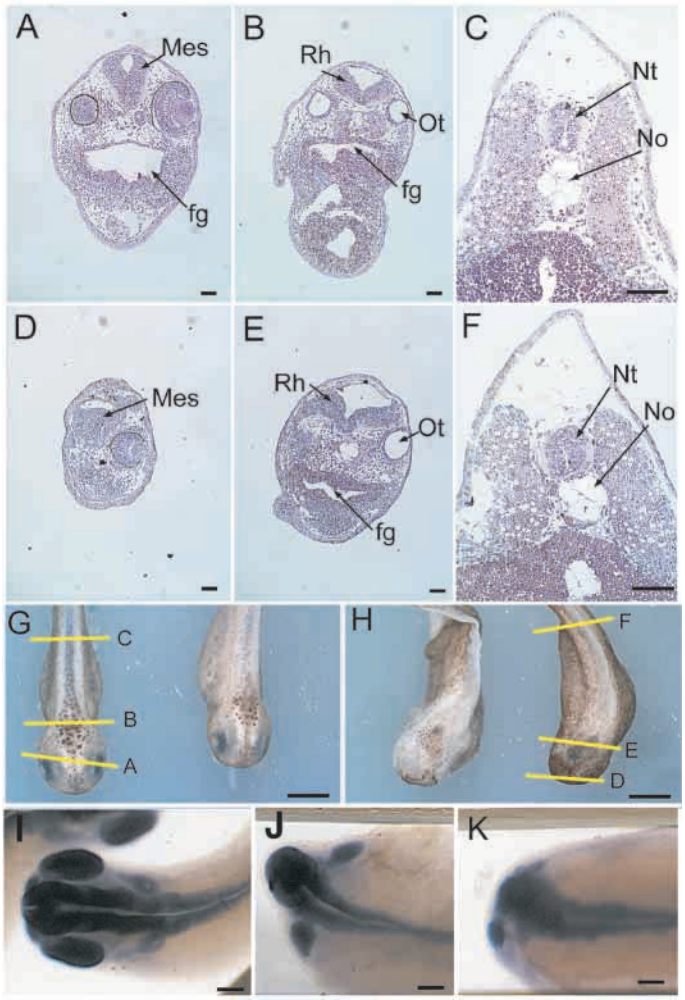

Fig. 8. Histological analysis of head-defective embryos. Control (A,B,C) and Xrel3Δ58 mRNA injected embryos (D,E,F) with clear head-defective phenotypes were serially sectioned and examined at comparable levels, as determined by anatomical landmarks. Embryo sections are through the mesencephalon (mes) or midbrain (A,D), rhombencephalon (Rh) or hindbrain (B,E), and trunk (C,F). fg, foregut; No, notochord; Nt, neural tube; Ot, Otocyst. Dorsal views of control (G) and Xrel3Δ58 (H) embryos are shown before sectioning with level of section indicated by yellow bars. Letters next to yellow bars correspond to histological sections in A to F. (I-K) Early tadpole embryos processed for whole-mount RNA in situ hybridisation using Nrp1 as a neural marker. An unaffected embryo (I) has a full complement of brain, eyes, otocysts and spinal cord, while there is progressive loss of anterior structure and organisation in type II (J) and V (K) embryo. Scale bars: 0.1 mm for A-F; 0.5 mm for G,H; 0.25 mm for I-K.

Our findings should be useful in determining the mechanism of oncogenesis by Rel/NF-κB proteins. The prototype Rel gene was the transforming viral oncogene v*Rel*, which causes leukaemia in birds (Gilmore, 1999), and Rel/NF-κB overexpression has been implicated in numerous lymphomas, leukaemias and solid tumours in humans (Rayet and Gelinas, 1999). That Rel/NF-κB activates Gli1 expression in these tumours and in Gli1 overexpressing cancers is an intriguing notion.

An alternative means to inhibit Xrel3 function besides dominant negative interference by Xrel3Δ58 would be to overexpress in embryos, the natural Rel/NF-κB inhibitor, IκB (e.g. Tannahill and Wardle, 1995). Our unpublished observations suggest that, unlike most Rel proteins, Xrel3 is not sequestered in the cytoplasm. Transient transfections of mammalian fibroblasts with a green fluorescent protein – Xrel3 fusion protein always resulted in fluorescent protein in nuclei and injection of GFP-Xrel3 plasmid DNA into embryos consistently indicated protein localisation to the nucleus. These observations indicate to us that Xrel3 is constitutively active in embryos and differentiated cells and, unusually, not under the control of a cytoplasmic inhibitor.

Our experiments provide some insight into the functional aspects of domains of the Rel protein itself. Crystallographic and biochemical studies place the DNA-binding and dimerisation domain in the (approx. 220 residue) N-terminal Rel homology domain (Chen and Ghosh, 1999), while the C-terminal region is required for the transactivating activity of the

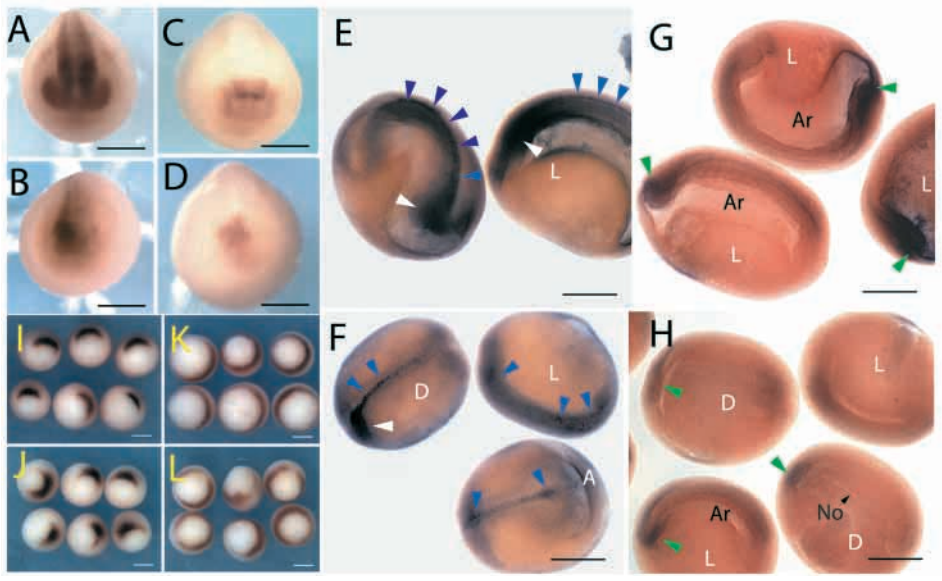

Fig. 9. Anterior-defective embryos have disrupted expression patterns of neural patterning markers in neurulae but normal expression of mesodermal markers at gastrulation. Control (A,C,E,G,I,K) embryos are compared against Xrel3Δ58 (B,D,F,H,J,L) injected embryos. Stage 15 embryos were stained for nrp-1 (A,B), Otx2 (C,D), Shh (E,F) and Gli1 (G,H). Stage 10-10.5 embryos were stained for chordin (I,J) and Xbra (K,L) and viewed from the vegetal pole. Blue arrowheads in E and F indicate specific expression (dark blue-black stain) of Shh in floor-plate and white arrows indicate expression of Shh in prospective brain. Similarly, green arrowheads in G and H indicate expression in prospective brain of Gli1. White letters indicate perspective views of embryos: A; anterior; D, dorsal; L, lateral. Embryos in A-D are shown at anterior end. Ar, archenteron; No, notochord. Scale bars=0.5 mm.

protein (Schmitz et al., 1995; Martin and Fresno, 2000). Interestingly, although our C-terminally deleted form of Xrel3 could not bind DNA, and interfered with the binding of wild-type Xrel3 to a κB consensus, it was still able to heterodimerise with the wild-type protein. This finding suggests that, like a closely related Rel family member, RelA (Zhong et al., 1998), the C-terminal domain may interact with the N terminus.

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