Int. J. Dev. Biol. 42: 67-77 (1998)

Original Article

Involvement of NF-κB associated proteins in FGF-mediated mesoderm induction

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ABSTRACT In this report, we have used mRNA injection to study the action of mutants of XrelA, a *Xenopus* homolog of the RelA (p65) component of NF-κB, on the induction of mesoderm in *Xenopus* embryos. A region of the rel homology domain of XrelA was deleted to create XrelAΔSP, which retains the dimerization and activation domains, but no longer binds to DNA. We also made an analogous derivative of mammalian NF-κB1 (p50). We show that both constructs have dominant inhibitory activity. When message encoding either is injected into eggs or oocytes, DNA binding of rel family members is suppressed, as is transactivation of a κB-dependent promoter in embryos. Expression of XrelAΔSP in animal caps blocks the induction of mesoderm by bFGF. In addition, this mutant prevents elongation movements generated by activin, but has little effect on posterior dorsal cytodifferentiation, which in marked contrast is blocked by inhibition of the FGF signal transduction pathway between the receptor and MAP kinase. The specificity of the XrelAΔSP effect on FGF signaling is shown by rescue of mesodermal marker expression when XrelAΔSP is co-expressed with a specific rel inhibitor. The target of these dominant negative constructs seems to be neither XrelA itself, nor p50, but rather some other molecule with which XrelA, rather than NF-κB1, heterodimerizes. We show that XrelAΔSP blocks FGF induction of mesoderm downstream of MAP kinase and *Xbra* expression. Thus it prevents the maintenance of *Xbra* expression by inhibiting its autoregulation by embryonic FGF (eFGF). We suggest that XrelAΔSP differs from other reported inhibitors of FGF signaling because it inhibits only gastrula stage FGF signaling and not the maternally programmed signaling at the blastula stage. Our results therefore suggest that zygotic FGF action is required for cell movements rather than dorsal differentiation.

KEY WORDS: *mesoderm, Xenopus, NF-κB, FGF, gastrulation, Brachyury*

Introduction

Although members of the rel family of transcription factors are perhaps more commonly associated with immune responses and apoptosis (for review see Baldwin, 1996), the potential of rel family members to act as developmental patterning agents is well illustrated by the pivotal role of *dorsal* in the formation of the dorsal-ventral axis of *Drosophila* (reviewed in Belvin and Anderson, 1996). Two members of the rel family of transcription factors, *XrelA*, related to mammalian *RelA*, and *Xrel2*, a novel member of the family, are known to be expressed during the early stages of *Xenopus* development (Kao and Hopwood, 1991; Richardson *et al*, 1994; Tannahill and Wardle, 1995). Maternally expressed XrelA protein is differentially localized to nuclei of the animal hemisphere and marginal zone from the mid to late blastula stages (Bearer, 1994). XrelA overexpression experiments have suggested the involvement of this factor both in patterning of the head and tail of the embryo (Richardson *et al.*, 1995) and also in dorsal-ventral development (Kao and Lockwood, 1996).

Much use has been made of dominant inhibitory mutant receptors in the assignment of developmental roles to a family of related genes (i.e., Amaya *et al.*, 1991, 1993; Hemmati-Brivanlou and Melton, 1992; Graff *et al.*, 1994). Like these receptors, the rel transcription factors also function as dimers, enabling a similar approach to be used to study their role in development. We have previously reported the developmental effects of expression of a dominant negative XrelA derivative with a deletion of the activation domain; this probably acts by titrating out κB sites (Richardson

Abbreviations used in this paper: BMP, bone morphogenetic protein; FGF, fibroblast growth factor; HIV-LTR, human immunodeficiency virus long terminal repeat; MAP kinase, mitogen activated protein kinase; NLS, nuclear localization sequence; RHD, rel homology domain; TGF, transforming growth factor.

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0214-6282/98/$10.00

Printed in Spain

et al., 1994, 1995). The results were consistent with a role for factors binding to κB sites in the posterior of the embryo, and to a lesser effect in the head. As factors unrelated to rel have been identified which bind to kB sites (Faisst and Meyer, 1992; Staehling-Hampton *et al.*, 1995), more specific dominant negatives, which act directly on endogenous XrelA protein, are needed to define its role in development. We therefore analyzed the phenotypic effects of rel proteins deficient in DNA binding. We report that although several such deletions have dominant negative activity, only one, XrelAΔSP, has any obvious phenotypic effects. Unexpectedly, this molecule blocks induction of mesoderm by FGF in the model animal cap system. However, the effects on activin signaling differ from those of blocking FGF action at the receptor level, reported by others (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). This enables us to make some conclusions about the roles of FGF in mesoderm formation and patterning.

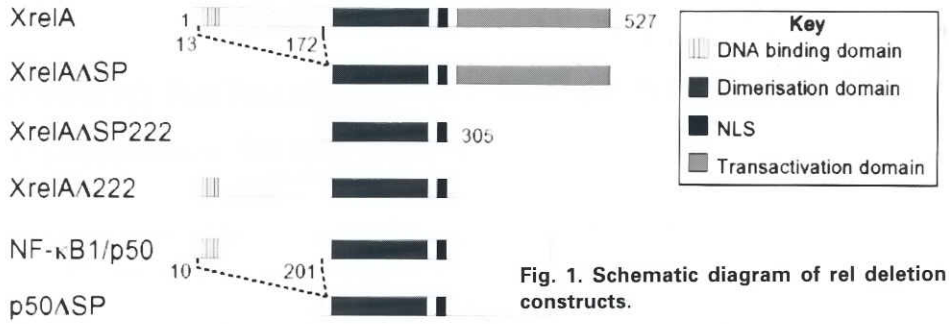

Fig. 1. Schematic diagram of rel deletion constructs.

It is now well known that the mesoderm is formed in morulae by primary signals from the vegetal blastomeres to the marginal zone cells and that this signaling differs dorsally from elsewhere (Dale and Slack, 1987; Jones and Woodland, 1987). The extreme dorsal mesoderm is specified to form notochord, and this region initially acts as a dorsal organizing center, the "Spemann organizer". In the rest of the marginal zone a homogeneous ventrolateral state is created, and this becomes more finely patterned by dorsalizing signals from the Spemann organizer. There are also local ventralizing signals, mediated by BMPs (Dale *et al.*, 1992; Jones *et al.*, 1992). Dominant negative receptor studies indicate that the primary signaling event depends absolutely on signals acting through activin receptors, or at least molecules capable of dimerizing with them, indicating that ligands of the TGF-β family are involved (Hemmati-Brivanlou and Melton; 1992; Schulte-Merker *et al.*, 1994). A dominant negative FGF receptor (XFD) has been used to show that for posterior dorsal mesoderm to form, FGF signaling via the ras to MAP kinase signal transduction pathway is also necessary (Amaya *et al.*, 1991, 1993; MacNicol *et al.*, 1993; Cornell and Kimelman, 1994; LaBonne and Whitman, 1994; Gotoh *et al.*, 1995; Umbauer *et al.*, 1995). There are two ways in which this might occur. Firstly, it has been reported that in the blastula there is a low level of signaling that sensitizes animal cap cells to activin (LaBonne *et al.*, 1995). It was found that levels of FGF which are sub-inducing for animal caps can induce vegetal cells to form mesoderm (Cornell *et al.*, 1995; Gamer and Wright, 1995). This suggests that the ratio of activin-like and FGF signaling defines the mesodermal and endodermal states. Secondly, it has been shown that the transcription factor *Xbra*, which is induced as an immediate early response to mesoderm inducers (Smith *et al.*, 1991), later causes the production of FGF in the gastrula which in turn induces expression of *Xbra* (Isaacs *et al.*, 1994; Schulte-Merker and Smith, 1995). This autoregulatory loop could be needed for production of dorsal mesoderm as is suggested by the fact that cell contact is needed for dorsal posterior cell differentiation markers to appear. The use of XFD does not easily distinguish these two kinds of role for FGF because it inhibits both. Using cycloheximide to inhibit protein synthesis, LaBonne *et al.* (1995) suggested that XFD could reduce immediate early activin induction of mesoderm. However, this result does not exclude the possibility that XFD also has later effects on via the FGF/Xbra autoregulatory loop. Our results using the mutant XrelAΔSP are consistent with the view that dorsal mesoderm cytodifferentiation depends on the early sensitizing effect of FGF

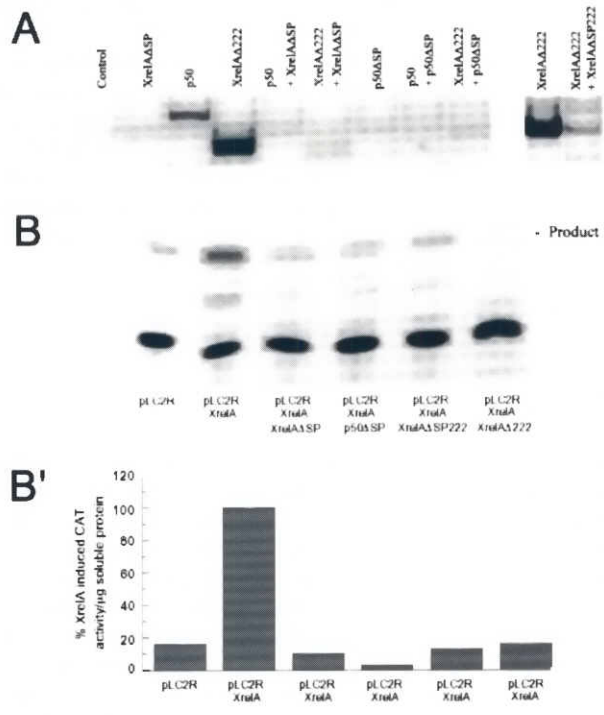

Fig. 2. Inhibition of XrelA and p50 function by mutants with dominant negative activity. (A) *Each of the DNA binding deficient deletion constructs was tested for ability to inhibit DNA binding of XrelAΔ222, which contains the wild-type XrelA rel homology domain, and of p50. The appropriate constructs were co-translated in oocytes, and DNA binding visualised using gel mobility shift assays. The ratio of dominant negative mRNA to wild-type RNA was 20:1 (4 ng:200 pg) in each case.* **(B)** *The same deletion constructs were also tested for the ability to inhibit transactivation by wild-type XrelA from an reporter construct (pLC2R) driven by the HIV LTR. DNA and RNA as shown were injected into 2-cell embryos and CAT activity measured at stage 11.* **(B')** *The product in B was quantified with respect to total soluble protein.*

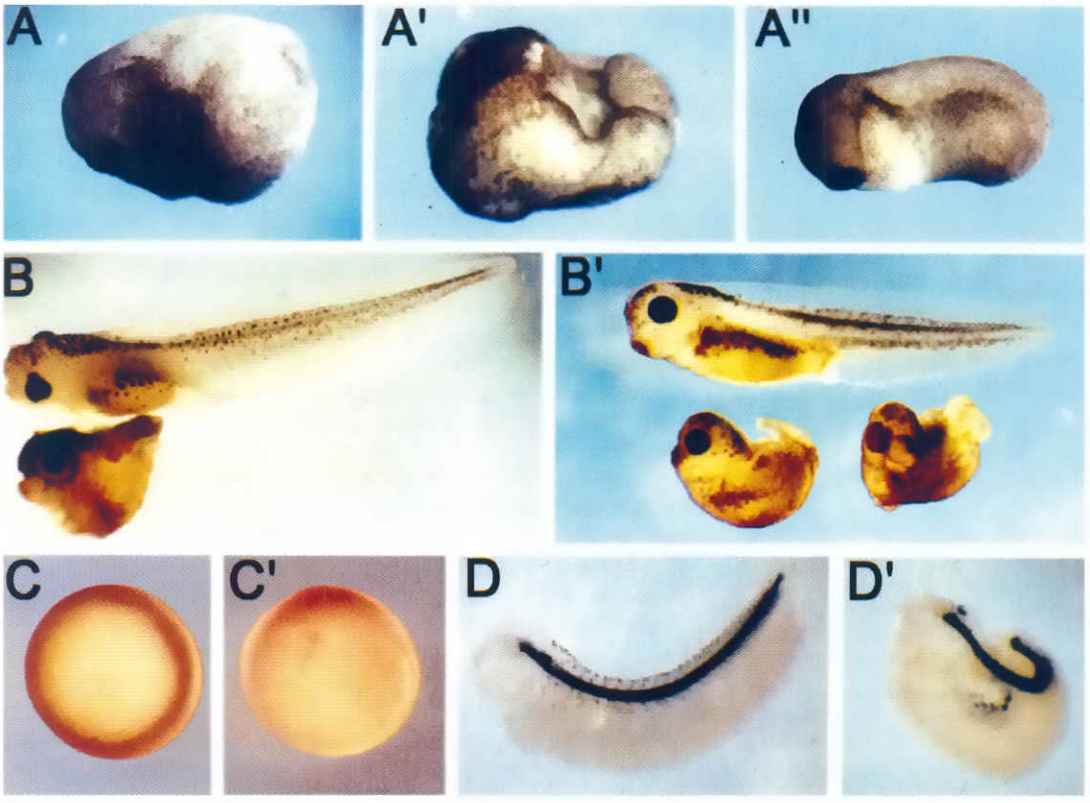

Fig. 3. Effects of rel deletion constructs on embryos. *(A, A', A'') Comparison of phenotypes at stage 25.* **(A)** *Representative embryo from a batch injected with 1 ng XrelAΔSP mRNA showing reduction in posterior structures.* **(A')** *More severely affected embryo from same batch showing axis split dorsally around an exposed yolk plug.* **(A'')** *Uninjected control sibling embryo.* **(B)** *Comparison of control stage 40 embryo (upper) with sibling embryo injected with 1ng XrelAΔSP mRNA (lower). Note the complete lack of tail formation in the lower embryo.* **(B')** *Comparison of typical embryo at stage 38 (upper) with 2 embryos from batches injected with 0.5 ng XFD mRNA (lower).* **(C and C')** *In situ hybridizations comparing expression of Xbra at stage 10 in an uninjected embryo (C) and in a sibling embryo bilaterally injected with 1 ng XrelAΔSP mRNA (C'). Note the reduction in Xbra expression to a small arc.* **(D and D')** *In situ hybridizations for the expression of the notochord marker collagen II. (D) Control embryo at stage 32 showing the normal development of the notochord and weaker staining in the somites. (D') Distribution of dorsal axial tissues around the exposed yolk plug in an cleared embryo injected with 1 ng XrelAΔSP RNA. Note that a notochord is present only on one side of the plug, while the other side of the plug has only unidentified loosely staining tissue (possibly either disorganized somite or notochord).*

and that the later role of FGF in the mesoderm is primarily to enable convergence and extension movements to occur.

Results

Characterization of rel deletion constructs

The various constructs used in this study are schematized in Figure 1. The wild-type clone XrelA possesses the typical attributes of a rel family member; its molecular properties were described by Richardson *et al.* (1994) and the biological effects of its overexpression reported by Richardson *et al.* (1995) and Kao and Lockwood (1996). XrelAΔ222, which lacks the activation domain and acts as a dominant negative, probably largely by titrating out B sites, has also been previously described (Richardson *et al.*, 1995). This report is principally concerned with XrelAΔSP, which lacks regions known from work on mammalian p65/XrelA to be necessary for DNA binding (Logeat *et al.*, 1991). The dominant negative interference activity of this construct would be expected to apply to the majority of rel family members, since it should be capable of forming inactive dimers with most of them. We also used an analogous dominant negative derivative of NFκB1, the other subunit of mammalian NF-κB, known as p50ΔSP. This construct is essentially the same as that described previously by Logeat and colleagues (Logeat *et al.*, 1991). All the constructs used in this study were tested for protein stability using ^{35}S methionine labeling (data not shown).

The DNA binding of the mutants was assessed by injecting capped synthetic messenger RNAs into oocytes and allowing translation overnight. The oocyte homogenates were then used in standard electrophoretic mobility shift assays (EMSA). Figure 2A shows that a 20-fold excess of XrelASP suppresses the binding of both XrelA and mammalian p50 to κB sites. In these analyses the activation domain deletion XrelAΔ222 was used as the target DNA binding protein because it produces a much sharper and stronger band than the full-length protein. However, similar results were obtained with full length XrelA (not shown). In addition, figure 2A shows that the double deletion clone XrelAΔSP222, which combines the deficiencies of XrelAΔSP and XrelAΔ222, also suppresses the DNA binding of XrelA. The construct p50ΔSP suppresses XrelA binding even more effectively than its RelA counterpart (Fig. 2A), probably because the XrelA/p50 heterodimers are more stable than the XrelA homodimers.

Figures 2B and B' show the effect of these mutants on transcriptional activation by XrelA. In this experiment the synthetic RNAs from the clones were co-injected with linearized pLC2R, a plasmid containing a CAT gene under the control of an HIV LTR. This promoter contains two κB sites and its transcription is strongly stimulated by XrelA (Richardson *et al.*, 1994). All the deletion constructs were seen to suppress transactivation by XrelA as well as preventing binding of this factor to DNA. XrelASP, XrelASPΔ222 and p50ΔSP can therefore be considered to act as dominant negatives against rel family members. In addition, these experi-

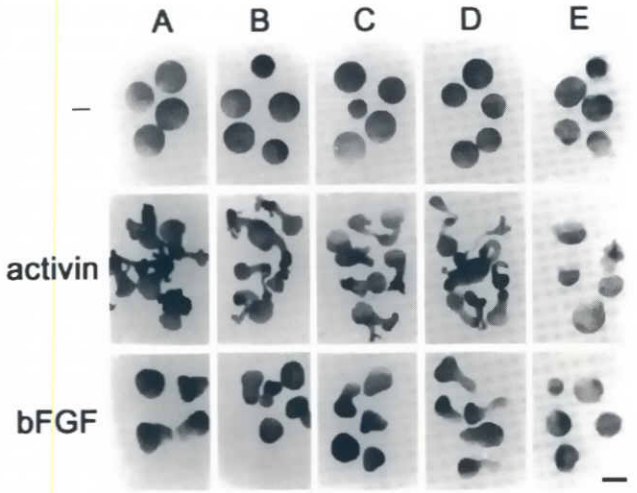

Fig. 4 A-E: Effect of XrelA and p50 dominant negative expression on the induction of morphological movements in animal caps. *Embryos were uninjected* **(A)**, *or injected with 1 ng of mRNA encoding the dominant negatives XrelAΔSP222* **(B)**, *XrelAΔ222* **(C)**, *p50ΔSP* **(D)** *or XrelAΔSP* **(E)** *at the two cell stage. Animal caps were isolated at stage 8.5 and cultured in Barth's medium alone, or containing either 1 x WIF or 50 ng/ml bFGF. Normal morphogenetic movements of the tissue, scored at control stage 17, are seen in response to both factors with the exception of caps taken from embryos expressing XrelAΔSP. Inhibition of FGF and activin induced extension movements by XrelAΔSP is highly reproducible. Bar, 250 μm.*

ments confirm the stability of all the mRNAs and their protein products both in oocytes (Fig. 2A) and embryos (Fig. 2B).

Effects of XrelA deletion constructs on whole embryos

Expression of levels of XrelAΔSP which enable dominant negative activity against its parent protein in EMSA and CAT assays severely perturbs gastrulation of *Xenopus* embryos (Fig. 3A,B). In contrast, neither the double mutant XrelAΔSP222 nor p50ΔSP had any significant effects on development (not shown). At low levels of XrelAΔSP expression (< 1 ng) extension of the dorsal axis is shortened, but embryos appear otherwise normal (Fig. 3A). Embryos expressing higher levels of XrelAΔSP (1ng) often fail to gastrulate normally. Although these embryos form a complete blastopore lip, closure of the blastopore ceases shortly after this point. During neurula stages the blastopore remains open behind the forming head of the embryo resulting in the split dorsal axis seen in figure 3A'. This gastrulation phenotype is superficially similar to that produced by overexpression of the dominant negative FGF receptor XFD (Isaacs *et al.*, 1994; Fig. 3B') and results in the formation of embryos with shortened trunks, split dorsally around an exposed yolk plug behind the head (Fig. 3B). At higher doses head development is also affected and cyclopia is common (not shown). Table I shows the combined results from 2 separate XrelAΔSP injection experiments.

Unlike XFD, XrelAΔSP expression does not lead to a loss of differentiated dorsal trunk tissues. This is illustrated by *in situ* hybridizations using a collagen II probe, a marker of notochord at the tailbud stage (Amaya *et al.*, 1993; Fig. 3D), which show that notochord development occurs on one side of the exposed yolk plug only (Fig 3D'). Transverse sections of embryos at tailbud stages confirm that notochord and segmented somites are present on one side of the plug with disorganized muscle on the other side (not shown). Nervous system is present on the same side as the notochord, but the neural plate appears reduced in size and frequently fails to fold up. Ventral and lateral mesoderm appears to form normally, but lateral plate mesoderm is usually thicker than in control embryos.

To investigate whether XrelAΔSP has a direct effect on mesoderm induction early gastrulae were assayed for expression of the pan mesodermal marker *Xbra*. XFD is able to inhibit expression of *Xbra* at this stage (Amaya *et al.*, 1993; LaBonne *et al.*, 1994). Normal expression of *Xbra*, shown in figure 3C, is severely reduced in the presence of XrelAΔSP, but complete knockout was never seen (Fig. 3C').

XrelAΔSP blocks morphological movements in animal caps treated with mesoderm inducing factors

The phenotype of XrelAΔSP embryos suggests that expressing this deletion may interfere with FGF signaling. The dominant negative FGF receptor construct XFD has been reported to eliminate the induction of morphological movements by either FGF or activin in isolated ectodermal explants (animal caps). We tested for comparable effects of XrelAΔSP on animal caps using embryos injected at the two cell stage with various doses of XrelAΔSP mRNA. The animal caps were excised at stage 8.5 and cultured in BX containing either 50 ng/ml bFGF or activin (WEHI cell culture medium; Albano *et al.*, 1990). Morphogenetic movements, if seen, began at around stage 11 and were scored at stage 17, after which no further movements occurred. Examples of typical animal caps

TABLE 1

PHENOTYPE PRODUCED BY INJECTION OF XRELAΔSP mRNA

mRNA injected	no. of embryos	surviving to tailbud stage	normal development	shortened posterior axis	split posterior axis	split posterior axis, no head	normal axis, no head
2.5 ng	85	39	0	2	7	29	1
1 ng	95	61	0	20	28	8	7
0.2 ng	74	61	9	41	8	3	0
uninjected	55	53	53	0	0	0	0

Scoring of embryos from 2 separate injection experiments. Embryos were scored for axis phenotype and head defects at stage 25. Short dorsal axis embryos were as Figure 3 A and split dorsal axis as Figure 3A'.

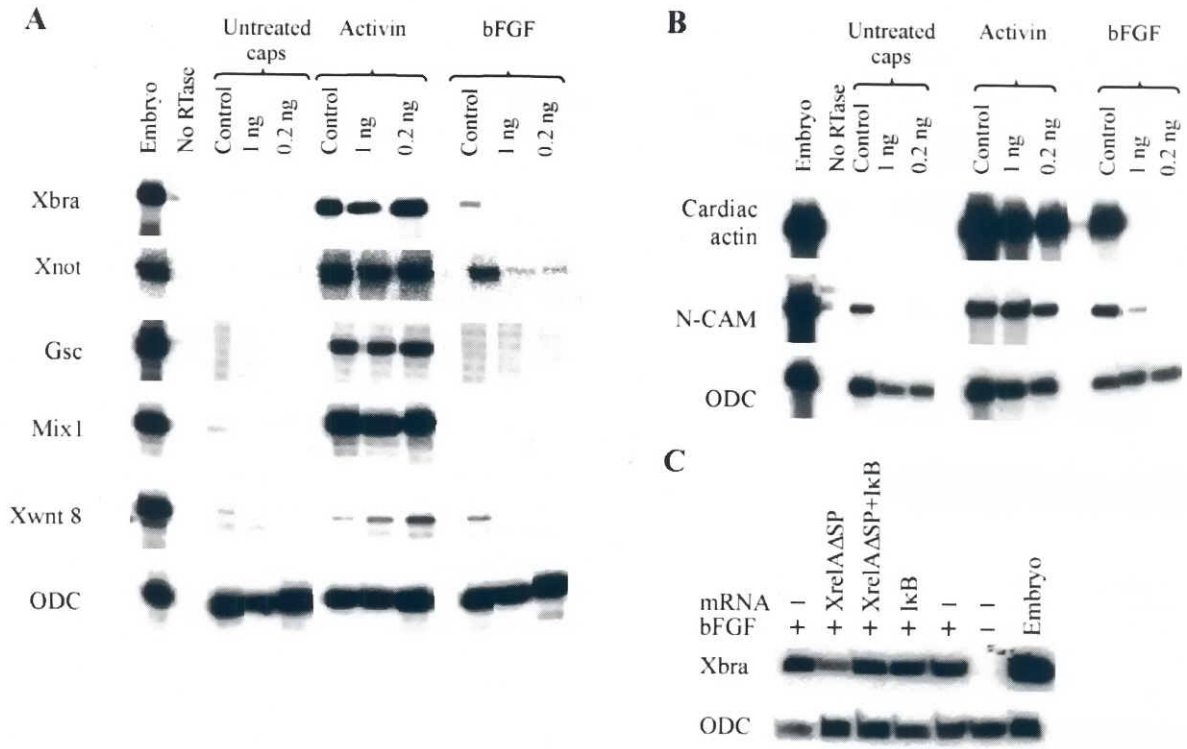

Fig. 5. Differential effects of XrelAΔSP on expression of mesodermal marker genes induced by FGF and activin. *Embryos were injected with XrelAΔSP mRNA as shown and animal caps were excised at stage 8.5 before treatment with mesoderm inducing factors.* **(A)** *Expression of marker genes at stage 11.* **(B)** *Expression of muscle and neural specific genes at stage 17.* **(C)** *Rescue of the effects of XrelAΔSP. Co-injection of 500 pg of XrelAΔSP mRNA with 1ng of mRNA encoding IκB, a specific inhibitor of NF-κB proteins, fully rescues the induction of Xbra by bFGF.*

are shown in figure 4, panels A-E. Animal caps from embryos injected with the dominant negative constructs XrelAΔ222, p50ΔSP or XrelAΔSP222 behaved in the same way as uninjected caps in response to either mesoderm inducing factor (Fig. 4 A-D). In contrast, animal caps from embryos injected with XrelAΔSP did not undergo visible morphogenetic movements in response to either FGF or activin (Fig. 4E). This supports the hypothesis that, like XFD, XrelAΔSP blocks FGF signaling. However, a lack of morphogenetic movements in animal caps does not necessarily imply a lack of mesoderm induction. Animal caps were therefore cultured to stage 40 for examination of histology. At mRNA doses as low as 200 pg per embryo, XrelAΔSP was found to eliminate the induction of histologically differentiated mesodermal tissues by bFGF (data not shown).

The effect of XrelAΔSP on the induction of early and late mesodermal marker genes

Previous reports have shown that inhibition of FGF signaling by XFD eliminates the induction in animal caps of a number of mesodermal marker genes by activin as well as FGF (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). In order to compare the effects of XrelAΔSP on marker induction to those reported for XFD, caps were taken from embryos injected with either 1 ng or 0.2 ng of XrelAΔSP mRNA and analyzed for expression of early and late marker genes using quantitative RT-PCR. Injection of 0.2 ng of XrelAΔSP mRNA eliminates FGF induction of the early marker genes *Xbra, Xnot* and *Xwnt 8* (Fig. 5A), consistent with the hypothesis that it acts by blocking FGF signaling. In contrast, expression of 1 ng of XrelAΔSP did not eliminate activin-mediated induction of any of the marker genes tested, although expression of *Xbra* was reduced to around 25% of levels in uninjected caps. *Xnot* and *Gsc* expression was reduced slightly (to around 75%) by high levels of XrelAΔSP, whereas the expression of *Mix.1* was totally unaffected and that of *Xwnt 8* considerably increased. FGF-induced expression of muscle specific cardiac actin and of the pan-neural marker *N-CAM*, which is presumably induced as a result of prior mesoderm induction, was eliminated by XrelAΔSP expression (Fig. 5B). However, the induction of these late markers of muscle and neural tissue by activin was unaffected. In contrast, XFD has been shown to eliminate expression of *Xbra*, *Xnot*, and muscle actin and N-CAM resulting from treatment of animal caps with activin (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994).

The specificity of the effect of XrelAΔSP expression on mesoderm induction by FGF can be demonstrated using a *Xenopus* IB which specifically inhibits the activity of XrelA *in vitro* and *in vivo* (A.M. Garcia Estrabot and H.R.W., unpublished results). Co-injection of XrelAΔSP with *Xenopus* IκB mRNA completely restores the induction of the mesoderm specific marker *Xbra* by bFGF (Fig. 5C).

The effects of XrelAΔSP on FGF-mediated mesoderm induction are not due to interference with endogenous XrelA function

We have shown that expression of XrelAΔSP inhibits the ability of the wild type XrelA protein to function (Fig. 2). Despite this, the

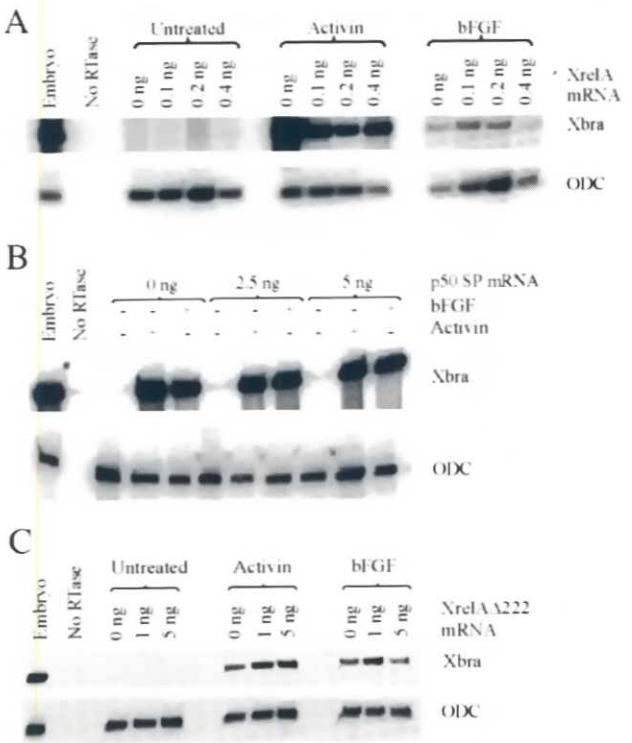

Fig. 6. Animal caps from embryos injected with XrelA, XrelAΔ222 or p50ΔSP were excised and treated as in Fig. 5 and analyzed for *Xbra* expression at stage 11. *Wild type XrelA* **(A)** *equally reduces induction of Xbra by both activin and FGF. The dominant negatives p50ΔSP* **(B)** *and XrelAΔ222* **(C)** *have no discernible effect on induction of Xbra by either factor.*

effects of XrelAΔSP on FGF-mediated mesoderm induction are unlikely to be a direct result of the inhibition of XrelA function in embryos. XrelA itself does not appear to have a role in the induction of mesoderm (see Fig. 6A), indeed expression of high levels in embryos somewhat reduces expression of a wide range of genes (Richardson *et al.*, 1995). In animal cap assays, XrelA was found to reduce both activin and FGF induced expression of the early mesodermal marker *Xbra* (Fig. 6A), as well as *Xwnt 8*, *Gsc* and *Mix.1* (data not shown) to a similar extent, suggesting that this may be part of a general suppression of transcription. As mentioned earlier, XrelAΔSP is the only rel deletion construct tested which appears to be capable of inhibiting FGF or activin induction of morphogenetic movements in animal caps (Fig. 4). p50ΔSP (Fig. 6B), XrelAΔ222 (Fig. 6C) or XrelAΔSP222 (data not shown) all failed to reduce *Xbra* induction by FGF or activin in animal caps and did not affect morphogenetic movements. We have shown that these three mutants and XrelAΔSP are equally effective as dominant negatives against XrelA (Fig. 1). As shown earlier, there is no difference in the stability of the proteins or their transport to the nucleus. These results therefore suggest that the effects of XrelAΔSP on FGF signaling are not mediated via interaction with endogenous wild type XrelA.

XrelAΔSP does not inhibit the induction of mesoderm by BMP-4

In addition to FGF and activin, a third *Xenopus* factor which has been shown to induce mesoderm in animal cap explants is bone morphogenetic protein 4 (BMP-4). Ectodermal explants from embryos overexpressing *Xenopus* BMP-4 generally form ventral mesoderm (Dale *et al.*, 1992, Jones *et al.*, 1992). It is not known whether XFD, or other inhibitors of FGF signaling, can affect the induction of mesoderm by BMP-4. We examined the effect of XrelAΔSP on ventral mesoderm induction by BMP-4 by analyzing the expression of several marker genes in isolated animal cap explants (Fig. 7A). Induction of the pan-mesodermal marker *Xbra* by BMP-4 occurred normally in the presence of doses of XrelAΔSP shown to be capable of eliminating induction by FGF. Similarly, BMP-4 induced expression of the ventral marker *Xwnt 8* and the posterior markers *Xpo* and *Xhox 3* was unaffected by XrelAΔSP expression. Animal caps expressing BMP-4 and XrelAΔSP were also examined by histology at stage 40 (Figs. 7B-D) and found to be identical to those expressing BMP-4 alone.

XrelAΔSP does not prevent activation of MAP kinase by FGF but blocks the autoregulatory eFGF/Xbra loop downstream of Xbra

MAP kinase activation occurs as a result of FGF signal transduction in *Xenopus* caps and is known to be both sufficient and essential for mesoderm induction by FGF (LaBonne *et al.*, 1995). XFD and other previously described inhibitors of FGF signaling have been shown to reduce the activation of MAP kinase (Gotoh *et al.*, 1995; LaBonne *et al.*, 1995). An assay for MAP kinase activity based on the phosphorylation of myelin basic protein by a 42 kDa protein has been described previously (Mason *et al.*, 1996). We used this assay to compare the activation of MAP kinase in animal caps expressing XrelAΔSP and XFD after treatment with FGF for 20 min (Fig. 8A). Whereas XFD eliminated MAP kinase activation, as previously reported (LaBonne *et al.*, 1995), expression of levels of XrelAΔSP known to eliminate induction of mesoderm by FGF failed to inhibit MAP kinase activation or to reduce basal levels of activation. This suggests that XrelAΔSP inhibits FGF-mediated mesoderm induction downstream of MAP kinase.

Maintenance of *Xbra* expression following its initial induction has been shown to depend on a feedback loop involving eFGF (Isaacs *et al.*, 1994; Schulte-Merker and Smith, 1995). To test the effect of XrelAΔSP on this autoinduction, mRNA encoding the mouse Brachyury protein was injected into *Xenopus* embryos with and without XrelAΔSP. Animal caps were isolated as before and assayed for the expression of *Xbra* and *eFGF* at stage 10.5 (Fig. 8B). As expected, mouse Brachyury induced expression of both genes in animal cap explants. Expression of XrelAΔSP blocked *Xbra* and *eFGF* induction suggesting that it interferes with the induction of *eFGF* by Xbra and therefore inhibits maintenance of *Xbra* by zygotic FGF signaling in the embryo.

The effect of XrelAΔSP on FGF-mediated mesoderm induction depends on the presence of the XrelA transactivation domain and absence of the dimerization domain

The use of several mutant constructs allows some insight into the nature of the XrelAΔSP block to FGF signaling. XrelAΔSP, which lacks the putative DNA binding domain, is the only mutant form of XrelA or p50 so far described which eliminates FGF signaling and blocks elongation of animal cap explants. Subsequent deletion of the transactivation domain, in the form of the deletion XrelAΔSP222, results in the loss of this ability (Figs. 4,6), without concurrent reduction in the stability or dimerization capability (Fig. 2). Similarly, a DNA binding deficient deletion of human

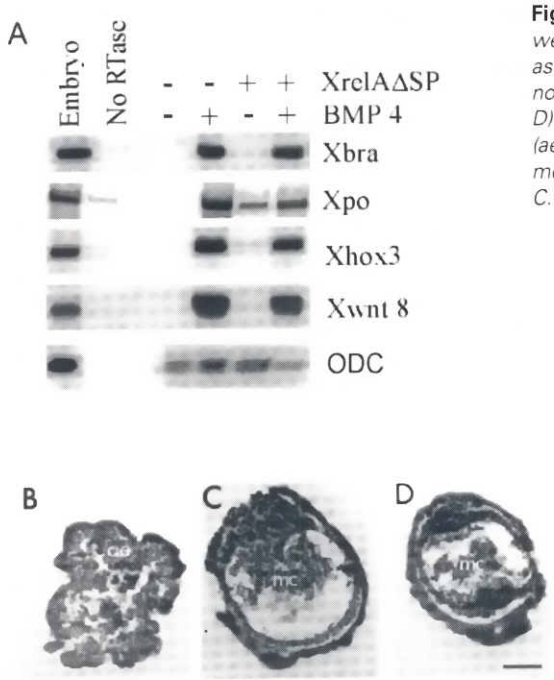

Fig. 7. XrelAΔSP does not inhibit the induction of mesoderm by BMP-4. *Animal caps were injected with 1 ng BMP-4 mRNA alone or in combination with 2 ng XrelAΔSP mRNA, as indicated.* **(A)** *RT-PCR analysis of marker expression showing that XrelAΔSP has little or no effect on the induction of ventral and intermediate mesodermal markers by BMP-4. (B-D) Histology of animal caps at stage 40.* **(B)** *uninjected caps contain only atypical epidermis (ae),* **(C)** *caps from embryos injected with BMP-4 mRNA develop vesicles containing mesenchyme (mc).* **(D)** *caps from embryos injected with BMP-4 and XrelAΔSP develop as C. Bar in D, 100 μm and applies to panels B-D.*

p50, which has no transactivation domain, has no effect on morphogenetic movements or mesoderm induction in induced animal caps (Figs. 4,6). These results suggest that the putative transactivation domain of XrelA is important for the ability of XrelAΔSP to block FGF-mediated mesoderm induction.

Discussion

Effect of XrelAΔSP on mesoderm induction in animal caps

We have described the effects of expressing XrelAΔSP, a dominant negative mutant of a *Xenopus* embryonic NF-κB subunit, which is deficient in DNA binding but retains dimerization and activation domains. XrelAΔSP blocks all evidence of mesoderm induction by FGF in animal caps, as judged by molecular and morphological criteria. However, this mutant has no effect on the induction of mesodermal markers by BMP-4 and its effects on activin induction appear to be specifically focused on cell movements, leaving cell differentiation markers relatively unaffected. The latter result contrasts with that observed by others who blocked the FGF signaling pathway at the receptor level using XFD, despite the superficial similarity of the phenotypes produced in whole embryos (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). XFD blocks both cell elongation movements and expression of dorsal posterior cytodifferentiation markers. In another study, Schulte-Merker *et al.* (1994) did note the formation of dorsal markers in the presence of XFD but attributed this to the anterior nature of the inductions, occurring as a result of injecting activin as mRNA. Our results cannot be explained in this way since dorsal markers are expressed in caps treated with a wide range of activin concentrations in the presence of XrelAΔSP (C.W.B and H.R.W, unpublished observations). FGF and Xbra do not appear to be required for the formation of the anterior dorsal tissues in *Xenopus*, as demonstrated by the presence of anterior somites in embryos expressing an Xbra dominant negative fusion protein or XFD (Amaya *et al.*, 1991, 1993; Isaacs *et al.*, 1994; Conlon *et al.*, 1996;).

Effects of XrelAΔSP on embryo phenotype

Expression of XrelAΔSP produces a phenotype which is superficially similar to that produced by blocking FGF signaling at the level of the receptor using XFD. This is particularly true with respect to the mode of gastrulation, which in both cases leads to splitting of the trunk dorsally around an exposed yolk plug behind the head. On closer examination however, XrelAΔSP embryos were seen to differ from those injected with XFD, most notably in the presence of dorsal mesoderm derivatives, somites and notochord, primarily on one side of the open blastopore. In accordance with the results of others we were unable to detect differentiated notochord in the vestigial trunk and tail of XFD embryos although anterior patches were seen in some cases (Amaya *et al.*, 1993). A phenotype more like that of XrelAΔSP embryos has been reported to result from the overexpression of a dominant negative deletion of the calcium dependent cell adhesion molecule C-Cad, known as C-trunc (Lee and Gumbiner 1995). C-trunc embryos also have notochord and somites around one side of the blastopore but neural tissue is less disrupted than in XrelAΔSP embryos. Like XrelAΔSP, C-trunc is able to inhibit convergence and extension movements in animal caps treated with activin, suggesting that C-Cad dependent cell adhesion is required for these movements to occur (Brieher and Gumbiner, 1994). However, although there is some evidence for an interaction between cadherins and FGF receptors (reviewed in Mason, 1994), there is no evidence that C-trunc inhibits FGF signaling. In contrast XrelAΔSP inhibits expression of genes induced by FGF as well as gastrulation movements, suggesting that the phenotype is not produced as a result of a direct effect on cell adhesion molecules alone.

The role of FGF in mesoderm induction by activin

As mentioned earlier, there are two phases of FGF expression in the blastula and gastrula. Both are currently thought to be important for the formation of mesoderm. Initially maternal FGF, at a low, sub-mesoderm inducing level, acts as a competence factor enabling the activin-type signal generated by the dorsal vegetal cells (the Nieuwkoop center) to induce the mesoderm of the dorsal organizer (Cornell *et al.*, 1995; Gamer and Wright, 1995; LaBonne *et al.*, 1995). Subsequently, during the early stages of gastrulation, zygotic eFGF forms an autocatalytic loop which functions to maintain expression of the transcription factor *Xbra* in posterior dorsal mesoderm (Isaacs *et al.*, 1994; Schulte-Merker and Smith, 1995). This later role of FGF may, at least in part, explain the need for cell:cell interactions in the patterning of the mesoderm (Green

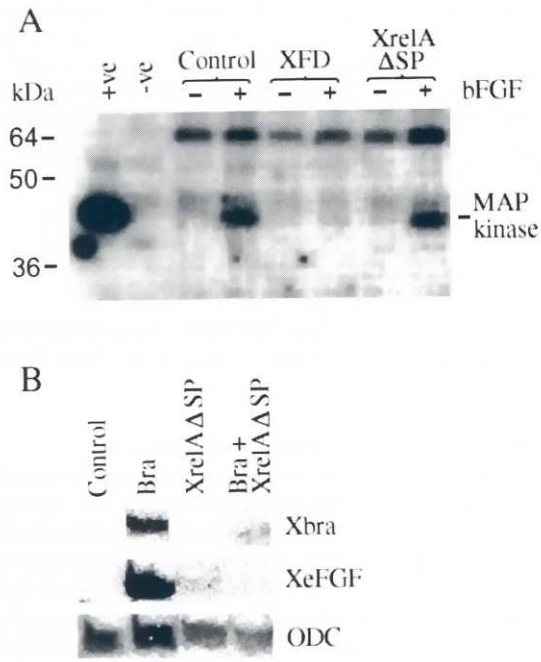

Fig. 8 (A). Assay for the activation of MAP kinase in response to FGF treatment of animal caps. *Lanes 1 and 2 are positive and negative controls respectively for the detection of active MAP kinase and correspond to extracts from matured (1) and immature (2) stage VI oocytes. Active MAP kinase is visible as a 42 kDa band. Caps were assayed for MAP kinase specific phosphorylation of myelin basic protein after 20 min contact with 50 ng/ml bFGF according to Mason et al., 1996. The lanes in 3 are extracts from uninjected animal caps, MAP kinase is activated by FGF. Caps from embryos injected with 400 pg of XFD mRNA (4) do not show an activation of MAP kinase in response to FGF. In contrast, injection of 2 ng XrelAΔSP does not affect the activation of MAP kinase by FGF in animal caps (5).* **(B) RT-PCR assay for the induction of Xbra and XeFGF by exogenous Brachyury.** *600 pg mRNA from the mouse Brachyury gene was injected into 2 cell Xenopus embryos with and without 1 ng XrelAΔSP mRNA. Animal caps were isolated at stage 9 and assayed for induction of Xbra and XeFGF at stage 11.*

et al., 1994; Wilson and Melton, 1994). To date experiments using XFD and other inhibitors of signal transduction from FGF to MAP kinase have been unable to completely distinguish between these two phases of FGF expression. However, it is possible to construct a model which could explain the differences between the effects of XFD and XrelAΔSP on the induction and patterning of mesoderm. We suggest that XrelAΔSP is able to inhibit the zygotic FGF maintenance signal, but not the specification of competence to respond to activin-like signals, which depends on maternal FGF expression (see Fig. 9). If this were the case, then the immediate early response to activin would be unaffected. In fact, the reduction in *Xbra* induction by activin due to the expression of XrelASP is similar in degree to that found by LaBonne and colleagues when caps of the same stage were treated with cycloheximide (LaBonne *et al.*, 1995). Cycloheximide would block the zygotic effects of FGF, which require translation, but leave the maternal signals intact.

On the basis of the results presented in this report, we propose a model for the action of XrelAΔSP, shown in figure 9. According to this model, the observation that XrelAΔSP permits dorsal cytodifferentiation in caps treated with activin and in embryos implies that the eFGF/Xbra autoregulatory loop is not essential for mesoderm patterning. It may therefore be the case that maternal FGF, acting as a competence factor, is more important for this aspect of early *Xenopus* development. However, maintenance of *Xbra* expression by zygotic FGF does appear to be essential for the normal convergence and extension movements of gastrulation to occur.

What is the target of XrelAΔSP?

We have shown that inhibition of FGF signaling by XrelAΔSP does not affect the activation of MAP kinase, but prevents autoinduction of *eFGF* by mouse Brachyury protein. Hence XrelAΔSP inhibits maintenance of *Xbra* expression. It has been found that MAP kinase activation is both necessary and sufficient for mesoderm induction by FGF (Gotoh *et al.*, 1995; LaBonne *et al.*, 1995; Umbauer *et al.*, 1995). Several dominant inhibitory rel constructs were able to block the binding of both NF-κB subunits to DNA. These include derivatives of both XrelA and mammalian p50 and can also block transactivation by XrelA (p50 alone is too weak a transcriptional activator to make these measurements). Notably only XrelAΔSP blocks FGF signaling, suggesting that neither zygotic XrelA nor a X*enopus* p50 equivalent are its targets. There remains however a slight question over maternal rel molecules, since newly synthesised XrelAΔSP may not be able to compete into preformed complexes.

Deletion of the activation domain from XrelAΔSP, forming the double deletion XrelAΔSP222, eliminates its ability to interfere with FGF signaling. This suggests a role for the activation domain in the inhibitory interaction. Interestingly, expression in embryos of a construct which retains only the activation domain and NLS resembles the phenotype of XrelAΔSP and can act as a dominant negative against XrelA (C.W.B and H.R.W., unpublished). Subsequent removal of the NLS disables both of these functions and strongly suggests that the target of XrelASP is nuclear and that the interaction requires regions of XrelA contained within the activation

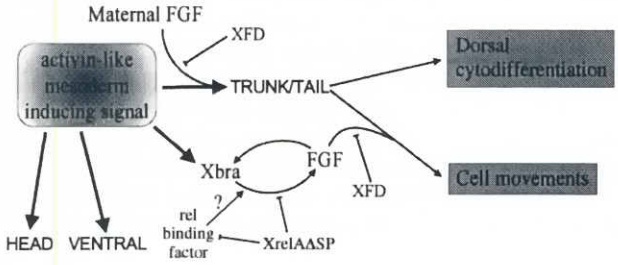

Fig. 9. Model showing possible points of action of XFD and XrelAΔSP in animal cap inductions. *This is simplified since it is clear that activin and FGF do more than induce Xbra, and the latter does more than induce XeFGF.*

domain. Although the target of XrelAΔSP is unknown, a number of interactions between rel family members and other DNA binding factors have been reported (Gonzalez-Crespo and Levine, 1993; Stein *et al.*, 1993a,b; Lehming *et al.*, 1994; Perkins *et al.*, 1994; John *et al.*, 1995). Further investigations using the various deletion constructs of XrelA and p50 documented here may enable the identification of the endogenous target of XrelAΔSP and confirm its role in the co-ordination of cell movements.

Materials and Methods

Construction and testing of dominant negatives

XrelA, XrelAΔ222 and XrelAΔSP were described previously (Richardson *et al.*, 1994). p50ΔSP is identical to the deletion made by Logeat *et al.* (1991). XrelAΔSP222 is a further deletion of XrelAΔSP truncated at aa 305. The constructs are shown in Figure 1.

Electrophoretic Mobility Shift Assays (EMSA)

Extracts for EMSA were made by homogenization in 5 μl per oocyte or embryo of extraction buffer (10 mM Hepes pH 8.0, 50 mM NaCl, 0.5 M sucrose, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 0.2% Triton X-100, 7 mM β-mercaptoethanol, 15% glycerol, 1 mM PMSF, 0.5 μg/ml leupeptin, 0.5 μg/ml aproteinin). Homogenates were cleared by centrifugation for 3 min at 13,000 rpm and the supernatant stored at -80°C until required.

A double-stranded oligonucleotide probe was made by annealing single κB site wild-type oligonucleotides (SKBW: CAACGGCAG**GGGACTTTCC**C-TCTCCTT). It contains a core κB site based on the HIV-LTR (bold) flanked by randomly chosen bases. Mutant competitor probe contains the same flanking regions, but has three mutations in the core κB site (SKBM: CAACGGCAG**CTCACTTTCC**CTCTCCTT. Probes were end-labeled with [3^2P] γ-ATP and T4 polynucleotide kinase.

Binding reactions (30 μl) consisted of 5 μl of protein extract with 4% glycerol, 50 mM NaCl, 10 mM Tris.Cl pH 7.5, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol and 0.5 μg/μl poly(dI-dC). Unlabeled mutant or wild-type oligonucleotide (5 ng) was added if required, and the reactions incubated at room temperature for 5 min before the addition of 50 pg labeled SKBW probe. After incubation for a further 5 min, the samples were analyzed on native 6% polyacrylamide gels (29 : 1 bis-acrylamide), in 0.25 x TBE at 200 volts, 4°C for 2 h.

Chloramphenicol acetyl transferase (CAT) assays

CAT assays were performed in triplicate, as described previously (Richardson *et al.*, 1994). Transcriptional activity was measured by exposing the chromatograms to a Molecular Dynamics PhosphorImager screen and quantified using Image Quant software.

Embryo and oocyte culture

Xenopus laevis oocytes and embryos were obtained, cultured and micro-injected as described previously (Wilson *et al.*, 1986; Old *et al.*, 1992). Micro-injection of mRNA was generally bilateral at the two cell stage, whereas unilateral injections of DNA and mRNA were used for CAT assays. For histology and *in situ* hybridizations embryos were fixed overnight in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde), with the vitelline membranes removed.

Dissection and culture of animal caps

Animal caps were removed from late blastula *Xenopus* embryos, between stages 8 and 9 incubated in Barth's medium (BX) containing 50 ng/ml human recombinant bFGF (Gibco BRL) or 1 x WEHI mesoderm inducing cell medium (WIF), which contains murine activin A (Albano *et al.*, 1990). Animal caps were either harvested at stages 11 and 17 for analysis of mRNAs, or cultured for 3 days, to control stage 40, for histological analysis.

Histology

Fixed animal cap explants were embedded in paraplast and sectioned to 8 μM before staining with hematoxylin and eosin.

Quantitative Reverse Transcription-PCR.

Total *Xenopus* mRNA was prepared essentially as described in Richardson *et al.* (1995), but modified slightly for explants. Groups of five animal caps were extracted using 150 μl of extraction buffer with 10 μg of glycogen as a carrier and all subsequent volumes adjusted accordingly. After the first phenol extraction and precipitation step, RNA was resuspended in 100 μl transcription buffer (such as SP6 buffer, Gibco BRL) containing 20 units DNase I and 12 units of placental RNase inhibitor and incubated for 15 min at 37°C, before re-extraction. Quantitative RT-PCR analysis of mRNAs was based on the method of Rupp and Weintraub (1991). 1-2 cap equivalents (0.5 μg of RNA) were used in reverse transcription reactions. RNA was first denatured at 75°C for 5 min, then cooled on ice. 30 μl reverse transcription reactions contained 3.3 μM random hexamers, 3 mM MgCl2, 500 μM dNTPs, 1 unit/l placental RNase inhibitor and 400 units MMLV reverse transcriptase in 1 x PCR buffer (Gibco BRL). Reactions were incubated for 1 h at 42°C and terminated by heating to 95°C for 5 min. PCR reactions in a 25 μl volume used 1 μl of reverse transcription reaction in 1 x PCR buffer with the addition of 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μCi α-[^{32}P]-dGTP, 1 μM each primer and 0.5 units of Taq DNA polymerase (Gibco BRL). Samples were denatured for 3 min at 94°C before cycling through 1 min at the appropriate annealing temperature, 1 min extension at 72°C and 30 sec at 94°C. The annealing temperature was 55°C for all primer sets except cardiac actin, which was annealed at 62°C to prevent cross reaction with cytoskeletal actin. Samples were resolved on 6% polyacrylamide gels containing urea, as for standard sequencing protocols. Cycle numbers were calculated from experiments similar to those described by Wilson and Melton (1994). 24 cycles were used for all primer sets except ODC, which was amplified for only 17 cycles.

RT-PCR Primer sequences

Primer sequences for Xwnt 8 and Xbra were taken from Wilson and Melton (1994), Gsc as in LaBonne and Whitman (1994) and cardiac actin as in Rupp and Weintraub (1991). Other primers used:- Mix.1: 5' AATGTCTCAAGGCAGAGG, 3' TGTCACTGACACCAGAA (bp 741-1140; Rosa, 1989). N-CAM: 5' CACAGTTCCACCAAATGC, 3' GGA-ATCAAGCGGTACAGA (bp 2817-3159; Kintner and Melton, 1987). ODC: 5' gGAGCTGCAAGTTGGAGA, 3' TCAGTTGCCAGTGTGGTC (bp 1482-1558; Bassez *et al.*, 1990). Xhox3: 5' TTACGCCTCACCTGCACA 3' GCCAACATGGTGTTCATC (bp 1002-1240; Ruiz i Altaba and Melton, 1989), Xpo: 5' GGTCTCACATTGCTATGC, 3' TCATCAGGTGCTGTGCTC (bp 1972-2195; Sato and Sargent, 1991).

Whole-mount in situ hybridizations

Whole-mount *in situ* hybridization reactions used the method of Harland (1991). *Xenopus Brachyury* (pXBra, Smith *et al.*, 1991) was kindly provided by Dr. Jim Smith and Collagen II (Amaya *et al.*, 1993) was kindly provided by Dr. Les Dale. Probes were was prepared as described in Richardson *et al.* (1995).

Detection of MAP kinase activity

Analysis of MAP kinase activity in animal caps used the method of Mason *et al.* (1996).

Acknowledgments

We thank David Stott for the mouse Brachyury expression construct used in the autoinduction experiment, Les Dale for the BMP-4 expression construct and collagen II probe, Harry Isaacs for XFD, and Jim Smith for the Xbra probe. WEHI medium was a kind gift of Liz Jones. We also thank Rosalind Friday for help with the autoinduction and rescue experiments and Melanie Lake and Bob Old for assistance with the MAP kinase assay. C.W.B. was supported by a Ph.D studentship from the MRC and D.J.S. by a Ph.D studentship from SERC.

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Received: March 1997
Accepted for publication: October 1997