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 XrelA:SP, 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 XrelA:SP 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 XrelA:SP effect on FGF signaling is shown by rescue of mesodermal marker expression when XrelA:SP 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 XrelA:SP 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 XrelA:SP 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., Arany et al., 1991, 1993; Hemmati-Brivanlou and Melton, 1992; Graft 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 et al., 1995).

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.
The results were consistent with a role for factors binding to xB 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, XrelAASP, 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 Kimmel, 1994; LaBonne and Whitman, 1994). This enables us to make some conclusions about the roles of FGF in mesoderm formation and patterning.

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 Kimmel, 1994; LaBonne and Whitman, 1994; Gotch 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 sensitzes 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 XrelAASP are consistent with the view that dorsal mesoderm cytodifferentiation depends on the early sensitizing effect of FGF.
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 kB sites. In these analyses the activation domain deletion XrelA222 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 XrelA222, which combines the deficiencies of XrelA and XrelA222, also suppresses the DNA binding of XrelA. The construct p50ASP 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 2' 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 kB 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, XrelASP222 and p50ASP can therefore be considered to act as dominant negatives against rel family members. In addition, these experi-
TABLE 1

<table>
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<th>mRNA injected</th>
<th>no. of embryos</th>
<th>surviving to tailbud stage</th>
<th>normal development</th>
<th>shortened posterior axis</th>
<th>split posterior axis</th>
<th>split posterior axis, no head</th>
<th>normal axis, no head</th>
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<td>2.5 ng</td>
<td>85</td>
<td>39</td>
<td>0</td>
<td>2</td>
<td>7</td>
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<tr>
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<td>95</td>
<td>61</td>
<td>0</td>
<td>20</td>
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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 3A and split dorsal axis as Figure 3A'.
are shown in figure 4, panels A-E. Animal caps from embryos injected with the dominant negative constructs XrelA222, p50ASp or XrelA222ASp behaved in the same way as un.injected caps in response to either mesoderm inducing factor (Fig. 4A-D). In contrast, animal caps from embryos injected with XrelAASp did not undergo visible morphogenetic movements in response to either FGF or activin (Fig. 4E). This supports the hypothesis that, like XFD, XrelAASp 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, XrelAASp was found to eliminate the induction of histologically differentiated mesodermal tissues by bFGF (data not shown).

The effect of XrelAASp 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 XrelAASp on marker induction to those reported for XFD, caps were taken from embryos injected with either 1 ng or 0.2 ng of XrelAASp mRNA and analyzed for expression of early and late marker genes using quantitative RT-PCR. Injection of 0.2 ng of XrelAASp mRNA eliminates FGF induction of the early marker genes Xbra, Xnot and Xwnt8 (Fig. 5A), consistent with the hypothesis that it acts by blocking FGF signaling. In contrast, expression of 1 ng of XrelAASp 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 uninjectected caps. Xnot and Gsc expression was reduced slightly (to around 75%) by high levels of XrelAASp, whereas the expression of Mix1 was totally unaffected and that of Xwnt8 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 XrelAASp 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 XrelAASp expression on mesoderm induction by FGF can be demonstrated using a Xenopus IκB 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 XrelAASp with Xenopus IκB mRNA completely restores the induction of the mesoderm specific marker Xbra by bFGF (Fig. 5C).

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

We have shown that expression of XrelAASp inhibits the ability of the wild type XrelA protein to function (Fig. 2). Despite this, the
The effects of XrelA:SP 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, XrelA:SP is the only deletion construct tested which appears to be capable of inhibiting FGF or activin induction of morphogenetic movements in animal caps (Fig. 4). p50:SP (Fig. 6B), XrelA:222 (Fig. 6C) or XrelA:SP222 (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 XrelA:SP 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 XrelA:SP on FGF signaling are not mediated via interaction with endogenous wild type XrelA.

**XrelA:SP 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 XrelA:SP 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 XrelA:SP 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 XrelA:SP expression. Animal caps expressing BMP-4 and XrelA:SP were also examined by histology at stage 40 (Figs. 7B-D) and found to be identical to those expressing BMP-4 alone.

**XrelA:SP 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 XrelA:SP 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 XrelA:SP known to eliminate induction of mesoderm by FGF failed to inhibit MAP kinase activation or to reduce basal levels of activation. This suggests that XrelA:SP 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 XrelA:SP on this autoinduction, mRNA encoding the mouse Brachyury protein was injected into Xenopus embryos with and without XrelA:SP. 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 XrelA:SP 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 XrelA:SP 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 XrelA:SP block to FGF signaling. XrelA:SP, 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 XrelA:SP222, 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
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**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 flexigal 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 latter role of FGF may, at least in part, explain the need for cell-cell interactions in the patterning of the mesoderm (Green...
A model which could explain the differences between the effects of XFD and XrelASP on the induction and patterning of mesoderm. We suggest that XrelASP 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 XrelASP, shown in figure 9. According to this model, the observation that XrelASP 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 XrelASP?**

We have shown that inhibition of FGF signaling by XrelASP does not affect the activation of MAP kinase, but prevents autoinduction of eFGF by mouse Brachyury protein. Hence XrelASP inhibits maintenance of Xbra expression. It has been found that MAP kinase activation is both necessary and sufficient for mesoderm induction by FGF (Gottoh 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-kB 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 XrelASP blocks FGF signaling, suggesting that neither zygotic XrelA nor a Xenopus p50 equivalent are its targets. There remains however a slight question over maternal rel molecules, since newly synthesised XrelASP may not be able to compete into preformed complexes.

Deletion of the activation domain from XrelASP, forming the double deletion XrelASP222, 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 XrelASP 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 domain.
domain. Although the target of XrelA △ SP 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 XrelA △ SP and confirm its role in the co-ordination of cell movements.

Materials and Methods

Construction and testing of dominant negatives

XrelA, XrelA △ 222 and XrelA △ SP were described previously (Richardson et al., 1994). p50 △ S is identical to the deletion made by Logeat et al. (1991). XrelA △ SP222 is a further deletion of XrelA △ SP truncated at aa 305. The constructs are shown in Figure 1.

Electrophoretic Mobility Shift Assays (EMSA)

Extracts for EMSAs 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 aprotinin). 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 xB site wild-type oligonucleotides (SKBW: CAACGCCAGGGACTTTCCCCTCCTCCTT). It contains a core xB 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 xB site (SKBM: CAACGCCAGGCTACTTTCCCTCCTCCTT). Probes were end-labeled with 32p-ATP and T4 polynucleotide kinase.

Binding reactions (30 μl) consisted of 5 μl of protein extract with 4% glycerol, 50 mM NaCl, 10 μM Tris.Cl pH 7.5, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol and 0.5 μg/ml poly(d-I-c). 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). Micron 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 MgSO4, 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/μl 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 MgCl2, 200 μM dNTPs, 0.5 μC/μl [32P]-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 15 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 15 cycles.

RT-PCR Primer sequences

Primer sequences for Xwnt 8 and Xbra were taken from Wilson and Melton (1994), Gac as in LaBonnew and Whitman (1994) and cardiac actin as in Rupp and Weintraub (1991). Other primers used:- Mix.1: 5‘ AATGTCCTCAAGGCGAGGG 3‘ TGTCACTGACACCAGAA (bp 1972-2195; Sata and Sargent, 1991). ODC: 5‘ gGAGCTGCAAGTTGGAGA 3‘ TCAGTTGCGAGTTGCT (bp 1482-1558; Bassez et al., 1990). Probes were prepared as described in Aichardson et al., 1994:

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 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).

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