Cell signalling and gene regulation in early *Xenopus* development

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October 1998

A thesis submitted to the University of London
for the degree of Doctor of Philosophy

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This thesis is dedicated to my parents, Margaret and Peter Neal
Acknowledgements

I am grateful to the Director of the Institute, Dr J. J. Skehel, and the Director of Studies, Dr R. W. King for giving me the opportunity to do my PhD at the NIMR. Thanks to the Medical Research Council who provided the funding for my PhD.

A big thank you to all the members of the Division of Developmental Biology, past and present, for their advice, discussion and friendship. They have made my time at the Institute stimulating and also great fun. Of all of them, I owe particular thanks to Branko Latinkic, whom I worked closely with during the first part of my PhD, and Niall Armes whose enthusiasm and encouragement I will never forget.

I am grateful to all my friends and colleagues around the Institute for all the good times that we have shared.

I would like to thank my parents and my sister Hilary for all their love and support, I feel so lucky to have them as my family. I am grateful to Alison Pryce for her friendship and for all the memorable days that we spent in the lovely Archway. A special thank you goes to Julian Mackintosh, without whom I wonder if this thesis would ever have got into print!

Finally I would like to thank my supervisor Dr Jim Smith. His wisdom, guidance and support have played a crucial role in my training as a scientist. Thank you!
Abstract

The two activin type I receptors ALK-2 and ALK-4 have distinct effects on gene expression in the *Xenopus* animal cap assay. Constitutively active ALK-4 (ALK-4*) can reproduce the effects of activin treatment including the dose dependent induction of progressively more dorso-anterior markers, whilst constitutively active ALK-2 (ALK-2*) induces only ventral mesodermal markers and counteracts the dorsalising effects of ALK-4. The dorsal inducing properties of ALK-4* can be transferred to ALK-2* by transfer of a small peptide loop (the β4-β5 loop) from the kinase domain of one receptor to the other. In contrast, I show, that the equivalent region of the ALK-2* receptor, when transferred to ALK-4*, cannot effectively counteract the dorsalising effects of ALK-4*, suggesting that other regions of type I receptors are also involved in determining signal specificity.

The chimeric receptor created by transfer of the ALK-2* GS domain and its flanking sequences into ALK-4* (C9), and the chimeric receptor created by transfer of the activation loop of the ALK-2* kinase (C11) into ALK-4* cannot induce the full range of ALK-4* responses, although they contain the ALK-4* β4-β5 loop. My results suggest that ALK-4* regulates at least two different signalling events, one of which is specified by C9 and C11.

The *Xenopus Brachyury* (*Xbra*) gene is induced in response to the mesoderm inducing factors FGF and activin (and by the activin receptors ALK-2* and ALK-4*). I have examined the transcriptional regulation of a pseudoallele of *Xbra*, *Xbra2*, in response to FGF. My results indicate that 381 bp 5' of the *Xbra* transcription start site are sufficient to confer responsiveness to FGF (and activin).

Examination of the *Xbra2* 5' flanking sequence revealed a serum response-like element. This suggested that activation of *Xbra2* in response to FGF may occur in a similar way to the activation of *c-fos*, through the activation of a ternary complex bound to an SRE-like element. My results show that this is unlikely. However, I provide preliminary evidence that a serum response-like factor functions as a negative regulator of mesoderm formation in *Xenopus* embryos.
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Chapter 1

Introduction

Formation and patterning of the mesoderm are fundamental to the establishment of the vertebrate body plan. For this reason, the way in which mesoderm is induced and patterned is an intense area of study. Work on embryos of the amphibian, *Xenopus laevis*, have provided researchers with the most insight into the cellular and molecular interactions that occur during these two processes.

The *Xenopus* embryo is an excellent system in which to study the mechanisms controlling mesoderm formation and patterning, for several reasons. *Xenopus* embryos can be obtained in large numbers and they develop outside the mother, making them accessible to experimental manipulation at all developmental stages. Furthermore, the embryos are large in size which makes them relatively easy to microinject and dissect into pieces that can be assayed for their inductive potential. In addition to this an efficient procedure for generating transgenic *Xenopus* embryos has recently been developed. Thus, transgenesis can be used to ectopically express genes of interest, to direct expression of modified gene products which interfere with the function of their endogenous, wild type counterparts, and to analyse the spatial expression of promoters in the embryo (Kroll and Amaya, 1996).

The first part of this thesis concerns the signalling specificities of ALK-2 and ALK-4 which are receptors for the mesoderm inducing factor activin. Constitutively active forms of these receptors transduce clearly distinct signals in *Xenopus* (Armes and Smith, 1997). The second part of the study is focused on the transcriptional regulation of *Xenopus Brachyury*, a target gene for the mesoderm inducing factors FGF and activin and the activin receptors ALK-2 and ALK-4.
Early *Xenopus* development

During its development in the mother, the *Xenopus* oocyte becomes polarised along its animal-vegetal axis, but remains radially symmetrical around this axis. The animal-vegetal polarisation is clearly visible by a difference in pigmentation, the animal hemisphere is dark and the vegetal hemisphere is light. Oocytes contain stores of potential regulatory molecules, proteins and RNAs that serve to control embryonic development prior to the onset of zygotic transcription, which begins at the mid-blastula transition (MBT) (Kirschner et al., 1985), when the embryo contains ~4000 cells. Several RNAs are known that are specifically localised to a thin layer of cytoplasm (the cortex) underlying the plasma membrane in the vegetal hemisphere, including the TGF-β family member Vg-1, which may be involved in mesoderm induction (Weeks and Melton, 1987), as will be discussed later.

The radial symmetry of the oocyte is broken at fertilisation as a result of sperm entry. This triggers a cytoplasmic shift, about half way through the first cell cycle, which involves movement of the subcortical cytoplasm relative to the plasma membrane and the cortex (Vincent and Gerhart, 1986; Gerhart et al., 1989). The ventral side of the embryo, where the sperm enters the oocyte, forms where the vegetal cytoplasm meets the animal cortex, giving rise to ventral structures like blood and mesenchyme. The dorsal side, which gives rise to axial structures, such as notochord, somites and neural tissue, forms where the animal cytoplasm meets the vegetal cortex.

**Mesoderm Induction and Dorsalisation**

At early cleavage stages *Xenopus* embryos can be considered to consist of two cell types: prospective ectoderm in the animal hemisphere and prospective endoderm in the vegetal hemisphere. Mesoderm arises during blastula stages as a result of inductive interactions in which cells from the vegetal half of the embryo act on cells in the equatorial region (marginal zone) of the embryo causing them to form mesoderm rather than ectoderm. Nieuwkoop demonstrated this interaction by culturing an animal pole
explant (animal cap) from a blastula stage embryo in contact with vegetal pole cells. Cultured alone, the animal cap forms atypical epidermis; in the presence of vegetal pole cells, it forms a variety of mesodermal cell types (Nieuwkoop, 1969).

Vegetal pole cells induce two types of mesoderm. Those from the dorsal side of the embryo tend to induce dorsal cell types, such as notochord and somites, while lateral and ventral vegetal blastomeres induce ventral structures such as blood and mesenchyme (Boterenbrood and Nieuwkoop, 1973; Dale et al., 1985). This is consistent with the finding that, at the 32-cell stage, only dorsal-vegetal cells are able to induce dorsal structures (Dale and Slack, 1987a) and to cause axial duplication when transplanted to the ventral side of a host 32-cell stage embryo (Gimlich, 1986). It seems likely that this activity represents a dorsal mesoderm inducing signal.

These data provided some insight into how mesoderm is formed and patterned in *Xenopus*. However, they appeared to contradict the fate map of *Xenopus* embryos which shows that about 60% of somitic muscle comes from the ventral half of the embryo (Dale and Slack, 1987a). In experiments in which blastulae were divided in half and cultured separately, ventral halves typically formed extreme ventral structures containing little or no muscle (Dale and Slack, 1987b), suggesting that a signal from the dorsal half of the embryo is required to produce the correct range of mesodermal tissues along the dorso-ventral axis. This process has been called 'dorsalisation' (Smith and Slack, 1983; Dale and Slack, 1987b). Evidence for the existence of a dorsal signal comes from experiments in which dorsal and ventral regions from early gastrulae are juxtaposed. Isolated dorsal marginal zone tissue forms notochord, with some muscle and neural tissue, while ventral marginal zone cells form blood, mesenchyme and mesothelium. But, if the two pieces are combined, although the dorsal tissue continues to differentiate as notochord, the ventral marginal zone forms large amounts of muscle (Slack and Forman, 1980; Dale and Slack, 1987b).

The source of the dorsalising signal in prospective mesoderm is the Spemann organiser, in the dorsal marginal zone. By transplanting the
organiser of an early gastrula stage embryo to the ventral side of another gastrula, it was discovered that the organiser is capable of respecifying the fate of host cells in the area, resulting in axial duplication (Spemann and Mangold, 1924; Smith and Slack, 1983). Due to the similarity of the axial duplications caused by transplant of either dorsal vegetal cells from a 32-cell stage embryo or the organiser region of an early gastrula stage embryo, it was reasoned that the dorsal organiser activity may be induced to form as a result of inductive signals emanating from underlying vegetal cells.

The processes of mesoderm induction and dorsalisation have been resolved into the ‘three signal model’ (Fig. 1.1) (Smith and Slack, 1983). According to this model, a signal from the ventral and lateral vegetal cells induces ventral mesoderm, which comprises about 300° of the marginal zone, and the dorso vegetal cells emit a signal that induces the formation of dorsal mesoderm, or organiser, which makes up the remaining 60° of the marginal zone. Originally these two signals were considered to occur early in development, before the onset of zygotic transcription at the MBT, with a third signal, acting later, passing from the organiser region to expand the size of the area of dorsal mesoderm (Fig. 1.1). Recently, however, there has been evidence to suggest that the initial inductive signals do not pass from the vegetal hemisphere to the animal hemisphere until after the MBT (Wylie et al., 1996). Wylie et al. have shown that when recombinants of animal and vegetal explants are cultured together for a short, one hour culture period, vegetal explants from late blastulae are able to induce competent animal caps to express MyoD. Vegetal explants from early (preMBT) blastulae, however, do not induce identical animal caps to express MyoD, over the same period of time (Wylie et al., 1996). This demonstrates that the cell interactions involving signals from the vegetal portion of the embryo that are required to induce MyoD occur only after zygotic transcription has started. It remains to be tested whether the same is true for other markers.

Since the three signal model was devised, intensive effort has been made to characterise the molecules involved in mesoderm induction and axial
Figure 1.1. The three signal model (modified from Smith, 1989). Embryological experiments described in the text predict the model shown. As a result of oogenesis and cortical rotation, three territories are established: the animal hemisphere, the ventro-vegetal blastomeres and the dorso-vegetal blastomeres. The vegetal blastomeres induce mesoderm of a corresponding character in the overlying animal cells. The dorsal mesoderm, or organiser, (O) then patterns the ventral mesoderm.
patterning. With the discoveries that have been made, it has been necessary to make modifications to the model. Notably, the three signal model regards ventral mesoderm as a 'ground state' that is adopted by lateral and ventral regions in the absence of dorsalising signals. However, it appears that ventral mesoderm formation requires active signalling and is not simply a passive process (Sive, 1993), as will be discussed later.

**Establishment of the dorso-ventral axis**

As mentioned above, the dorso-ventral axis is established at fertilisation as a result of sperm entry which triggers cortical rotation. Ultraviolet (UV) irradiation is effective at blocking the formation of the dorso-ventral axis when applied at two different times: firstly when applied to the vegetal pole of mature ovarian oocytes, and secondly when applied to fertilised eggs shortly after sperm entry, thereby preventing cortical rotation (Holwill et al., 1987; Elinson and Pasceri, 1989). Both treatments result in radially symmetrical embryos that, at post gastrula stage, consist of three concentric germ layers, the ectoderm, mesoderm and endoderm, that are of ventral character (Fig 1.2). The natures of the pre-fertilisation and post-fertilisation dorsalising activities are not understood, although they are clearly separable; normal development can be restored in embryos that have been subjected to UV irradiation just after fertilisation by tipping them so that cortical rotation is re-imposed (Scharf and Gerhart, 1980), while eggs that are UV irradiated as oocytes undergo normal cortical rotation and cannot be rescued by tipping (Elinson and Pasceri, 1989). As UV irradiation of the vegetal pole of mature oocytes prevents the formation of a dorso-ventral axis, it seemed possible that a component(s) of the dorsal pathway, or a factor that influences this pathway, is established during oogenesis and localised to the cortex of the vegetal pole before oocyte maturation. The vegetal localisation of an oocyte dorsalising activity has been confirmed by cytoplasmic transfer experiments (Holowacz and Elinson, 1995), however the molecular character of this activity is not known.

Several lines of evidence indicate that, in the post-fertilisation UV-sensitive period, the UV target is cytoskeletal. UV irradiation disrupts cortical
Figure 1.2. Two events pattern the early embryo (taken from Heasman, 1997). UV irradiation experiments reveal that at least two events pattern the embryo: (A) a UV-insensitive event establishes a post-gastrula-stage embryo consisting of three concentric germ layers - ectoderm, ventral mesoderm and ventral endoderm and (B) a UV-sensitive event produces tissue of dorsal type, including neural tissue, somites, notochord and dorsal endoderm, and also produces dorso-ventral and anterior-posterior axes in the embryo. LPM, lateral plate mesoderm.
cytoplasmic movements by disorganising microtubules, thereby preventing the normal displacement of a dorsalising activity to the dorsal side of the embryo (Gerhart et al., 1989; Houliston, 1994). It is likely that the manual tipping of a 1-cell stage embryo that has been UV irradiated post-fertilisation restores the displacement of a dorsalising activity to the dorsal side of the embryo, thereby restoring the dorso-ventral axis.

A surprising number of molecules that are expressed in early embryos have been identified as sufficient to ‘rescue’ (restore the dorso-ventral axis) the UV-ventralised phenotype (Table 1.1). In such experiments candidate proteins are overexpressed by injecting mRNA, or dominant negative forms of those mRNAs, into early cleavage stage UV-ventralised embryos. Results from experiments like this have suggested roles in dorsal axis formation for all the molecules listed in Table 1.1. For example Xwnt-8 and a processed form of Vg-1 (see later) are sufficient to induce the formation of a complete dorso-ventral axis (Thomsen and Melton, 1993), and activin and goosecoid are capable of inducing an incomplete dorso-ventral axis (Cho et al., 1991; Smith and Harland, 1991). It is important to note that most of the molecules shown in Table 1.1 (marked *) are absent or expressed at very low levels as maternal mRNAs, and are only expressed strongly after the onset of zygotic transcription (after MBT). Thus, it seems likely that these molecules function downstream of UV-sensitive maternal events and that some of them may represent the dorsalising activity of the organiser region. Further research is necessary to distinguish which, if any, of these factors are normally involved in establishing the dorso-ventral axis, and of those involved, what relationship they share with each other.

Recently some of the essential components of the dorsal, UV-sensitive pathway have been identified. β-catenin is maternally expressed at the RNA and protein level (DeMarais and Moon, 1992). The protein is asymmetrically localised, accumulating in dorsal, but not ventral nuclei by the 16-32 cell stage (Larabell et al., 1997). When ectopically expressed in UV ventralised embryos, β-catenin is sufficient to mimic the endogenous dorsal-determining activity by inducing the formation of complete dorso-ventral
Table 1.1 Molecules that rescue the UV phenotype, (adapted from Heasman, 1997).

<table>
<thead>
<tr>
<th>Complete axis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Xwnt-8</td>
<td>(Smith and Harland, 1991)</td>
</tr>
<tr>
<td>*noggin</td>
<td>(Smith and Harland, 1992a)</td>
</tr>
<tr>
<td>Vg1 (modified form)</td>
<td>(Thomsen and Melton, 1993)</td>
</tr>
<tr>
<td>Xwnt-8b</td>
<td>(Cui et al., 1995)</td>
</tr>
<tr>
<td>*Xnr1 and Xnr2</td>
<td>(Jones et al., 1995)</td>
</tr>
<tr>
<td>*Siamois</td>
<td>(Lemaire et al., 1995)</td>
</tr>
<tr>
<td>*chordin</td>
<td>(Sasai et al., 1994)</td>
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<thead>
<tr>
<th>Incomplete axis</th>
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<tr>
<td>*goosecoid</td>
</tr>
<tr>
<td>activin</td>
</tr>
<tr>
<td>*Xbra</td>
</tr>
<tr>
<td>Xdsh</td>
</tr>
<tr>
<td>Xwnt11</td>
</tr>
<tr>
<td>dom. neg. BMP receptor</td>
</tr>
<tr>
<td>*Xnr4</td>
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<td>*Xnr3</td>
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axes (Funayama et al., 1995; Guger and Gumbiner, 1995). Evidence to suggest that β-catenin is vital for the establishment of the dorso-ventral axis in \textit{Xenopus} embryos comes from experiments that show that depletion of maternal β-catenin transcripts from \textit{Xenopus} oocytes prevents the formation of a dorso-ventral axis (Heasman et al., 1994a). The dorsal deficiencies of such embryos can be rescued by ectopic expression of β-catenin mRNA (Heasman et al., 1994a).

β-catenin is a homologue of the \textit{Drosophila} protein Armadillo (Peifer et al., 1992). Armadillo is a component of the \textit{Drosophila} Wingless signal transduction pathway (Fig 1.3), suggesting that β-catenin may itself lie downstream of a \textit{Xenopus} Wnt (Xwnt) signal. In keeping with this, the dorsal deficiencies of β-catenin depleted embryos, that can be rescued by the injection of β-catenin mRNA, cannot be rescued by Xwnt-8, even though Xwnt-8 RNA has strong dorsalising activity in wild-type embryos (Heasman et al., 1994b). It is not known which endogenous Xwnt, if any, functions upstream of β-catenin. One possible candidate is Xwnt8b which is maternally expressed and has potent axis forming ability (Cui et al., 1995) (Table 1.1). A second Xwnt, Xwnt11, is maternally expressed with transcripts localised to the vegetal hemisphere. However it does not have strong axis forming capacity (Ku and Melton, 1993) (Table 1.1), which argues against a role for Xwnt11 upstream of β-catenin (Ku and Melton, 1993).

It is likely that the ability of β-catenin to alter gene expression and cell fate involves its interaction with LEF/Tcf class transcription factors (Behrens et al., 1996). One such factor, XTcf-3, is a ubiquitous protein in early \textit{Xenopus} embryos, and it has been shown to associate with β-catenin using the yeast two-hybrid system (Behrens et al., 1996; Molenaar et al., 1996). Injection of a dominant negative form of XTcf-3 into the dorsal side of \textit{Xenopus} embryos blocks the formation of the endogenous dorso-ventral axis and, moreover, blocks the ability of ectopic β-catenin to induce secondary axes (Molenaar et al., 1995), placing XTcf-3 alongside or downstream of β-catenin (Fig. 1.3). The \textit{Drosophila} LEF/Tcf family member \textit{pangolin} is required \textit{in vivo} for Wingless signal transduction. The LEF/Tcf homologue encoded by \textit{pangolin}
Figure 1.3. A comparison of the *Drosophila* and *Xenopus* Wnt signal transduction pathways (adapted from Heasman, 1997). Wnt signalling promotes the inactivation of zw3/GSK3, which leads to the accumulation of Armadillo/β-catenin. Armadillo/β-catenin may then interact with HMG-box transcription factors which regulate the expression of specific genes. ? indicates uncertain components. Although pangolin is depicted here in the nucleus, its positioning remains hypothetical at the moment.
interacts with Armadillo and functions downstream of Armadillo to transduce the Wingless signal (Fig 1.3) (Brunner et al., 1997).

The serine-threonine kinase zeste-white-3 (zw3) (Peifer et al., 1994) acts as an inhibitor of the Wingless pathway in Drosophila by altering the stability of soluble Armadillo (Pai et al., 1997). A dominant-negative form of the Xenopus homologue of zw3, GSK3, causes ectopic axis formation when expressed in Xenopus embryos, but is unable to rescue β-catenin-deficient embryos (Wylie et al., 1996). Thus, GSK3 appears to act in a similar upstream position to β-catenin as zw3 does to Armadillo in Drosophila (Fig 1.3) (Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1996). None of the endogenous signalling components that lie upstream of GSK3 and β-catenin are known.

It is not certain whether UV treatment of oocytes or embryos damages the GSK3/β-catenin pathway directly or indirectly, although there is evidence to suggest that the effects are indirect. Firstly, UV irradiation of the vegetal poles of fertilised eggs causes the localisation of Siamois, a transcription factor downstream of β-catenin, in the vegetal rather than the equatorial region of the embryo (Brannon and Kimelman, 1996). This supports the idea that UV irradiation of the egg disrupts cytoplasmic movements, rather than the GSK3/β-catenin pathway directly. Secondly, normal development can be restored in UV irradiated fertilised eggs by manual tipping before the first cell division (Scharf and Gerhart, 1980). This suggests that the dorsalising pathway is not irreparably damaged by UV treatment of fertilised eggs. Thirdly, like wild-type oocytes, UV-irradiated oocytes can be dorsalised (made to form exaggerated dorsal and anterior structures), by lithium treatment at the 32-cell stage, which again suggests that the dorsalising pathway is not irreparably damaged by UV treatment of the oocyte (Elinson and Pasceri, 1989).

Classical experiments have shown that the exposure of embryos, between early cleavage stages and MBT, to lithium ions causes dorsalisation (Kao et al., 1986; Kao and Elinson, 1989). Lithium has been thought to act by inhibiting the inositol 1, 4, 5-trisphosphate (IP$_3$) pathway as it inhibits the
enzyme inositol monophosphatase (Berridge et al., 1989). Indeed, there is evidence to suggest that the IP$_3$ pathway is involved in dorso-ventral patterning in *Xenopus* embryos (Busa and Gimlich, 1989; Kume et al., 1997). Recent evidence, however, suggests that dorsalisation caused by lithium treatment may be due to its effects on the GSK3/β-catenin pathway. Lithium inhibits the enzyme, GSK3, resulting in the accumulation of β-catenin and consequently causes dorsalisation (Klein and Melton, 1996; Stambolic et al., 1996).

**Mesoderm inducing factors**

A number of signalling molecules have been identified that have the capacity to respecify prospective ectodermal tissue to form mesoderm rather than ectoderm. These factors mimic the activity of endogenous mesoderm inducing factor(s) found in vegetal pole cells. They include members of the fibroblast growth factor (FGF) family and members of the TGF-β superfamily (Smith, 1995). The exact nature of the endogenous mesoderm inducing factor(s) remains obscure. However, the mesoderm inducing factors that have been identified provide useful tools to investigate how mesoderm is induced and patterned in early *Xenopus* embryos.

The best characterised mesoderm inducing factors are activin, a member of the TGF-β superfamily purified from medium conditioned by a *Xenopus* cell line, and FGF (Sive, 1993; Smith, 1993). Addition of either factor to isolated animal pole explants, which normally form ectodermal tissue, results in their differentiation into mesodermal cell types. These two factors exert different effects, with high concentrations of activin inducing dorsal and axial structures, and FGF inducing ventral tissues (Green et al., 1990). In terms of the three signal model, it did seem conceivable that FGF acts as the ventral vegetal inducing signal and activin as the dorsal vegetal inducing signal.

**FGF family members**

*Xenopus* embryos expressing a truncated FGF tyrosine kinase receptor, that acts in a dominant-negative manner, lack a notochord and posterior
mesoderm, indicating that FGF signalling is required in the formation of mesoderm in *Xenopus* embryos (Amaya et al., 1991; Amaya et al., 1993). This suggested that an FGF family member may be a mesoderm inducing factor, which was supported by the findings that FGF-2 and eFGF are both expressed maternally (Kimelman et al., 1988; Isaacs et al., 1992). However, when a dominant negative form of an activin receptor is ectopically expressed in *Xenopus* embryos, the resulting embryos contain no embryonic mesoderm at all (Hemmati-Brivanlou and Melton, 1992), even though animal caps expressing the same dominant negative activin receptor can still be induced to form ventral mesoderm by FGF (Hemmati-Brivanlou and Melton, 1992). This indicates that FGF does not require an intact activin signalling pathway to induce mesoderm. Together these data suggest that, in normal development, FGF is not a mesoderm inducing factor; if it were, embryos injected with truncated activin receptors should still be able to form the ventral mesoderm that FGF is capable of inducing.

Although FGF may not be acting as a mesoderm inducing factor, evidence suggests that FGF-signalling is required for normal mesoderm formation. Expression of a truncated FGF receptor prevents exogenous activin from inducing the full spectrum of mesodermal genes in animal caps (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994), suggesting that FGF may have a role in activin-mediated mesoderm induction *in vivo*.

A role for FGF signalling during gastrulation seems likely. Several FGFs are expressed during gastrulation (Isaacs et al., 1992; Tannahill et al., 1992), including eFGF which is expressed in the blastopore lip and later in the dorsoposterior mesoderm. Dissociated gastrula mesoderm cells cultured in the presence of eFGF retain mesodermal character that is otherwise lost in culture (Isaacs et al., 1992), suggesting that FGF is involved in maintenance of mesodermal cell types. More recent experiments indicate that FGF is involved in maintaining expression of the mesoderm specific gene, *Xenopus Brachyury* (Xbra), and in the maintenance of posterior mesodermal cell types (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). The most convincing evidence for this has come from experiments using transgenic *Xenopus* embryos that ubiquitously express a truncated FGF
receptor under the control of a CMV promoter which drives expression from the early gastrula stage. In these embryos, mesoderm-specific genes, like Xbra, were activated normally, but their expression was transient and had disappeared by the mid-gastrula stage (Kroll and Amaya, 1996). These transgenic embryos resembled those produced by injection of mRNA encoding a truncated FGF receptor into fertilised eggs, supporting the idea that FGF signalling is required for the maintenance and differentiation of mesoderm, rather than the induction of mesoderm.

It has been demonstrated that FGF exerts its effects through the Ras/ERK (extracellular signal regulated kinase) MAPK (mitogen-activated protein kinase) pathway (Gotoh et al., 1995; LaBonne et al., 1995; Umbhauer et al., 1995). This pathway serves to link signals from the cell surface to cytoplasmic and nuclear events. The Ras/ERK MAPK pathway is one example of what are generically termed 'MAPK' pathways. All such pathways have a three-component protein kinase cascade consisting of a serine/threonine protein kinase, MAPKKK (mitogen-activated protein kinase kinase kinase), which phosphorylates and activates a dual-specificity protein kinase, MAPKK (mitogen-activated protein kinase kinase), which in turn phosphorylates and activates another serine/threonine protein kinase, MAPK, which translocates to the nucleus and regulates transcriptional responses to growth factor stimulation (Fig. 1.4) (Marshall, 1995). In the Ras/ERK MAPK pathway Raf corresponds to MAPKKK, MEK corresponds to MAPKK and ERK corresponds to MAPK (Fig. 1.4) . The expression of active forms of MEK or MAPK in animal caps induces the formation of mesoderm of the kind elicited by FGF. Furthermore activated MEK, like FGF, induces different types of mesoderm in a dose-dependent manner (Green et al., 1992; Umbhauer et al., 1995). Expression of a Xenopus MAPK phosphatase, on the other hand, blocks the induction of mesoderm by FGF and prevents the formation of notochord and posterior mesoderm in whole embryos (Umbhauer et al., 1995). It appears that activation of MAPK (ERK kinase) is critical for all aspects of FGF induced ventral mesoderm formation.
Figure 1.4. The FGF signalling pathway. Schematic representation of the events that follow FGF binding to the tyrosine kinase FGF receptor. Ligand binding stimulates the activity of the tyrosine kinase receptor which activates ras by causing it to exchange GDP for GTP. Activated ras phosphorylates the serine/threonine kinase raf, which in turn phosphorylates MAP Kinase Kinase (MEK, for MAPK/ERK Kinase), which then phosphorylates MAP Kinase (ERK).
TGF-β superfamily members

Activin

As mentioned above, activin induces animal pole tissue of blastula stage *Xenopus* embryos to form mesoderm rather than ectoderm (Smith, 1995b) and ectopic expression of a dominant-negative activin receptor results in the development of embryos containing little or no mesoderm (Hemmati-Brivanlou and Melton, 1992). Together these data provided strong support for a role for activin as an endogenous mesoderm inducing factor. However, it has since been found that this particular truncated activin receptor does not specifically block activin signalling, it also blocks that of BMP and a processed form of Vg-1 (Kessler and Melton, 1994; Schulte-Merker et al., 1994a). Thus, TGF-β signalling is vital for the formation of mesoderm in *Xenopus* embryos, but the extent to which activin itself is involved cannot be assessed from these experiments. More recently, use of a new design of dominant-negative activin receptor that specifically inhibits activin, but not Vg-1 or any other known mesoderm inducer, suggests that activin has an essential role in early *Xenopus* development (Dyson and Gurdon, 1996). Embryos expressing this new design of dominant-negative activin receptor have reduced anterior structures, indicating that activin signalling is required for normal development. Looking at the time at which mesoderm is induced, expression of this dominant negative receptor slightly delays the onset of expression of the pan-mesodermal marker *Xenopus Brachyury*, and reduces initial expression of this gene to 20% of the wild-type level (Dyson and Gurdon, 1996), implicating activin in mesoderm induction. In keeping with this, activin protein is present in *Xenopus* eggs and early embryos (Fukui et al., 1994; Oda et al., 1995).

In conflict with a role for activin as an endogenous mesoderm inducing factor in *Xenopus* embryos, overexpression of the activin-binding protein follistatin, at levels sufficient to block the effects of ectopic activin administration, does not perturb mesoderm formation (Schulte-Merker et al., 1994a). If activin is to be considered as a candidate for an endogenous
mesoderm inducing factor it must be present in the embryo in a form inaccessible to follistatin. In addition to this, mouse embryos with homozygous mutations in both activin A and activin B form mesoderm normally (Matzuk et al., 1995; Smith, 1995a), suggesting that activin signalling is not required for mesoderm formation in the mouse, and, by extension, that it may not be required in any vertebrate embryo. Whether activin is required for mesoderm induction in *Xenopus* is still not known. However, if not activin itself, it does seem likely that an activin-like activity is required for mesoderm induction in *Xenopus* embryos.

**Concentration-dependent effects of activin**

One remarkable property of activin is that it is capable of inducing different mesodermal cell types at different concentrations. Low concentrations of activin induce the formation of ventral and posterior cell types, and activate the expression of ventral and posteriorly-expressed genes, such as *Xbra*, while high concentrations induce the formation of dorsal and anterior tissues and activate the expression of dorsal and anteriorly-expressed genes, such as *goosecoid* (Green and Smith, 1990; Green et al., 1992; Green et al., 1994; Gurdon et al., 1994; Symes et al., 1994; Wilson and Melton, 1994; Gurdon et al., 1995). This property of activin was first demonstrated in experiments using disaggregated-reaggregated animal cap explants (Green et al., 1994; Symes et al., 1994; Wilson and Melton, 1994). If dispersed animal caps are exposed to a wide range of concentrations of activin and analysed after only a few hours, genes such as *Xbra* are expressed over a wide range of concentrations. However, if cells are reaggregated and cultured to neurula stages, *Xbra* expression becomes refined to a narrow window of activin doses. Thus, precise interpretation of activin concentrations in disaggregated-reaggregated animal cap explants requires cell-cell interactions and takes some time to occur (Green et al., 1994; Symes et al., 1994; Wilson and Melton, 1994). It appears that a more direct and rapid interpretation of activin concentration is made in intact animal caps. When activin soaked beads are sandwiched between animal cap explants, cells that are close to the activin source, and therefore receive high doses of activin, express the dorso-
anterior marker goosecoid, whilst those that are further away activate Xbra (Gurdon et al., 1994; Gurdon et al., 1995; Jones et al., 1996a).

The simplest interpretation of these results is that a concentration gradient of activin is established in the responding animal cap explants, within which animal cap cells respond to predetermined thresholds of activin activity by expressing particular mesodermal markers and adopting particular cell fates (Fig. 1.5A). Alternatively it could be that the long-range effects of activin are mediated by a relay of short-range signals. For example, activin may diffuse over a short range to induce the expression of goosecoid, but a second signal released by these goosecoid-expressing cells is responsible for inducing Xbra in adjacent cells (Fig. 1.5B).

Recently there have been several lines of evidence to support the model that activin can act as a morphogen and does not initiate a cell-cell signalling relay in Xenopus tissues. Firstly, Gurdon et al. performed experiments in which non-competent cells (endoderm from a stage-40 tadpole) were inserted between activin mRNA-expressing vegetal tissue and responding animal tissue (Gurdon et al., 1994). These non-competent cells cannot induce Xbra in an animal cap sandwich, nor can they express Xbra in response to mesoderm inducing signals. However, an Xbra-inducing signal from the activin expressing vegetal tissue was transmitted successfully through the non-competent layer to the animal tissue (Gurdon et al., 1994). This result was not dependent on the non-competent cells synthesising a secondary inducing factor, since the same results were obtained in experiments in which the layer of non-competent cells was treated with the protein synthesis inhibitor cycloheximide (Gurdon et al., 1994). Secondly, it was found that a constitutively active activin receptor (ALK-4*), in the absence of ligand, was unable to generate long-range signalling in conjugated animal caps (Jones et al., 1996a). Thirdly, experiments using dominant negative forms of an activin receptor demonstrated that the most distant responses to activin require a functional activin signalling pathway (McDowell et al., 1997). Finally, it has been shown that activin can diffuse through solid tissue (McDowell et al., 1997). In this experiment beads coated with radiolabelled activin were sandwiched between two animal cap
Figure 1.5. Models for long-range signalling by activin (taken from Dale, 1997). (A) Morphogen gradient. In this model, activin diffuses throughout the layer of cells and forms a concentration gradient, with high levels close to the source and low levels distally. At the lowest level of activin, cells differentiate as epidermis (yellow cells), but above a prespecified concentration (threshold 1) they activate expression of $Xbra$ (red cells). As the concentration increases, a second prespecified value (threshold 2) is reached, and cells activate expression of $gsc$ (blue cells). While this same threshold may also repress $Xbra$, it is also possible that $Xbra$ is repressed by $gsc$. (B) Signal relay. There are many variants of this model but, in the one illustrated here, activin (signal 1) diffuses over a short range and induces $gsc$; activin also causes a second signal to be released (signal 2), which acts over a short range to induce $Xbra$ in adjacent cells. The essential feature of this model is that $Xbra$ is not induced by activin directly, but by a secondary signal initiated by activin.
explants and, after a few hours, the conjugates were sectioned and the
distribution of radiolabelled activin observed by autoradiography. It was
found that activin had formed a concentration gradient within the animal
cap, diffusing at least 120 μM (approximately seven cell diameters) away
from the source (McDowell et al., 1997). Together these data provide
compelling evidence that activin, or an activin-like activity, can diffuse
through solid tissue and signal over a long-range.

The mechanism by which cells distinguish between different concentrations
of activin is unknown. However recently there has been a gain in our
understanding of how cells sense their position in a morphogen gradient.
Dyson and Gurdon have shown that increasing occupancy of one single
receptor type with activin can cause the same kind of cell to express different
genes (Dyson and Gurdon, 1998). 2% receptor occupancy is sufficient to
induce the expression of Xbra, which is expressed in response to low doses of
activin, while a three fold increase in receptor occupancy causes a switch in
gene response, inducing genes like goosecoid, which is expressed in response
to high doses of activin (Dyson and Gurdon, 1998). A knowledge of the
regulation events that follow different levels of receptor occupancy will
provide further insight into the mechanism by which a cell interprets its
position in a morphogen gradient.

It appears that a gradient of an activin-like activity is not sufficient to
regulate the expression of all mesodermal markers in Xenopus embryos.
Studies on the goosecoid promoter have identified two response elements
in the 5' flanking sequence; one is responsive to activin-signalling and the
other to Wnt-signalling (Watabe et al., 1995), indicating that the expression
pattern of goosecoid is regulated by a combination of signals, rather than by a
morphogen gradient alone. Indeed, recent evidence suggests that co­
operation between activin and Wnt signalling pathways is required for
expression of organiser specific genes, such as Siamois, chordin and
goosecoid, but not that of pan-mesodermal markers such as Xbra (Crease et
al., 1998).
**Vg-1**

The TGF-β family member Vg-1 is the best candidate, to date, for an endogenous mesoderm inducing factor. The Vg-1 gene encodes a maternal mRNA that is localised to the vegetal region of the oocyte and early embryo (Rebagliati et al., 1985; Weeks and Melton, 1987). It is thus expressed at the right time and in the right place to act as an endogenous mesoderm inducing factor. However, it is not known if Vg-1 protein is secreted and processed *in vivo*.

TGF-β-like proteins are synthesised as inactive precursors which form disulphide-linked dimers and are proteolytically cleaved, releasing a mature C-terminal bioactive dimer (Massague et al., 1994). Endogenous Vg-1 accumulates as an unprocessed precursor and little or no mature Vg-1 has been detected in *Xenopus* embryos (Dale et al., 1989; Tannahill and Melton, 1989; Thomsen and Melton, 1993). Consistent with this, injection of embryos with Vg-1 mRNA produces high levels of precursor, but no processed protein and, consequently, neither mesoderm induction or developmental effects are observed (Dale et al., 1989; Tannahill and Melton, 1989; Dale et al., 1993; Thomsen and Melton, 1993). In contrast, chimeric proteins consisting of the pro-regions of BMP-2, BMP-4 or activin fused to the C-terminal mature region of Vg-1 have been shown to be biologically active (Fig 1.6) (Dale et al., 1993; Thomsen and Melton, 1993; Kessler and Melton, 1995).

Like activin, chimeric Vg-1 induces differentiation of dorsal, but not ventral mesodermal tissues, and the dose dependent activation of both dorsal and ventral mesodermal markers (Dale et al., 1993; Thomsen and Melton, 1993; Kessler and Melton, 1995). Furthermore, chimeric Vg-1 can restore a complete dorso-ventral axis in UV-ventralised embryos (Table 1.1) (Dale et al., 1993; Thomsen and Melton, 1993). Consistent with a role in mesoderm induction, the activin binding protein follisatin, which does not block endogenous mesoderm formation, does not interfere with chimeric Vg-1 signalling (Kessler and Melton, 1995). For Vg-1 to function as an endogenous mesoderm inducing factor, it remains to be determined whether, and if so,
Figure 1.6. Chimeric Vg1 constructs. Schematic representation of the TGF-β family members activin βB, BMP4 and Vg1, and chimeric BMP4-Vg1 and activin-Vg1. These proteins contain a signal sequence, a pro-region, tetrabasic cleavage site and mature region. Activin and BMP4, but not Vg1, form disulphide linked dimers that are subsequently cleaved, releasing the mature C-terminal peptide as a bioactive dimer. The chimeric constructs, fused downstream of the cleavage site, are designed to facilitate processing and secretion of mature Vg1.
how Vg-1 is processed in the early embryo and which regions, if any, contain active Vg-1 protein.

**Xenopus nodal-related 1-4**

Mouse embryos that are homozygous for a null mutation in the TGF-β gene *nodal* arrest development at early gastrulation and contain little or no embryonic mesoderm (Conlon et al., 1994). Four homologues of nodal have been isolated in *Xenopus*, *Xenopus nodal related 1-4* (Xnr1-4) (Jones et al., 1995; Smith et al., 1995; Joseph and Melton, 1997). It appears that none of these nodal-related genes encode proteins that are candidates for endogenous mesoderm inducing factors, since their expression begins after the onset of zygotic transcription. Nonetheless, Xnr1 and Xnr2, like nodal itself, are dose-dependent inducers of dorsal and ventral mesoderm in animal cap explants (Jones et al., 1995) and Xnr4 induces ventral mesoderm (Joseph and Melton, 1997). At gastrulation Xnr1-4 are all expressed in the marginal zone, with highest levels of expression on the dorsal side (Jones et al., 1995; Smith et al., 1995; Joseph and Melton, 1997). There are subtle differences in the expression patterns of these genes and in the effects of the proteins they encode (Table 1.1). However, all four proteins share the ability to dorsalise ventral mesoderm at the early gastrula stage (Jones et al., 1995; Smith et al., 1995; Joseph and Melton, 1997). Thus, the products of the nodal-related genes are good candidates for endogenous factors that regulate the dorsalisation of ventral mesoderm at gastrula stages. As yet, it is not possible to decide which of these genes, if any, plays the most important part in this process.

**Bone Morphogenetic Proteins (BMPs)**

The Bone Morphogenetic Protein family members BMP-2, BMP-4 and BMP-7 can induce animal pole tissue of blastula stage embryos to form ventral mesoderm (Dale et al., 1992; Jones et al., 1992). mRNAs encoding these factors are present maternally, but they are not localised in the egg or early blastula, which argues against a role for them as endogenous mesoderm inducing factors (Dale et al., 1993; Fainsod et al., 1994; Hemmati Brivanlou
and Thomsen, 1995). Of the three, BMP-4 has been most widely studied. Injection of mRNA encoding BMP-4 promotes the development of ventral mesoderm in *Xenopus* embryos. These ventralising effects override both endogenous mesoderm inducing signals and those produced by activin (Dale et al., 1992; Jones et al., 1992), suggesting that ventral mesoderm formation requires active signalling and is not simply a passive process. This contrasts with the ‘three signal model’ in which ventral mesoderm is viewed as a ‘ground state’ that is adopted by ventral and lateral sectors of the marginal zone in the absence of dorsalising signals (Sive, 1993).

In support of a role for BMP-4 in ‘ventralisation’, the gene is expressed in ventral and lateral marginal zones of the early gastrula and is absent from the organiser region (Fainsod et al., 1994; Schmidt et al., 1995). Furthermore, expression of a dominant negative BMP-4 receptor in *Xenopus* embryos causes loss of ventral structures and an increase in dorsal tissue (Graff et al., 1994; Maeno et al., 1994; Suzuki et al., 1994). Over-expression of BMP-4 does not affect the initial response to mesoderm induction, but does cause rapid down-regulation of organiser specific genes immediately after the onset of gastrulation (Jones et al., 1996b). This, together with the observation that embryos injected with BMP-4 cannot be rescued by grafts of Spemann’s organiser, demonstrates that BMP-4 can act at gastrula stages to promote ventralisation of *Xenopus* embryos (Jones et al., 1996b).

Since the discovery of the ventralising properties of BMP-4, a model for dorso-ventral patterning of *Xenopus* embryos has emerged which involves interactions between dorsal and ventral cells. The secreted proteins noggin and chordin are transcribed in the Spemann organiser region at gastrula stages. Like the *Xenopus* nodal-related proteins, these are dorsalising molecules, in that they can convert ventral mesodermal tissue into dorsal mesoderm when ectopically expressed on the ventral side of the embryo (Smith and Harland, 1992a; Sasai et al., 1994). As well as affecting the fate of equatorial cells, noggin and chordin also affect the differentiation of cells of the animal hemisphere. Both factors induce the differentiation of neural and endodermal tissue in animal pole explants, without causing the formation of mesoderm (Smith and Harland, 1992b; Sasai et al., 1995; Sasai et
al., 1996). It has been demonstrated recently that noggin and chordin interact directly with BMP-4 in vitro and can block the binding of BMP-4 to its receptor (Piccolo et al., 1996; Zimmerman et al., 1996), suggesting that these molecules may function in vivo as BMP-4 inhibitors.

Recent evidence suggests that the secreted metalloprotease Xolloid acts upstream of BMP receptor signalling, blocking the effects of chordin, but not noggin (Piccolo et al., 1997; Mullins, 1998). In biochemical studies, soluble Xolloid protein has protease activity, cleaving chordin at two specific sites and inactivating its BMP-antagonising activity (Piccolo et al., 1997). Digestion of an inactive complex of BMPs and Chordin with Xolloid results in the recovery of BMP biological activity. Furthermore, overexpression of a dominant negative form of Xolloid indicates that Xolloid protein activity is required in vivo for correct dorso-ventral patterning in Xenopus (Piccolo et al., 1997). Taken together, these results suggest a role for proteolytic processing in the establishment of different cell fates along the dorso-ventral axis.

Chordin shows strong sequence homology with the Drosophila gene short gastrulation, the product of which inhibits the function of decapentaplegic (dpp), a close relative of BMP-4 (François et al., 1994; Holley et al., 1995). Xolloid is a close relative of the Drosophila gene tolloid, which is a positive regulator of Dpp activity (see Mullins, 1998). Thus, it appears that there is remarkable conservation in the systems responsible for dorso-ventral patterning in insects and vertebrates.

**TGF-β signalling in early Xenopus development**

The TGF-β superfamily comprises a large number of secreted signalling polypeptides implicated in a diverse set of functions (Kingsley, 1994). As described above, TGF-β signalling is vital for patterning of the early Xenopus embryo. In fact, TGF-β superfamily members are required during development for morphogenesis, patterning and differentiation in vertebrates, Drosophila and C. elegans. In adult tissues they are involved in
Figure 1.7. Model for activin/TGF-β receptor activation. Type II receptors are constitutively active kinases capable of binding ligand alone. Type I receptors bind ligand co-operatively in the presence of type II receptors. Following ligand-induced activation of the two receptor classes, the Type II receptor phosphorylates the type I receptor on serine and threonine residues in a critical regulatory region, the GS domain. This phosphorylation event activates the type I receptor, which then mediates downstream signalling events.
processes such as tissue repair (Moulin, 1995; O’Kane and Ferguson, 1997) and bone and cartilage formation.

TGF-β family members act by binding to two transmembrane serine/threonine kinases, termed the type I and type II receptors (Heldin et al., 1997). These receptors are structurally similar, with small cysteine-rich extracellular regions and intracellular portions consisting mainly of the kinase domains. Current models, based on studies of the receptors for TGF-β, propose that ligand first binds to the constitutively active type II receptor serine/threonine kinase. Then, the type I receptor, which is unable to bind ligand in the absence of the type II receptor, is recruited into the complex (Fig 1.7). Following ligand-induced association of the two receptor classes, the type II receptor phosphorylates the type I receptors on serine and threonine residues in a critical regulatory region known as the GS domain, and this phosphorylation event activates the type I receptor which then mediates downstream signalling events (Fig 1.7) (Wrana et al., 1994a). Analysis of 125I-labelled TGF-β1 crosslinked to its receptors has suggested that the signalling complex is a heterotetramer consisting of two type I receptors and two type II receptors (Yamashita et al., 1994). This receptor activation model predicts that the type II and type I receptors act in sequence, which is supported by the findings that a single amino acid substitution in the GS domain of type I receptors results in constitutive ligand-independent activation of the downstream signalling cascade (Wieser et al., 1995; Attisano et al., 1996; Hoodless et al., 1996).

Seven different type I receptors have been characterised in vertebrate cells (Table 1.2), and I am going to focus on two of these, ALK-2 (ActR-I) and ALK-4 (ActR-IB) (Attisano et al., 1993; Carcamo et al., 1994; ten Dijke et al., 1994; Tsuchida et al., 1996). Both of these receptors have been shown to bind activin in tissue culture binding assays, and they are both expressed in the early Xenopus embryo (Kondo et al., 1996; Chang et al., 1997). It has been demonstrated that constitutively active forms of ALK-2 and ALK-4, termed ALK-2* and ALK-4*, induce prospective ectodermal tissue to form mesoderm, but that the two receptors elicit different responses (Armes and Smith, 1997). The effects of ALK-4* resemble those of activin, in that it can,
Table 1.2. TGF-β family members, their receptors and signalling molecules (adapted from Heldin et al., 1997). Note the non-specificity of ligands, type I and type II receptors.

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>TGF-β</th>
<th>Activin</th>
<th>BMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Examples of ligands</td>
<td>TGF-β 1</td>
<td>Activin A</td>
<td>BMP-2</td>
</tr>
<tr>
<td></td>
<td>TGF-β 2</td>
<td></td>
<td>BMP-4</td>
</tr>
<tr>
<td></td>
<td>TGF-β 3</td>
<td></td>
<td>BMP-7</td>
</tr>
<tr>
<td>Type II receptors</td>
<td>TβR-II</td>
<td>ActR-II</td>
<td>BMPR-II</td>
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<tr>
<td></td>
<td></td>
<td>ActR-IIB</td>
<td>ActR-II</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>ActR-IIB</td>
</tr>
<tr>
<td>Type I receptors</td>
<td>ALK-5/TβR-I</td>
<td>ALK-2/ActR-I</td>
<td>ALK-3/BMPR-IA</td>
</tr>
<tr>
<td></td>
<td>ALK-4/ActR-IB</td>
<td></td>
<td>ALK-6/BMPR-IB</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ALK-2/ActR-I</td>
</tr>
<tr>
<td>Pathway-restricted SMADs</td>
<td>Smad2</td>
<td>Smad2</td>
<td>Smad1</td>
</tr>
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<td>Smad3</td>
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<td>Smad8</td>
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</tbody>
</table>
in a dose dependent manner, induce expression of the general mesodermal marker \textit{Xbra} (Smith et al., 1991) as well as the dorsoanterior marker \textit{goosecoid} (Cho et al., 1991). The response to ALK-2* resembles BMP-4 (Dale et al., 1992; Jones et al., 1992), in that it induces only ventral markers and causes ventralisation of intact embryos. In addition to this ALK-2* is capable of counteracting the dorsalising effects of ALK-4* (Fig 1.8), an activity reminiscent of the ability of BMP-4 to ventralise the response to activin (Dale et al., 1992; Jones et al., 1992). However, ALK-2* and BMP-4 differ in their ventralising effects because the action of ALK-2* is immediate (Armes and Smith, 1997), whilst that of BMP-4 is delayed (Jones et al., 1996b).

Experiments to identify the regions of ALK-2* and ALK-4* that control receptor signalling specificity have implicated the loop between kinase subdomains IV and V (the \(\beta_4-\beta_5\) loop or IB1) in mediating the strong dorsal gene inducing properties of ALK-4*. The 7 amino acids that make up this loop are capable, when transferred from ALK-4* to ALK-2*, of carrying with them the ability to induce dorsal markers (Armes et al., 1998). An analogous result has been obtained recently using the TGF-\(\beta\) receptor T\(\beta\)R1/ALK-5 and TskL/ALK2 in a tissue culture system (Feng and Derynck, 1997). Similarly, a chimeric receptor with the reverse transfer of the \(\beta_4-\beta_5\) loop from ALK-2* to ALK-4* retains the ability to induce \textit{Xbra} and loses the ability to induce dorsal genes (Armes et al., 1998). Experiments presented in Chapter 3 investigate whether the \(\beta_4-\beta_5\) loop of ALK-2 is capable, when transferred from ALK-2* to ALK-4*, of counteracting the dorsalising effects of ALK-4*, or if another region(s) of ALK-2* is required to elicit this effect.

The \(\beta_4-\beta_5\) loop of ALK-4* is necessary for the induction of dorsal genes in the context of ALK-2*/ALK-4* chimeric receptors (Armes et al., 1998). However, it is not always sufficient to mediate dorsal gene induction in the context of these chimeric receptors (Armes et al., 1998), suggesting that other regions of ALK-4* are also involved in controlling ALK-4* signalling specificity. This is investigated further in Chapter 4.
Figure 1.8. ALK-2* counteracts the dorsalising effects of ALK-4*. The ventralising effects of ALK-2* are immediate, therefore it must block ALK-4* dorsal inducing signals either by targeting the ALK-4* downstream signalling cascade, or ALK-4* directly.
**Smads: mediators of TGF-β family signalling**

The discovery of the Smad proteins two years ago has provided a breakthrough in the understanding of how signals are transduced from activated type I serine/threonine kinase receptors to the nucleus. The product of the *Drosophila* gene *Mothers against decapentaplegic* (*Mad*), which was identified as being required for signalling by the BMP homologue Decapentaplegic (*Dpp*), is the founder member of the Smad family (Raftery et al., 1995; Sekelsky et al., 1995; Newfeld et al., 1996; Wiersdorff et al., 1996). The related *Sma-2, Sma-3* and *Sma-4* genes of *C. elegans* are required for dauer larva formation, their mutant phenotypes resembling that resulting from mutation of *daf-4*, a type II TGF-β receptor (Savage et al., 1996). At least nine genes homologous to *Mad* and *sma* have been identified in vertebrates and these have been termed Smads (for references see Heldin et al., 1998).

The Smad family can be divided into three subgroups on the basis of structural and functional criteria. The first group comprises those Smads that are direct substrates of the TGF-β family receptor kinases. The second group consists of Smads that are not direct receptor substrates, but participate in signalling by associating with receptor-activated Smads. The third group includes proteins that inhibit Smad activation and are known as anti-Smads.

Current models propose that, following activation of type I-type II receptor complexes in response to TGF-β ligand stimulation, receptor regulated Smads associate with type I receptors, are phosphorylated on specific serine residues and released from the type I receptor to form a stable complex with a common-mediator Smad (Fig 1.9). This complex translocates from the cytoplasm to the nucleus where it regulates transcriptional responses to TGF-β ligands. An additional level of control is achieved by anti-Smads which appear to inhibit TGF-β signalling by preventing the activation of pathway-restricted Smads. Evidence that has led to the formation of this model is described below (for reviews see Kretzschmar and Massague, 1998; Heldin et al., 1997).
Figure 1.9. Model showing positive and negative regulation of TGF-β/Smad signalling (adapted from Kretzschmar, 1998), see text for details.
Pathway-restricted Smads

Different members of the Smad family have different roles in signalling. Smad2 and Smad3, which are structurally very similar, are phosphorylated and translocated to the nucleus after stimulation by TGF-β or activin (Chen et al., 1996b; Eppert et al., 1996; Zhang et al., 1996; Nakao et al., 1997b). Smad1, and presumably its close homologues Smad5 and Smad8, are phosphorylated and translocated into the nucleus after stimulation by BMP-2 or BMP-4 (Hoodless et al., 1996; Liu et al., 1996; Kretzschmar et al., 1997b). Thus, different Smads become phosphorylated by specific type I receptors and thereby act in a pathway-restricted fashion.

The first indication that different Smads elicit different effects came from experiments in which *Xenopus* Smad proteins were ectopically expressed in *Xenopus* animal pole explants. Like BMPs and ALK-2*, Smad1 and Smad5 induce ventral mesoderm (Graff et al., 1996; Suzuki et al., 1997). *Xenopus* Smad2, like activin and ALK-4*, acts in a concentration-dependent manner to induce the expression of dorsal genes (Graff et al., 1996). Smad proteins are well conserved across species, for example, *Drosophila* Mad (Newfeld et al., 1996) and rat Smad8 (Chen et al., 1997b) have ventralising activity, like Smad1, and mouse and human Smad2 have dorsalising activity (Baker and Harland, 1996; Eppert et al., 1996), like *Xenopus* Smad2. In these overexpression experiments receptor regulated Smads somehow bypass the requirement for receptor activation to elicit transcriptional responses.

Analysis of mouse embryos that are homozygous null for Smad2 show that Smad2 signals are essential for mesoderm formation and the establishment of anterior-posterior polarity (Nomura and Li, 1998; Waldrip et al., 1998). Nomura and Li report the complete absence of mesoderm in Smad2 deficient mice (Nomura and Li, 1998), and Waldrip et al. see extraembryonic mesoderm without formation of embryonic mesoderm (Waldrip et al., 1998). These differences in phenotype may be due to differences in genetic background or in the strategy used by the two groups to target the *Smad2* gene for deletion. Smad2 is also required to establish anterior-posterior polarity, probably because of its function in restricting the site of primitive-
streak formation (Waldrip et al., 1998). (In mice, gastrulation begins with the formation of the primitive streak – a localised thickening of the epiblast occurs, marking out the posterior end of the mouse embryo. As gastrulation proceeds, the primitive streak extends anteriorly and epiblast cells move through the primitive streak to form mesoderm). Thus, Smad2 signalling is one of the earliest-known events required for mesoderm formation and anterior-posterior patterning in the mouse. Given that overexpression of Smad2 in *Xenopus* animal pole explants induces the differentiation of mesodermal tissue, it appears that critical aspects of Smad2 function are shared between frogs and mammals.

Pathway-specific Smads bind directly to type I receptors and this has been demonstrated by co-immunoprecipitation of Smad2 or Smad3 with type I-type II receptor complexes affinity crosslinked with $^{125}$I labelled TGF-β (Macias Silva et al., 1996; Zhang et al., 1996; Nakao et al., 1997b). This interaction requires ligand-induced activation of the receptor complex, but is only observed with the kinase inactive form of ALK-5/TβR-1; interaction is not detectable with ALK-5/TβR-1. This is likely to be due to the transient nature of the interaction between pathway-restricted Smads and wild-type receptors. Phosphorylation of pathway-restricted Smads by type I receptors triggers their activation. Smad1 is phosphorylated directly *in vitro* by highly purified bacterially expressed ALK-6/BMPR-1B (Kretzschmar et al., 1997b), Smad2 is phosphorylated by immunoprecipitated TGF-β receptor complexes (Macias Silva et al., 1996), and Smad3 is phosphorylated by an ALK-5/TβR1 kinase domain preparation (Zhang et al., 1996). Phosphorylation of Smad1 and Smad2 *in vivo* and *in vitro* occurs at the two most C-terminal serine residues of the C-terminal motif SSXS, which is present in all pathway-restricted Smads (Macias Silva et al., 1996; Kretzschmar et al., 1997b). Mutation of these C-terminal serines causes Smad2 to bind stably to type I receptors and to elicit dominant negative effects (Macias Silva et al., 1996). Mutation of these residues in Smad 1 and Smad2 not only abolishes their ligand-induced phosphorylation (Macias Silva et al., 1996; Kretzschmar et al., 1997b), but prevents the association of these Smads with the common mediator Smad, Smad4 (see below) (Kretzschmar et al., 1997b), and their
subsequent accumulation in the nucleus (Macias Silva et al., 1996; Kretzschmar et al., 1997b). Furthermore, it prevents ligand induced activation of transcriptional responses via Smad1 or Smad2 (Kretzschmar et al., 1997b). Taken together, these data show that receptor-regulated Smad proteins directly transmit TGF-β family signals from activated type I-type II receptor complexes into the nucleus (Fig. 1.9).

Common-mediator Smads

Smad4 is the only common-mediator Smad identified in vertebrates to date. It was first identified as the product of the deleted in pancreatic carcinoma locus 4 (DPC4), a tumour suppressor gene that is mutated in a high proportion of pancreatic cancers and a smaller proportion of other cancers (Hahn et al., 1996). Smad4 homologues are present in Drosophila (Padgett et al., 1997) and C. elegans (Sma-4) (Savage et al., 1996).

Although Smad4 is similar in overall structure to the pathway-restricted Smads, it lacks the C-terminal SSXS motif. Consistent with this, Smad4 does not bind to, nor is it phosphorylated by, TGF-β or BMP receptors (Lagna et al., 1996; Macias Silva et al., 1996; Zhang et al., 1996; Nakao et al., 1997b). It has been reported, however, that Smad4 is phosphorylated in response to activin stimulation (Lagna et al., 1996). The functional significance of this is not yet clear.

Following ligand stimulation and phosphorylation of pathway-restricted Smads, Smad4 forms hetero-oligomers with receptor-activated Smads, and these complexes translocate to the nucleus and activate transcriptional responses (Fig 1.9)(Lagna et al., 1996; Zhang et al., 1996; Wu et al., 1997). In mammalian cells Smad4 complexes with Smad1, and possibly Smad5 and Smad8 as well, after activation of BMP type I receptors (Lagna et al., 1996; Kretzschmar et al., 1997b), whereas it forms complexes with Smad2 and Smad3 after activation of TGF-β or activin type I receptors (Lagna et al., 1996; Nakao et al., 1997b; Wu et al., 1997). In unstimulated cells, Smad4 forms homo-oligomers (Lagna et al., 1996).
Expression of Smad4 in *Xenopus* animal pole explants induces the formation of mesoderm (Lagna et al., 1996; Zhang et al., 1997). BMPs induce the formation of Smad4-Smad1 hetero-oligomers and activin induces the formation of Smad4-Smad2 hetero-oligomers. Consistent with this, Smad4 synergises with Smad1 to induce more ventral mesoderm in *Xenopus* animal caps than is induced by Smad1 alone, and Smad4 synergises with Smad2 to induce more dorsal mesoderm than is induced by Smad2 alone (Zhang et al., 1997). It appears that maximum transcriptional effect of pathway-restricted Smads requires their co-operation with Smad4 (Lagna et al., 1996; Zhang et al., 1996; Nakao et al., 1997b).

The involvement of Smad4 in a broad range of functions has been suggested by experiments that indicate that Smad4 is required for certain TGF-β responses in mammalian cells (Lagna et al., 1996; Zhang et al., 1996), and in experiments in *Xenopus* embryos using a truncated form of Smad4 (ΔSmad4) (Lagna et al., 1996). In *Xenopus* animal pole explants, ΔSmad4 blocks the induction of dorsal mesoderm by Smad2 and ventral mesoderm by Smad1. ΔSmad4 also slightly reduces induction of ventral mesodermal markers in animal caps in response to activin (Lagna et al., 1996).

Smad4 deficient mouse embryos have reduced size, fail to gastrulate and show abnormal visceral endoderm development (Sirard et al., 1998). However, aggregation of mutant ES cells with wild-type tetraploid morulae rescues the gastrulation defect (Sirard et al., 1998), indicating that Smad4 is initially required for the differentiation of the visceral endoderm and that the gastrulation defect is secondary and non-cell autonomous. Rescued embryos show severe anterior truncations indicating a second role for Smad4 in anterior patterning during embryogenesis (Sirard et al., 1998). However, unlike Smad2 (Waldrip et al., 1998), Smad4 does not appear to be involved in anterior-posterior axis formation in mouse embryos, providing evidence for the existence of Smad4-independent signalling by pathway-specific Smad proteins.
Smad protein domains and their function

Both structurally and functionally, Smad proteins consist of three domains: the highly conserved N-terminal and C-terminal domains, known as MH1 and MH2 respectively, which are separated by a more divergent linker region (Fig. 1.10) (for reviews see Kretzschmar and Massague, 1998; Heldin et al., 1997). As well as containing the SSXS motif, the MH2 domain of pathway-restricted Smads appears to act as an effector domain. As such, when fused to a GAL4 DNA binding domain, the MH2 domain is sufficient to induce a transcriptional response (Liu et al., 1996), and, when expressed in Xenopus animal pole explants, the MH2 domain of Smad2 induces the full range of activin responses in the absence of the MH1 domain (Baker and Harland, 1996). In addition to this, the MH2 domains of Smad1, Smad2, Smad3 and Smad4 mediate homomeric interactions and receptor-activation-induced interactions between these pathway-restricted Smads and Smad4 (Hata et al., 1997; Wu et al., 1997; Zhang et al., 1997). In Smad2, the MH2 domain is also responsible for interaction with the transcription factor FAST-1 (Chen et al., 1997a) (see below).

Two different activities have been ascribed to the MH1 domain. Firstly, in the basal state, the MH1 domain inhibits the effector functions of the MH2 domain (Baker and Harland, 1996; Liu et al., 1996). The MH1 domains of Smad2 and Smad4, for example, prevent the association of their corresponding MH2 domains (Hata et al., 1997). This inhibition is achieved as a result of a direct physical interaction of the MH1 and MH2 domains (Hata et al., 1997). It appears that phosphorylation of the C-terminal SSXS motif in pathway-restricted Smads removes this inhibition. Secondly, it has been demonstrated that the MH1 domain of Drosophila MAD has specific DNA binding activity which is required for Dpp-induced activation of an enhancer within the vestigial wing-patterning gene (Kim et al., 1997). Interestingly, binding of Mad to the vestigial enhancer is only observed when its MH2 domain has been removed (Kim et al., 1997). Thus, for binding of the Mad MH1 domain to the vestigial enhancer, the MH2 domain appears to have a repressor function.
Figure 1.10. Different functional domains in Smads (adapted from Heldin et al., 1997). The conservation of MH1 and MH2 domains in Smads suggests that these domains may have similar functions in different members of the Smad family, but it remains to be shown to what extent observations made on individual members can be generalised.
Transcriptional regulation by Smads

TGF-β family members mediate a wide variety of effects by eliciting transcriptional responses in many target genes. In addition to the specific DNA-binding activity of the MH1 domain of Drosophila Mad that is required for Dpp-induced activation of an enhancer within the vestigial gene (Kim et al., 1997)(see above), studies on the activation of Xenopus Mix.2 has provided strong evidence for a function of Smads as transcriptional modulators in response to TGF-β signalling. The homeobox gene Mix.2 is expressed as an immediate early response to activin-like members of the TGF-β superfamily (Chen et al., 1996a); that is, expression of Mix.2 in response to such factors does not require new protein synthesis. Transcriptional activation of Mix.2 in response to activin is associated with the formation of the activin response factor (ARF), a protein complex with specific DNA binding activity that recognises a 50 bp regulatory element, the ARE, in the Mix.2 promoter (Chen et al., 1996a). The first component of the ARF to be identified was a novel winged-helix transcription factor, Forkhead-like activin signal transducer-1 (FAST-1), that was identified by its ability to interact with a hexanucleotide repeat present in the ARE (Chen et al., 1996a). FAST-1 is a nuclear protein that associates with Smad2 in response to activin stimulation (Chen et al., 1996a). The association with FAST-1 is mediated through the MH2 effector domain of Smad2 (Chen et al., 1997a; Liu et al., 1997).

It has recently been demonstrated that Smad4 is essential for the transcriptional activity of the ARE (Liu et al., 1997). Gel mobility shift experiments have shown that FAST-1, Smad2 and Smad4 are all part of the ARF that is formed in response to TGF-β or activin treatment (Chen et al., 1997a; Liu et al., 1997). Smad4 is not required for nuclear translocation of receptor-activated Smad2; rather it has a dual role in the nucleus as a component of the ARF. The MH1 domain of Smad4 promotes binding of the Smad2/Smad4/FAST-1 complex to DNA, and its MH2 domain provides an activation function that is required for Smad2 to stimulate transcription (Liu et al., 1997).
In addition to the ARE of Mix.2, activin response elements have been identified for several other genes that are expressed in *Xenopus* animal caps in response to activin in a protein synthesis-independent manner. These include *goosecoid* (Watabe et al., 1995) and XFKH1/XFD1', a pseudo allele of *Pintallavis* (Kaufmann et al., 1996; Howell and Hill, 1997). The activin response elements of these genes bear little sequence similarity to each other or to that of Mix.2, suggesting that different Smad-containing transcriptional complexes, which show different DNA-binding specificities, might be formed in response to activin stimulation.

**Inhibitory Smads**

Identification of the inhibitory Smads (Smad6 and Smad7 and the *Drosophila* protein Dad) demonstrated that TGF-β superfamily members are both positively and negatively regulated by members of the Smad family. Smad6 and Smad7 are the most divergent of the vertebrate Smads and, like Smad4, they lack the C-terminal SSXS phosphorylation motif (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al.; 1997a; Hata et al., 1998). Smad6 and Smad7 have an overall domain structure typical of Smads, with MH1 domains that are highly divergent from other Smads (Hayashi et al., 1997; Nakao et al., 1997a; Hata et al., 1998).

Analysis of Smad7 function has demonstrated that it can block TGF-β-mediated responses in mammalian cells (Hayashi et al., 1997; Nakao et al., 1997a), and activin/TGF-β signalling in *Xenopus* embryos (Nakao et al., 1997a). Smad7 appears to exert its inhibitory effect by interacting stably with activated TGF-β receptor complexes, thereby blocking the association, phosphorylation and activation of Smad2 and Smad3 (Hayashi et al., 1997; Nakao et al., 1997a). As a consequence of this, active heteromeric complexes between these pathway specific Smads and Smad4 are not formed.

Smad6 has been shown to associate with a number of different type I receptors, including TGF-β, activin and BMP receptors, when overexpressed in mammalian cells (Imamura et al., 1997; Hata et al., 1998). Smad6 overexpression has also been shown to block Smad2 (but not Smad3) and
Smad1 phosphorylation mediated by the TGF-β and BMP receptors, respectively (Imamura et al., 1997), and this has led to a model in which Smad6 blocks signalling in response to a variety of TGF-β ligands. However, in contrast to this, results have been obtained in experiments with Xenopus embryos that show Smad6 exerting a very specific inhibition of BMP signalling, but not of TGF-β or activin signalling (Hata et al., 1998). When expressed in Xenopus embryos, the effects of Smad6 resemble those of the secreted Spemann organiser proteins noggin and chordin on BMP4 (see earlier). Firstly, Smad6 dorsalises ventral mesoderm when expressed on the ventral side of 8 cell-stage embryos (Hata et al., 1998), and secondly, when expressed in animal pole explants, it induces the differentiation of neural tissue (Hata et al., 1998). Studies in mammalian cells have also suggested an alternative mechanism of inhibition in which Smad6 specifically competes with Smad4 for binding with receptor-activated Smad1, yielding an apparently inactive Smad1-Smad6 complex (Hata et al., 1998). It remains to be determined which of these two models of Smad6 action is most physiologically relevant (Fig 1.9).

As with Smad6 and Smad7, overexpression of Dad in Drosophila blocks Dpp and activated Mad signalling (Tsuneizumi et al., 1997), suggesting that Dad may directly interfere with the function of Mad.

Smad6 and Smad7 are induced in vascular endothelial cells in response to laminar fluid shear stress (Topper et al., 1997). Expression of Smad7 mRNA is also induced in response to TGF-β treatment of cells (Nakao et al., 1997a), and Dad expression is upregulated in response to Dpp in Drosophila (Tsuneizumi et al., 1997). Thus, inhibitory Smads may act as autoregulatory negative-feedback signals in response to TGF-β signals.

**Inhibition of Smads by MAP kinase pathways**

Another mechanism of regulation of the BMP/Smad1 pathway was identified following the discovery of in vivo phosphorylation sites other than those targeted by typeI-typeII receptor complexes (Kretzschmar et al., 1997a). Mitogenic growth factors which signal via receptor tyrosine kinases
(RTKs) and the ERK MAP kinase pathway, such as epidermal growth factor (EGF) or hepatocyte growth factor (HGF), rapidly induce phosphorylation of Smad1 at multiple serines in the linker region of the protein (Kretzschmar et al., 1997a). This phosphorylation is catalysed directly by the ERK MAP kinases in vitro and in vivo and it occurs independently of receptor-mediated C-terminal phosphorylation (Kretzschmar et al., 1997a). ERK-mediated phosphorylation specifically inhibits nuclear accumulation of Smad1 and this inhibition is observed even in the presence of BMP receptor activation (Kretzschmar et al., 1997a). Thus, BMP/Smad1 responses which depend on nuclear accumulation and transcriptional activity of Smad1, can be antagonised by mitogenic growth factors through the ERK MAP kinase pathway (Fig. 1.9) (Kretzschmar et al., 1997a). Growth factor/RTK-mediated phosphorylation has also been observed for the TGF-β/activin mediators Smad2 and Smad3 (M Kretzschmar, J. Massague, unpublished data; see (Kretzschmar et al., 1997b)). Therefore, it seems possible that Smad regulation by ERK MAP kinases may be a general phenomenon controlling signalling by all members of the TGF-β superfamily.

**Receptor-interacting proteins**

In addition to Smads, a number of other proteins have been shown to associate with the intracellular domains of TGF-β family type I receptors. The FK506-binding immunophilin FKBP12 interacts with unstimulated TβR-1/ALK-5 and other type I receptors (Charng et al., 1996; Wang et al., 1996b; Chen et al., 1997c). It does not mediate TGF-β signalling, rather it inhibits it. FKBP12 binds to a Leu-Pro sequence in the GS domain of type I receptors (Fig. 1.9) (Charng et al., 1996; Chen et al., 1997c), and counteracts phosphorylation of type I receptors by type II receptors (Wang et al., 1996b). It is released from type I receptors following ligand-induced receptor activation (Wang et al., 1996b). Thus, FKBP12 prevents spontaneous ligand-independent activation of TβR-1/ALK-5 by TβR-II.

The α-subunit of farnesyl transferase (FT-α) has also been shown to interact with the cytoplasmic domain of TGF-β family type I receptors (Kawabata et al., 1995; Ventura et al., 1996; Wang et al., 1996a). Farnesyl transferase plays a
critical role in the activation of p21\textsuperscript{RAS} by attaching a farnesyl group and aiding its membrane association. FT-\(\alpha\) has been shown to be phosphorylated by T\(\beta\)R-1/ALK-5 (Wang et al., 1996a), but the physiological relevance of this is unclear.

**Other mediators of TGF-\(\beta\) signalling**

Another pathway that acts downstream of TGF-\(\beta\) signalling involves TAB1 (Shibuya et al., 1996), a protein that regulates the activity of TAK1 (Yamaguchi et al., 1995), which functions as a MAPKKK. It has been demonstrated that TAK1 is involved in at least one TGF-\(\beta\)-induced transcriptional response, and its kinase activity is stimulated in response to TGF-\(\beta\) or BMP4 (Yamaguchi et al., 1995). The *Xenopus* homologues of TAK1 and TAB1, xTAK1 and xTAB1 respectively, induce differentiation of ventral mesoderm in *Xenopus* animal pole explants (Shibuya et al., 1998). Furthermore, expression of a ‘kinase dead’ version of xTAK1 reduces mesoderm induction by Smad1, Smad5 and BMP4 (Shibuya et al., 1998). Thus, xTAK1 and xTAB1 may function in the BMP signal transduction pathway in *Xenopus* embryos in co-operation with Smad signalling.

**Xenopus* Brachyury**

In addition to the search for endogenous mesoderm inducing factors and the elucidation of signal transduction pathways that are required for mesoderm formation, much can be learnt about the induction and patterning of mesoderm by studying the target genes of mesoderm inducing factors. One example is *Brachyury* (or *T*), which is particularly interesting for several reasons. Firstly, *Brachyury* is expressed throughout the mesoderm at the onset of gastrulation in mouse, zebrafish, *Xenopus* and chick embryos (Fig 1.11) (Herrmann et al., 1990; Wilkinson et al., 1990; Smith et al., 1991; Schulte-Merker et al., 1992; Kispert et al., 1995b; Knezevic et al., 1997). As gastrulation proceeds, transcripts are lost from prospective lateral and ventral mesoderm, but persist in the notochord and posterior mesoderm (Fig 1.11). Secondly, loss of functional *Brachyury* protein results in the loss of the tissues in which *Brachyury* is expressed at the highest levels for the
Figure 1.11. Whole mount *in situ* hybridisation showing expression of *Xbra* in the marginal zone of an early gastrula (left hand side) and in the notochord and posterior domains of a late gastrula (right hand side).
longest time. Thus, the mouse T mutant and the zebrafish no tail mutant, that both lack Brachyury function, characteristically lack a notochord and posterior mesodermal structures (Herrmann et al., 1990; Schulte-Merker et al., 1994b). In Xenopus, overexpression of a dominant-negative Brachyury construct produces a similar phenotype (Conlon et al., 1996).

Finally, mis-expression of the Xenopus homologue of Brachyury, Xbra, in Xenopus animal pole explants induces the differentiation of mesoderm (Cunliffe and Smith, 1992). In the same way that increasing amounts of FGF causes the formation of different types of mesoderm in prospective ectoderm (Green et al., 1992), low concentrations of Xbra mRNA induce the formation of ventral mesoderm, while high concentrations induce dorsal tissues including muscle (Cunliffe and Smith, 1992). Although Xbra is expressed in, and is required for, notochord formation, expression of Xbra alone in animal pole explants is not sufficient to induce notochord. However, notochord is formed if Xbra is co-expressed with the secreted protein noggin (Smith and Harland, 1992b), which inhibits BMP signalling (Zimmerman et al., 1996), or with the transcription factor Pintallavis, a homologue of HNF-3β (Ruiz i Altaba and Jessell, 1992; O’Reilly et al., 1995). Together, these results indicate that Brachyury plays a key role in vertebrate mesoderm formation.

**Activation of Xbra expression**

The mesoderm inducing factors activin and FGF, and components of their signal transduction pathways, can activate expression of Xbra in an immediate early manner (Smith et al., 1991). In the case of activin, overexpression of constitutively active type I activin receptors, notably ALK-4*, strongly activates expression of Xbra in animal pole explants (Jones et al., 1996a; Armes and Smith, 1997), as do some of the receptor-regulated members of the Smad family (Graff et al., 1996).

Experiments mentioned earlier suggest that signalling through the ERK MAP kinase pathway is critical for the formation of mesoderm and activation of Xbra expression by FGF (Fig 1.4) (Gotoh et al., 1995; LaBonne et
al., 1995; Umbhauer et al., 1995). Expression of active forms of p21RAS or MEK1 or MAP kinase is sufficient to activate expression of Xbra in prospective ectodermal tissue, while inhibition of MAP kinase signalling prevents induction of Xbra in response to FGF and upstream components of the MAP kinase pathway (Gotoh et al., 1995; LaBonne et al., 1995; Umbhauer et al., 1995).

**Xbra is a transcriptional activator**

Brachyury is the founder member of the T-box family of transcription factors. The N-terminal region of Brachyury defines a conserved domain known as the T-box (Bollag et al., 1994; Herrmann, 1995; Kavka and Green, 1997; Smith, 1997). Brachyury is a nuclear protein (Schulte-Merker et al., 1992; Cunliffe and Smith, 1994; Kispert and Herrmann, 1994), and binding-site selection experiments demonstrated that the T-box binds to a 20 bp palindromic sequence (Kispert et al., 1995a). More recently it has been shown that Xbra can bind to a non palindromic site that is identical to half of the 20 bp palindromic Brachyury site previously identified (Casey et al., 1998).

Brachyury is capable of activating transcription. Deletion analysis of mouse Brachyury revealed two transcription activation domains and two repressor domains in the C-terminal half of the protein (Kispert et al., 1995a), while similar experiments with Xenopus and zebrafish Brachyury only identified a single activation domain (Conlon et al., 1996). More detailed analysis is necessary to determine whether these additional domains are present in Xenopus or zebrafish Brachyury.

The Brachyury activation domain is absent in two zebrafish Brachyury mutants suggesting that it is required for Brachyury function. This was tested in Xenopus using an Xbra construct in which the activation domain of Xbra is replaced by the repressor domain of the Drosophila engrailed protein (creating Xbra-EnR) (Conlon et al., 1996). Transient transfection experiments confirmed that this Xbra-EnR construct efficiently inhibits transcriptional activation caused by Xbra (Conlon et al., 1996). Furthermore, overexpression of this fusion protein in Xenopus and zebrafish embryos
resulted in a phenotype that resembled the genetic mutants of \textit{Brachyury} in mouse and zebrafish (Conlon et al., 1996). Thus, the function of Brachyury during vertebrate gastrulation is to activate transcription of mesoderm-specific genes.

**Maintenance of \textit{Xbra} expression by an indirect autocatalytic loop involving eFGF**

FGF signalling is required to maintain \textit{Xbra} expression. Evidence for this came from experiments in which prospective mesoderm of the \textit{Xenopus} embryo was cultured either as an intact piece of tissue or as dispersed cells. If cultured as an intact piece of tissue \textit{Xbra} expression persisted, but if cultured as dispersed cells, \textit{Xbra} expression declined. This decline could be prevented, however, if the dispersed cells were cultured in the presence of FGF (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). These results, together with the observations that ectopic expression of \textit{Xbra} in animal pole explants activates the expression of eFGF (Isaacs et al., 1994), that \textit{Xbra} and eFGF are co-expressed (Isaacs et al., 1995), and that inhibition of FGF function causes a down-regulation of \textit{Xbra} expression (Amaya et al., 1993; Isaacs et al., 1994; Kroll and Amaya, 1996), led to a model in which \textit{Xbra} and eFGF are components of an indirect autoregulatory loop in which \textit{Xbra} induces expression of eFGF and eFGF maintains expression of \textit{Xbra}. Consistent with this model, \textit{Xbra} cannot induce mesoderm in prospective ectodermal explants expressing a truncated FGF receptor that blocks FGF signalling (Schulte-Merker and Smith, 1995).

A prediction of the autoregulatory loop model is that expression of \textit{Xbra} should be down regulated in embryos injected with \textit{Xbra-En\textsuperscript{8}}. In fact, if \textit{Xbra} function is blocked using \textit{Xbra-En\textsuperscript{8}}, expression of the gene is down-regulated in the notochord and dorsal mesoderm, but persists in the marginal zone, suggesting that the autoregulatory loop operates predominantly in the notochord and dorsal mesoderm (Conlon et al., 1996). Consistent with this, expression of zebrafish \textit{Brachyury, no tail}, declines in the notochord of \textit{no tail} mutant embryos, but persists in the germ ring (Schulte-Merker et al., 1994b). Evidence from experiments involving chimeric mouse embryos
suggest that an autoregulatory loop is not required for maintenance of *Brachyury* expression in the primitive streak (Schmidt et al., 1997). When *Brachyury*-mutant ES cells carrying a *Brachyury*-promoter *lacZ* construct (Clements et al., 1996) are introduced into wild type embryos, *lacZ* activity is detected in the primitive streak in these cells, even in the absence of functional Brachyury protein, and even at a distance of five to eight cell diameters from wild-type cells that might be providing FGF to maintain *Brachyury* expression (Schmidt et al., 1997). Although it seems unlikely that the expression of *Brachyury* in the primitive streak, germ ring or marginal zone of the embryo requires an autoregulatory loop, it is still possible that such a loop does function in the notochord (Schmidt et al., 1997).

In an effort to define downstream targets of Xbra involved in its autoinduction, an inducible version of Xbra, *Xbra-GR* was used (Tada et al., 1997). This construct comprises the *Xbra* open reading frame fused to the ligand binding domain of the human glucocorticoid receptor. When expressed in *Xenopus* embryos this construct is completely inactive unless dexamethasone is added to the culture medium. This makes it possible to inject *Xenopus* eggs with RNA encoding *Xbra-GR*, to dissect animal caps at the late blastula stage and then to treat them for a desired short, defined time (Tada et al., 1997). Thus, in contrast to micro-injecting wild type RNA into *Xenopus* embryos, which is translated and is capable of exerting its effect virtually immediately, the activity of the *Xbra-GR* protein can be controlled, by the addition of dexamethasone, to correspond with the time at which *Xbra* expression is activated in *Xenopus* embryos, making it possible to identify direct targets of Xbra. The *Xbra-GR* system confirmed that *Xbra* autoinduction is indirect (Tada et al., 1997) and that Xbra activates expression of *eFGF* directly (Casey et al., 1998). *Xbra-GR* induces expression of *eFGF* in dispersed cells, and in the presence of both cycloheximide and a dominant negative FGF receptor. Thus, induction of *eFGF* by Xbra is cell autonomous, does not require new protein synthesis, and nor does it require an intact FGF signalling pathway (Casey et al., 1998).

Examination of the upstream regulatory region of *eFGF* revealed a 10 bp element approximately 1 kb upstream of the transcriptional start site (Casey
et al., 1998) that is identical to half of the 20 bp palindromic Brachyury binding site previously identified (Kispert and Herrmann, 1993). This site is also present within 1 kb of the transcriptional start sites of mouse and human FGF4, which are close relatives of eFGF (Isaacs et al., 1992), suggesting that this sequence is involved in the regulation of eFGF/FGF4 expression. Consistent with this, gel shift analysis demonstrates that this sequence is specifically bound by monomers of Xbra and experiments in Xenopus oocytes show that it is sufficient for Xbra-dependent reporter gene expression (Casey et al., 1998). Together these data show that eFGF is a direct target of Xbra, and they support the model for autoregulation of Xbra in which Xbra activates expression of eFGF which in turn maintains expression of Xbra in notochord and dorsal mesoderm.

**Transcriptional regulation of Xbra**

Understanding how expression of Brachyury is regulated will provide insight into how mesoderm is formed and patterned in vertebrate embryos. Part of the regulatory region of the mouse Brachyury gene has been identified within 500 bp of the transcription start site. This is sufficient to drive expression of a reporter gene only in the primitive streak, suggesting that additional elements are required to control expression of Brachyury in the notochord (Clements et al., 1996). The expression of ascidian Ciona intestinalis Brachyury is restricted to the notochord (Corbo et al., 1997b). A 434 bp enhancer has been identified that is sufficient to direct notochord-restricted expression of reporter genes.

As a first step towards understanding the transcriptional control of Xbra, the promoter region of the gene was isolated (Latinkic et al., 1997). The gene cloned appears to be a pseudoallele of Xbra and has been designated Xbra2. The existence of two Brachyury genes, Xbra and Xbra2, is consistent with the pseudotetraploid nature of the Xenopus genome (Kobel and Du Pasquier, 1986). The expression pattern of Xbra2 is similar to that of Xbra (Smith et al., 1991), suggesting that they play similar roles in mesodermal differentiation.
2.1 kb of Xbra2 5' flanking sequence drives mesoderm-specific expression of reporter constructs in Xenopus embryos (Latinkic et al., 1997). Reporter gene expression can be observed in early gastrula embryos throughout the marginal zone and, as gastrulation proceeds, expression is lost in the notochord (Walter Lerchner, personal communication). The loss of reporter gene expression in the notochord indicates that, as in mouse embryos, additional Xbra promoter elements are required for expression in the notochord. In experiments using animal cap tissue it was shown that 2.1 kb of Xbra2 5' flanking sequence is sufficient to confer responsiveness to low doses, but not high doses, of activin (Latinkic et al., 1997). There is evidence to suggest that Xbra2 expression is down-regulated in response to high concentrations of activin as a result of suppression of transcription by homeobox containing genes such as goosecoid (Latinkic et al., 1997), which is expressed in response to high doses of activin.

In addition to responding to low doses of activin, 2.1 kb of Xbra2 5' flanking sequence can confer responsiveness to FGF. FGF signals through the ERK MAP kinase pathway to induce the expression of Xbra/Xbral (Fig. 1.4) (LaBonne et al., 1995; Umbhauer et al., 1995). It is not known, however, how MAP kinase exerts its effects on the Xbra2 promoter. In mammalian cells, the ERK MAP kinase pathway provides a common route by which signals from different growth factor receptors converge at a major regulatory element of the promoters of c-fos and other immediate early genes, the serum response element (SRE) (Treisman, 1995). In unstimulated cells, c-fos SRE binds a ternary complex comprising of serum response factor (SRF) and a member of the ternary complex factor (TCF) family of Ets domain proteins. The TCF is the major target of the ERK MAPK pathway in the c-fos promoter. Thus in response to growth factor stimulation, activated ERK MAPK phosphorylates and activates TCF, thereby potentiating transcriptional activation of c-fos (Gille et al., 1992; Janknecht et al., 1993; Marais et al., 1993; Treisman, 1994; Price et al., 1995). Examination of the upstream regulatory region of Xbra2 revealed an SRE-like element, suggesting that the ERK MAPK pathway may activate transcription of Xbra2.
in response to FGF treatment through the activation of a ternary complex bound to an SRE-like element (see Chapter 6).

**This study**

Constitutively active forms of the type I activin receptors ALK-2 and ALK-4 (designated ALK-2* and ALK-4*) transduce clearly distinct signals in *Xenopus* embryos. One feature of ALK-2* is that it is capable of counteracting the ability of ALK-4* to induce dorsal mesodermal markers in *Xenopus* animal pole explants. Chapter 3 presents experiments investigating the dominant ventralisation signal of ALK-2*, using chimeras of ALK-2* and ALK-4*.

The loop between kinase subdomains IV and V (the β4-β5 loop) of ALK-4* has been implicated in mediating the strong dorsal gene inducing properties of ALK-4*. Although this loop is necessary for the induction of dorsal genes in the context of ALK-2*/ALK-4* chimeric receptors, it is not always sufficient for this. Experiments presented in Chapter 4 investigate the involvement of other regions of ALK-4* in controlling ALK-4* signalling specificity.

*Xbra* is a target gene for the mesoderm inducing factors FGF and activin. Chapter 5 presents experiments designed to identify elements in the upstream regulatory region of *Xbra* responsible for mediating its activation by these factors.

Experiments presented in Chapter 6 investigate the possibility that FGF activates transcription of *Xbra* through the activation of a ternary complex bound to an SRE-like element in the *Xbra* 5' flanking sequence.
Chapter 2

Materials and Methods

EMBRYOS AND EMBRYO MANIPULATIONS

Obtaining *Xenopus* embryos

*Xenopus* embryos were obtained by artificial fertilisation as described by Smith and Slack (Smith and Slack, 1983). Briefly, *Xenopus* embryos were obtained from adult females that had been injected 12 hours previously with 500-1000 units of human chorionic gonadotrophin (HCG), and transferred to a 90 mm petri dish. The eggs were fertilised by rubbing them with testes dissected from a sacrificed male. Five minutes later the eggs were flooded with 10% Normal Amphibian Medium (NAM). The embryos were dejellied using 2% cysteine hydrochloride (pH 7.9-8.1), and staged according to The Normal Table of *Xenopus* Development (Nieuwkoop and Faber, 1967).

Microinjection of *Xenopus* embryos

Dejellied embryos were transferred into 75% NAM containing 4% Ficoll in 35 mm petri dishes lined with 1% agarose. Embryos were injected using an air-driven injection system (Inject+Matic). Typically, volumes of 10 nl were delivered into one-cell stage embryos and 5 nl into 2- or 32-cell stage embryos using a glass needle. The injection volume was calculated by injecting the liquid into oil and measuring the diameter of the drop using a graticule. By treating the drop as a sphere, the injected volume was determined.

Occasionally it was necessary to inject specific blastomeres. The dorsal and ventral halves of the embryo can be distinguished from the 4-cell stage, because the dorsal half of the embryo is light whereas the ventral side is darker. In cases where specific blastomeres of the 32-cell embryo were to be injected, only those embryos showing both the typical cleavage patterns of this stage and clear pigmentation differences were injected.
Embryos that were not used for animal cap assays were transferred to 10% NAM at stage 8 (mid-blastula).

**Animal cap dissection**

Animal pole explants (animal caps) were dissected from embryos at stage 8. The vitelline membrane surrounding the embryo was removed manually using sharpened number 5 watchmakers forceps (supplied by BDH). A square of tissue from the animal-most 20-25% was cut by using the forceps as scissors. The tissue was placed in 75% NAM on agarose-coated dishes until sibling embryos had reached the desired stage.

For growth factor treatments animal caps were placed in 75% NAM supplemented with 0.1% bovine serum albumin (BSA). The growth factors used were FGF-2 (recombinant human basic FGF, supplied by Sigma) and Activin A. FGF was used at 50 ng/ml. A crude preparation of recombinant human activin A was prepared from the conditioned medium of COS cells transfected with a human inhibin βA cDNA. The cells were the gift of Dr Gordon Wong (Genetics Institute Inc, Cambridge Massachusetts). Activin A was used at 8 units/ml. A unit of mesoderm activity is defined as the smallest amount of mesoderm inducing factor present in 1 ml of medium that is needed to induce mesoderm (Cooke et al., 1987).

**Whole mount antibody staining**

For antibody staining, embryos were removed from their vitelline membrane, fixed for 2 hours in MEMFA and then transferred into methanol for long-term storage. All procedures were performed in 5 ml screw top glass vials (Phase Separation). Embryos were transferred into a solution of 80% methanol and 10% hydrogen peroxide for bleaching, after which they were rehydrated in PBT (phosphate buffered saline (PBS) containing 0.1% Triton-X-100). Non-specific epitopes were blocked by incubating the embryos in 10% lamb serum mixed with PBT for 1 hour before the incubation with the primary antibody. For this study an antibody recognising notochord (MZ15; (Smith and Watt, 1985)) was used. This antibody was used at a 1:750 dilution.
in PBT and 10% lamb serum. Incubation of this antibody was performed overnight at 4°C.

The primary antibody was rinsed by 6 X 15 minute washes in PBT. The bound antibody was then revealed using an antibody, conjugated to horseradish peroxidase, derived against the primary antibody. A goat anti-mouse antibody was used and the incubation and washing conditions for this secondary antibody were the same as for the primary antibody. The horseradish peroxidase was stained using Diaminobenzidine (DAB; Sigma) and 0.01% hydrogen peroxidase. The embryos were then dehydrated in methanol and placed in Murray's clear (2 volumes of benzyl benzoate, 1 volume of benzoic acid) which made the embryos transparent.

MOLECULAR BIOLOGY TECHNIQUES

All molecular biology procedures were carried out essentially as described in Sambrook et al. (Sambrook et al., 1989).

DNA quantification and manipulation

DNA and RNA were quantified either by spectrophotometry at 260 nm (O.D.=1 equates to 50 mg/ml DNA, 40 mg/ml RNA) or by electrophoresis on agarose gels in the presence of ethidium bromide alongside standards of known concentration.

Concentration of DNA was performed by precipitation. NaOAc was added to a final concentration of 0.3 M, 2.5 volumes of cold ethanol was added and it was put at -20°C for about 10 minutes. Centrifugation at >20 000 g for 5-20 minutes was performed to pellet the DNA which was then washed in 70% ethanol, dried and resuspended in TE or water.

Restriction enzyme digests were performed essentially as described by Sambrook et al. 1989. They were performed at either 37°C or room temperature using commercially supplied restriction enzymes and buffers (Promega). The enzyme component of the reaction never comprised more than 10% of the reaction volume. For enzyme digests using more than one restriction enzyme, the buffer suggested by the manufacturer was used.
Intermolecular ligations were performed in small volumes, generally 10 μl for a total DNA content of 1 μg. For ligations involving plasmid sequences a 1:10 ratio of plasmid:insert was typically used. When the plasmid was capable of self-ligation, the compatible ends were dephosphorylated before use (see below). Ligations were performed overnight at 14°C (blunt ends) or for 4 hours at room temperature (sticky ends), using T4 DNA polymerase (Promega) in ligation buffer (Promega).

Dephosphorylation of DNA was performed using calf intestinal phosphatase as described by Sambrook et al. 1989.

**Preparation and storage of competent bacteria**

The DH5α strain of *Escherichia coli* was rendered competent for transformation by treatment with CaCl$_2$. A single colony was placed in 50 ml of L-broth and shaken at 37°C overnight. 10 mls of this culture were inoculated into 100 mls of P-medium (15.9mM K$_2$PO$_4$, 6.3mM KH$_2$PO$_4$, 15mM (NH$_4$)$_2$SO$_4$, 10mM MgSO$_4$, 1.8μM FeSO$_4$, 1% casamino acids and 0.2% glucose) and cells were grown to an OD$_{600}$ of 0.4. After washing in 100 mls of 10mM NaCl at 4°C, cells were repelleted. Bacteria were resuspended in 100 mls of 50 mM CaCl$_2$ and incubated at 4°C for 15 minutes. Finally bacteria were pelleted and resuspended in 10 mls of 50mM CaCl$_2$, 16% (v/v) glycerol, aliquotted and quickly frozen in dry ice before being stored at -80°C.

**Plasmid transformation of competent bacteria**

Frozen competent bacteria (as prepared above) were thawed and up to 100 ng of DNA was added to 100 μl of the cells on ice. The bacteria were kept on ice for 20-30 minutes and then heat shocked at 42°C for 90 seconds. Bacteria were cooled on ice, 800 μl of LB was added and the mixture incubated at 37°C for 45 minutes. Bacteria were then plated out onto LB plates containing 100 μg/ml ampicillin and placed in a 37°C incubator overnight.

For defined plasmid recovery, 10 μl of competent bacteria were added to 250 ng of plasmid DNA and placed at 37°C for 90 seconds after which time 100 μl
of L-broth was added and the mixture plated out onto LB plates containing 100 μg/ml ampicillin and placed in a 37°C incubator overnight.

**Small scale preparation of plasmid DNA**

From an overnight culture 1.5 ml was transferred to a 1.5 ml microfuge tube and spun for 45 seconds. The supernatant was tipped off and the pellet resuspended in 150 ml of solution I. 200 μl of solution II was then added and mixed followed by 150 μl of ice cold solution III followed by careful mixing. The tube was then spun for 5 minutes at 4°C. 400 μl of the supernatant was then transferred into a fresh microfuge tube and extracted with an equal volume of a 1:1 mix of phenol:chloroform. After centrifugation for 10 minutes, 350 μl of the upper layer was transferred into a new tube and extracted with chloroform. Following another 10 minute centrifugation the upper layer was transferred to a new tube and precipitated with 1 ml of 100% ethanol. After centrifugation the pellet was washed in 70% ethanol, pelleted, dried and resuspended in TE with 0.1 mg/ml RNase A.

Solution I 50 mM glucose, 10 mM EDTA,
25 mM Tris.Cl pH 8.0

Solution II 0.2 M NaOH, 1% SDS

Solution III 4 M KOAc pH 4.8

Alternatively, DNA was often prepared by a more rapid procedure called a TENS prep. The pellet from centrifugation of 1.5 ml of overnight culture was resuspended by vortexing in the LB (~50 μl) remaining after turning the tube upside down. 300 μl of TENS solution (0.1 M NaOH, 0.5% SDS) was added and shaken briefly. 150 μl of 3M NaOAC (pH 5.5) was then added and shaking was repeated. After a two minute centrifugation at room temperature in a microfuge the supernatant was removed (450 μl) and 900 μl of ice cold 100% ethanol was added. After a further two minute centrifugation the pellet was washed once in 70% ethanol and resuspended in TE with 0.1 mg/ml RNase A.
Large scale preparation of plasmid DNA

1 ml of plasmid bacterial culture was placed in 100 ml of LB containing 100 µg/ml ampicillin, and shaken at 37 °C overnight. The Qiagen maxi-prep kit (Hybaid) was then used to isolate the maxi-prep DNA, according to the protocol suggested by the manufacturer's.

Polymerase chain reaction

PCR reactions were carried out using Vent (exo+) polymerase (New England Biolabs) according to the manufacturer's recommendations. The reactions were set up using the buffers supplied in a total volume of 50 µl. dNTPs were added to a final concentration of 0.2 mM each, and primers were added to a final concentration of 1 µM. The reaction mix was overlaid with a light mineral oil (Sigma) to prevent evaporation. PCR reactions were performed on a Perkin-Elmer Thermal Cycler. Details of specific primers and conditions used are given in the plasmid construct section of this chapter.

Agarose gel electrophoresis of DNA and RNA

DNA fraction and size estimation were performed by agarose gel electrophoresis. Gels were prepared by dissolving agarose in 0.5 x TBE to a final concentration of 0.8% - 1.6% depending on the expected size of the DNA fragment. To visualise the DNA, 0.5 mg/ml ethidium bromide was added to the gel. DNA samples were mixed with gel loading buffer and electrophoresis was performed at 5 - 20 volts/cm of gel length, until the appropriate resolution was achieved. The resolved DNA was visualised using ultraviolet light at 340 nm, and the size was estimated by comparison with known size markers such as Lambda DNA digested with Hind III or BstE II or the 1 kb size markers (Gibco BRL).

Purification of specific DNA fragments from gels

In order to purify DNA fragments of interest, size fractionated DNA was purified using the QAIEX system (Hybaid) of isolating DNA from agarose gel pieces.
Digested DNA was subjected to agarose gel electrophoresis and the region of the gel containing the appropriate band was excised. This slice was mixed with solution A (a solution of sodium perchlorate) and 5 μl of glass beads for every μg of DNA in the gel slice, and then incubated at 50°C for 10 minutes. After incubation, the glass beads were spun down and washed three times in solution B. Finally, all solution B was removed and the pellet was resuspended in water or TE, and incubated for 15 minutes at room temperature before centrifugation for 1 minute and removal of the supernatant containing purified DNA.

**DNA sequence analysis**

All DNA sequence analysis was performed by the dideoxy chain termination method using a sequencing kit (Sequenase v.2 – USB), according to the manufacturer’s instructions.

**In vitro transcription**

RNA for injection into *Xenopus* embryos was transcribed from constructs containing a promoter from bacteriophage SP6, T3 or T7. The promoters from these bacteriophage are highly specific; for example, RNA polymerase from SP6 does not promote RNA synthesis from any DNA sequence except the SP6 promoter. The transcription method used allowed for synthesis of capped RNA, which is necessary for efficient translation of the RNA. The exact constructs used during this study are described in Table 2.1.

Transcription reactions were assembled as described below:

- 10 μl 5 X Transcription buffer (Promega)
- 5 μl 0.1M DTT
- 5 μl 10mM ATP, 10mM CTP, 10mM UTP mix
- 5 μl 1mM GTP
- 5 μl 5mM Cap analogue (m7-G; New England Biolabs)
- 5 μl RNAsin (Promega)
<table>
<thead>
<tr>
<th>gene</th>
<th>expression plasmid</th>
<th>linearisation site</th>
<th>RNA polymerase</th>
<th>Reference</th>
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<tr>
<td>ALK-2*</td>
<td>pSP64T</td>
<td>Bam HI</td>
<td>SP6</td>
<td>(Armes and Smith, 1997)</td>
</tr>
<tr>
<td>C2 and C4</td>
<td>pSP64T</td>
<td>Bam HI</td>
<td>SP6</td>
<td>(Armes et al., 1998)</td>
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<td>ALK-4*</td>
<td>PT-19R</td>
<td>Hind III</td>
<td>T7</td>
<td>(Armes and Smith, 1997)</td>
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<tr>
<td>C9 and C11</td>
<td>PT-19R</td>
<td>Hind III</td>
<td>T7</td>
<td>(Armes et al., 1998)</td>
</tr>
<tr>
<td>ALK-4*S^{172→A}</td>
<td>PT-19R</td>
<td>Hind III</td>
<td>T7</td>
<td>This thesis</td>
</tr>
<tr>
<td>ALK-4*T^{178→V}</td>
<td>PT-19R</td>
<td>Hind III</td>
<td>T7</td>
<td>This thesis</td>
</tr>
<tr>
<td>Smad1</td>
<td>pSP64 TEN</td>
<td>Xba I</td>
<td>SP6</td>
<td>(Graff et al., 1996)</td>
</tr>
<tr>
<td>Smad2</td>
<td>pSP64 TEN</td>
<td>Xba I</td>
<td>SP6</td>
<td>(Graff et al., 1996)</td>
</tr>
<tr>
<td>Smad4</td>
<td>pSP64 TEN</td>
<td>Bam HI</td>
<td>SP6</td>
<td>(Lagna et al., 1996)</td>
</tr>
<tr>
<td>ΔSmad4</td>
<td>pSP64 TEN</td>
<td>Bam HI</td>
<td>SP6</td>
<td>(Lagna et al., 1996)</td>
</tr>
<tr>
<td>Smad5</td>
<td>pSP64 TEN</td>
<td>Bam HI</td>
<td>SP6</td>
<td>(Suzuki et al., 1997)</td>
</tr>
<tr>
<td>Elk-1 Δ307</td>
<td>pT7βplink</td>
<td>Xba I</td>
<td>T7</td>
<td>(Hill et al., 1993)</td>
</tr>
<tr>
<td>Elk-1 ΔB Δ307</td>
<td>pT7βplink</td>
<td>Xba I</td>
<td>T7</td>
<td>Gift from Richard Treisman</td>
</tr>
<tr>
<td>Elk-1 VP16</td>
<td>pT7βplink</td>
<td>Xba I</td>
<td>T7</td>
<td>(Hill et al., 1994)</td>
</tr>
<tr>
<td>Elk-1 ΔB VP16</td>
<td>pT7βplink</td>
<td>Xba I</td>
<td>T7</td>
<td>Gift from Richard Treisman</td>
</tr>
<tr>
<td>SRF VP16</td>
<td>pT7βplink</td>
<td>Xba I</td>
<td>T7</td>
<td>(Dalton and Treisman, 1992)</td>
</tr>
<tr>
<td>SRF En&lt;sup&gt;R&lt;/sup&gt;</td>
<td>pSP64 T</td>
<td></td>
<td>SP6</td>
<td>Gift from Tim Mohun</td>
</tr>
<tr>
<td>engrailed</td>
<td>pSP64 T</td>
<td>Bam HI</td>
<td>SP6</td>
<td>(Badiani et al., 1994)</td>
</tr>
<tr>
<td>NKX 2.5 En&lt;sup&gt;R&lt;/sup&gt;</td>
<td>pB-SK</td>
<td>Xba I</td>
<td>T7</td>
<td>Gift from Tim Mohun</td>
</tr>
<tr>
<td>globin</td>
<td>T7A90</td>
<td>Xba I</td>
<td>T7</td>
<td>Gift from Richard Treisman</td>
</tr>
</tbody>
</table>
2.5 μl SP6/T7/T3 polymerase (Promega)
5 μl Linearised template DNA (1 mg ml⁻¹)

The reaction mixture was made up to 50 μl with diethyl pyrocarbonate (DEPC) treated water. The reaction was incubated for 30 minutes at 37°C, after which 2.5 μl of 10mM GTP was added and the reaction was incubated for a further 90 minutes at 37°C. The DNA template was destroyed by the addition of 5 μl of RNAase-free DNAase I (Promega) and this reaction was incubated at 37°C for 15 minutes. The mixture was then subjected to phenol/chloroform extraction, after which free nucleotides were removed by passing the reaction through a spin column (Chromaspin spin-100; Clontech).

To determine the efficiency of translation, synthetic RNA was translated using the rabbit reticulocyte system (Promega). Briefly, 200 ng of synthetic RNA was added to 8 μl of reticulocyte lysate, 0.5 μl ³⁵S methionine and 0.5 μl RNAsin. The mix was incubated at 30°C for 1 hour. Reaction products were analysed by separation on 12% or 15% SDS-PAGE gels before drying and exposure to autoradiographic film. Typically 1/20th of the reaction was loaded per lane. Protein size was estimated by comparison with pre-stained Rainbow molecular weight markers (Amersham).

PLASMID CONSTRUCTS

Luciferase-based vectors
Vincent Cunliffe subcloned a fragment of Xbra2 genomic DNA containing 53 bp of exon 1 and 2100 bp of the 5' flanking sequence into the promoterless luciferase vector, pGL2 Basic (Promega), using the Nhei and BglII sites in its polylinker, to generate -2100Xbra2pGL2. He obtained deletion derivatives of this construct by digesting -2100Xbra2pGL2 with either Pst I and Kpn I, or with SacI alone, generating -1000Xbra2pGL2 and -150Xbra2pgl2 respectively (Latinkic et al., 1997).

A series of deletions within the 1 kb of upstream flanking sequence of the Xbra2 gene was generated by PCR using the same 3' (downstream) primer
(nucleotides +34 to +48 relative to the transcriptional start site) and various 5' (upstream) primers representing a series of deletions in the Xbra2 promoter region. The 3' primer contained a BglII restriction site at its 5' terminus and the 5' primers contained a NheI restriction site at their 5' termini for directional cloning into the promoterless luciferase reporter construct, pGL2 Basic (Promega). Sequences of the PCR primers are listed below.

Downstream primer:

5' CGAAGATCTGCAGGTAGTAAATCC-3' (BglII site underlined).

Upstream primers:

-866Xbra2pGL2, 5' GCTAGCTAGCCGGTAGAATCCACAT-3'
-700Xbra2pGL2, 5' GCTAGCTAGCGTGGGACCACAAAC-3'
-617Xbra2pGL2, 5' GCTAGCTAGCCTAAACTAGGGGATG-3'
-527Xbra2pGL2, 5' GCTAGCTAGCGGGGATGTAAAATTGAGG-3'
-440Xbra2pGL2, 5' GCTAGCTAGCGTACCTATTATTTTG-3'
-381Xbra2pGL2, 5' GCTAGCTAGCCATCTGCCATTATACC-3'
-300Xbra2pGL2, 5' GCTAGCTAGCGTTCTTACTGGATGT-3'
-231Xbra2pGL2, 5' GCTAGCTAGCGCAGCTCTTTGATAG-3' (NheI site underlined). The conditions for amplification were: 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. The reactions were digested with NheI and BglII and ligated into pGL2 Basic, which had been digested with the same enzymes.

The vector sequence was removed from all Xbra2 luciferase reporter constructs by digestion with BamHI and NheI, except for -2100Xbra2pGL2 which was digested with BamHI alone to remove the vector sequence.

**EF-1α based vector**

The pXEX vector (Krieg et al., 1989), which contains the EF-1α promoter, was digested with BamHI, the ends were polished with Klenow fragment, and a
HindIII linker was ligated in its place. The $\beta$-galactosidase gene was excised from pSP6 nuc$\beta$-gal (Picard and Yamamoto, 1987) with HindIII and this was ligated into HindIII-digested pXEX (with the HindIII linker), generating pEF-1$\alpha$ $\beta$-galactosidase.

**ALK-4* point mutant constructs: ALK-4*S\textsuperscript{172}$\rightarrow$A and ALK-4*T\textsuperscript{178}$\rightarrow$V**

Serine 172 of ALK-4* was mutated to alanine, creating ALK-4*S\textsuperscript{172}$\rightarrow$A. Threonine 178 of ALK-4* was mutated to valine, creating ALK-4*T\textsuperscript{178}$\rightarrow$V. These ALK-4* point mutant constructs were made using a kit (QuikChangeTM Site-Directed Mutagenesis Kit; Stratagene), according to the manufacturer’s recommendations.

The primers used to make ALK-4*S\textsuperscript{172}$\rightarrow$A are as follows:

Upstream primer, 5’ CTTGCGAGATGTGTCTCGCCAAAGACAAGACG 3’
Downstream Primer, 5’ CGTCTTGTCTTTGGCGAGACACATCTCGCAAG 3’.

The primers used to ALK-4*T\textsuperscript{178}$\rightarrow$V make are as follows:

Upstream primer, 5’ CTCTCCAAAGACAAAGTTGTCTCGCAAG 3’
Downstream Primer, 5’ CGAGATCCTGGAGACACCATCTCGCAAG 3’.

**RNA ISOLATION AND ANALYSIS**

**RNA isolation**

Animal caps were collected, medium was removed and the samples were frozen on dry ice. RNA was isolated from animal caps using the acid phenol/guanadimium isothiocyanate procedure (Chomczynski and Sacchi, 1987). Briefly, tissue was homogenised in a solution of acid-phenol and guanadimium isothiocyanate. The phases were separated by adding 0.2 volumes of chloroform. The aqueous upper phase was then precipitated using an equal volume of isopropanol, after which the pellet was washed in 70% ethanol.

To guard against RNAase activity, all solutions were either filtered or treated with diethyl pyrocarbonate (DEPC) and then autoclaved.
RNAase protection

The constructs used to make probes for RNAase protection analysis are listed in Table 2.2. After a probe template construct was linearised with the appropriate restriction enzyme, the probe transcription reaction was set up at room temperature, as follows (essentially as described by Green et al; (Green et al., 1990)):

<table>
<thead>
<tr>
<th>Volume (µl)</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5 X Transcription buffer (Promega)</td>
</tr>
<tr>
<td>0.5</td>
<td>0.2M DTT</td>
</tr>
<tr>
<td>1</td>
<td>10mM Nucleotides (-UTP)</td>
</tr>
<tr>
<td>0.5</td>
<td>RNAsin (Promega)</td>
</tr>
<tr>
<td>5</td>
<td>$^{32}$P-UTP</td>
</tr>
<tr>
<td>1</td>
<td>Linearised template DNA</td>
</tr>
<tr>
<td>1</td>
<td>RNA polymerase</td>
</tr>
</tbody>
</table>

The reaction was incubated for 90 minutes at 37°C, after which 30 µl of formamide-containing loading dye was added. The reaction was denatured by heating to 80°C for 2 minutes and then size fractionated using a short polyacrylamide gel (6% polyacrylamide, 7M Urea). This gel was run at 35 W for approximately 1 hour, until the xylene cyanol dye had migrated two-thirds of the distance of the gel. The gel was wrapped in cling film and the desired band was cut out from the gel after being visualised with a 15 second exposure to X-ray film. The gel piece was eluted in 400 µl of elution buffer for 2–3 hours at 50°C. The eluate was then precipitated and resuspended so that 1 µl of probe contained at least 5 X 10^5 counts. The EF-1α riboprobe was labelled to one fifth of the activity by including some cold UTP in the transcription reaction (Sargent and Bennett, 1990), thereby preventing the occlusion of the final result by a strong EF-1α signal.
Table 2.2. Constructs used as templates for RNAase protection probes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Linearisation site</th>
<th>RNA Polymerase</th>
<th>Probe length</th>
<th>Protected Length</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF-1α</td>
<td>Hinf I</td>
<td>SP6</td>
<td>150</td>
<td>114</td>
<td>(Krieg et al., 1989)</td>
</tr>
<tr>
<td>xbra</td>
<td>Hind III</td>
<td>T7</td>
<td>281</td>
<td>232</td>
<td>(Armes et al., 1998)</td>
</tr>
<tr>
<td>xbra2</td>
<td>Bam HI</td>
<td>T7</td>
<td>370</td>
<td>222</td>
<td>(Latinkic et al., 1997)</td>
</tr>
<tr>
<td>Pintallavis</td>
<td>Bgl II</td>
<td>T3</td>
<td>384</td>
<td>290</td>
<td>(Ruiz I Altaba and Jessel, 1992)</td>
</tr>
<tr>
<td>goosecoid</td>
<td>Xba I</td>
<td>T3</td>
<td>440</td>
<td>367</td>
<td>(Cho et al., 1991)</td>
</tr>
</tbody>
</table>
Labelled probes were added to RNA (isolated from *Xenopus* animal caps) that was dissolved in 70% formamide and 1 X Hyb salts (see later). This mix was then heated to 85°C for 5 minutes. Following this hybrids were allowed to form for 8-14 hours at 50°C. After hybridisation, non-homologous sequences were degraded by digestion with RNAase T1 at 37°C for 30 minutes. RNAase T1 was then destroyed by incubation with proteinase K for 15 minutes at 37°C. The whole reaction was then subjected to phenol/chloroform extraction followed by ethanol precipitation. The resulting pellet was resuspended in 2 μl of DEPC-treated water. Formamide loading dyes were then added to the solution and the mixture was loaded on a large 6% polyacrylamide gel containing 7M urea. The gel was run until the xylene cyanol had migrated 75% of the total gel length. The gel was then fixed using a solution containing 10% methanol and 10% acetic acid in water, after which it was dried and exposed to X-ray film at -70°C, with intensifying screens.

**LUCIFERASE AND β-GALACTOSIDASE ASSAYS**

Animal caps were collected, medium was removed and the samples were frozen on dry ice. They were lysed in 3 μl/cap of 1X Lysis Buffer (Promega) and the lysate clarified by brief centrifugation. Supernatant was transferred to a clean tube: 40% was assayed for luciferase activity and the remaining 60% for β-galactosidase activity. The Luciferase Assay System of Promega (Madison,WI) and a Berthold luminometer were used (Latinkic et al., 1997). β-galactosidase activity was assayed as followed: 270 μl of 1.1mM MgCl₂, 50mM β-mercaptoethanol 25 mM sodium phosphate (pH 7.5), 1 mg/ml ONPG was added to 30 μl of extract. The reaction was carried out at 37°C and stopped by adding 500 μl of 1M Na₂CO₃. Optical density was measured at a wavelength of 420 nm (Latinkic et al., 1997).
FORMULATION OF FREQUENTLY USED SOLUTIONS

NAM (normal amphibian medium; (Slack, 1984))

10 X NAM:  
1.1M NaCl, 20mM KCl, 10mM Ca(NO₃)₂, 10mM MgSO₄, 1mM Na₂EDTA

10 X NAM (for 1 litre):  
65 g NaCl, 1.5 g KCl, 2.4 g Ca(NO₃)₂, 2.5 g MgSO₄, 0.37 g Na₂EDTA

75% NAM:  
37.5 ml 10 X NAM, 10 ml 0.1M Na phosphate (pH 7.4), 5 ml 0.1M NaHCO₃, 2.5 ml 10 mg ml⁻¹ gentamycin

10% NAM:  
5 ml 10 X NAM, 10 ml 0.1M Na phosphate (pH 7.4), 2.5 ml 10 mg ml⁻¹ gentamycin

MEMFA

10 X MEM salts:  
1M MOPS, 20 mM EGTA, 10mM MgSO₄

MEMFA (for 50 ml):  
5 ml 10 X MEM salts, 5 ml 37% formaldehyde

PBS:  
1% w/v NaCl, 0.025% w/v KCl, 0.14% w/v Na₂HPO₄, w/v 0.025% KH₂PO₄

TE:  
1mM EDTA, 10mM Tris.Cl pH 7.4

1 X TBE:  
89mM Tris-Base, 89mM Boric acid, 2mM EDTA (pH 8.0)

1 X TAE:  
40mM Tris acetate, 2mM EDTA (pH 8.0)

10 X gel load buffer:  
25% w/v Ficoll, 50mM EDTA (pH 8.0), 0.1% w/v bromophenol blue

Solutions for RNAse protection assays

Riboprobe elution buffer: 0.5M NH₄Acetate, 1mM EDTA, 0.1% SDS

5 X Hyb Salts:  
2M NaCl, 0.2M PIPES (pH 6.4), 5mM EDTA

RNA digestion buffer:  
10mM Tris (pH 7.5), 5mM EDTA, 300mM NaCl

Formamide loading buffer: 10mM NaOH, 1mM EDTA, 80% v/v formamide, 0.02% w/v bromophenol blue, 0.02% w/v bromophenol blue, 0.02% w/v xylene cyanol
FORMULATION OF FREQUENTLY USED BACTERIAL GROWTH MEDIA

**L-Broth:** 1% w/v Bacto-trypitone, 0.5% w/v Bacto-yeast extract, 1% w/v NaCl

**L-agar:** L-Broth supplemented with 1.5% Bacto-agar
Chapter 3

Investigation of the ALK-2* dominant ventralisation signal

INTRODUCTION

Activin is a member of the TGF-β superfamily and signals through a heteromeric complex of type I and type II serine-threonine kinase receptors (reviewed by Mathews, 1994). Activin induces animal pole tissue of early blastula stage *Xenopus* embryos to form mesoderm rather than ectoderm (Sive, 1993, Slack, 1994). It has been shown previously that constitutively active forms of the type I activin receptors ALK-2 and ALK-4 (designated ALK-2* and ALK-4*) also induce prospective ectodermal tissue to form mesoderm, but that the two receptors elicit different responses (Armes and Smith, 1997). The effects of ALK-4* resemble those of activin itself, in that it can, in a dose dependent manner, induce expression of the general mesodermal marker *Xbra* (Smith et al., 1991) as well as the dorsoanterior marker *goosecoid* (Cho et al., 1991). ALK-2* induces only ventral markers and causes ventralisation of intact embryos. ALK-2* is also capable of counteracting the dorsalising effects of ALK-4* (Armes and Smith, 1997).

The differential effects of ALK-2* and ALK-4* are likely to be mediated by structural differences within the receptors themselves, probably within the intracellular signalling domains of the molecules. To investigate the question of type I receptor signal specificity Niall Armes performed detailed sequence analysis to identify regions of ALK-2 and ALK-4 which may define particular downstream signalling events (Armes et al., 1998). All type I TGF-β receptors possess similar intracellular domain structures with a short poorly conserved juxtamembrane domain, a highly conserved GS domain, and a well conserved serine-threonine kinase domain (the kinase domains of ALK-2 and ALK-4 are 62% identical and 70% similar) (Fig. 3.1). A multiple sequence alignment was generated between the sequences of human ALK-1 to ALK-5 (ten Dijke et al., 1993; ten Dijke et al., 1994), and their likely orthologues in *Drosophila melanogaster*: the products of the *thick veins*
Figure 3.1. Structure of type I receptor kinase and alignment of the three regions selected for analysis. (A) 3-dimensional representation of the type I receptor kinase, based on crystal structures of other kinases (Hanks and Hunter, 1995). The position of the IB1 region (the β4-β5 loop) on the small lobe of the kinase, and the position of the IB3 region (the activation loop of the enzyme), are marked and coloured. The relative position of the GS domain is also shown, although the structure of this region is not known. (B) schematic representation of the type I receptor kinase showing the relative locations of the GS domain, the IB1 region and the IB3 region. Sequences are aligned from human ALK-1 to ALK-5 receptors and the Drosophila thick veins, saxophone and Atr-1 receptors. Identical residues are shaded in the same colour, regions showing almost 100% identity such as the central core of the GS domain are not included.
(tkv), and saxophone (sax) genes (Brummel et al., 1994), and the activin-like receptor Atr-1 (Wrana et al., 1994b). Analysis of the sequence alignment revealed three divergent regions between the vertebrate receptors that retain notable homology with tkv, sax and Atr-1 (Fig. 3.1). The most N-terminal region encompasses the GS domain. The second two regions were classified as Interest Boxes (IB) 1 and 3. IB1 is a short loop between kinase subdomains β4 and β5, and IB3 corresponds to the activation loop of the enzyme.

Figure 3.2 shows a series of chimeric receptors that were generated containing combinations of all three candidate specificity regions in constitutively active ALK-2* and ALK-4* backbones. Experiments with these chimeras highlighted the importance of IB1, a short loop on the lobe of the kinase, the β4-β5 loop. The 7 amino acids that make up this loop are capable, when transferred from ALK-4* to ALK-2*, of carrying with them the ability to induce dorsal markers (Armes et al., 1998). An analogous result has been obtained recently using the TGF-β receptor TβR1/ALK-5 and Tsk7L/ALK-2 in a tissue culture system (Feng and Derynck, 1997). Similarly, a chimeric receptor with the reverse transfer of the β4-β5 loop from ALK-2* to ALK-4* retains the ability to induce Xbra and loses the ability to induce dorsal genes (Armes et al., 1998). However, results presented in this chapter show that, in contrast to wild type ALK-2*, this chimeric receptor is unable effectively to counteract the dorsalising effects of ALK-4*. This result suggests that signal specificity does not reside wholly within the β4-β5 loop, and that there may be several signal transduction pathways leading from type I receptors.

One signalling pathway that is known to act downstream of type I receptors involves proteins of the Smad family (for reviews see Kretzschmar and Massague, 1998; Heldin et al., 1997). Over-expression of Smad proteins in prospective ectodermal tissue of early Xenopus embryos causes mesoderm formation. As with the type I receptors, different Smads elicit different responses: Smad2, like ALK-4*, acts in a concentration-dependent manner to induce the expression of dorsal genes (Baker and Harland, 1996), while Smad1 and Smad5, like ALK-2*, induce ventral mesoderm (Graff et al., 1996; Hoodless et al., 1996; Liu et al., 1996; Thomsen, 1996; Suzuki et al., 1997). As
Figure 3.2. Chimeric receptors. A schematic representation of the 16 clones containing combinations of all three candidate specificity regions in ALK-2* and ALK-4* backbones (Armes et al., 1998). Exchanged regions derived from ALK-4* are shown in green and regions from ALK-2* are shown in red.
the dorsal inducing effects of the chimeric ALK-2* receptor created by transfer of the ALK-4* β4-β5 loop into ALK-2* resemble those of Smad2, we postulate that the specificity of the signal regulated by the β4-β5 loop is realised by activation of specific Smad signalling pathways (Armes et al., 1998). Indeed, recent evidence, from experiments with the BMP receptor ALK-3 and the TGF-β receptor ALK-5, suggests that the IB1 from type I receptors feeds directly into the Smad signalling pathway (Chen et al., 1998). If the IB1 from these receptors are switched, the receptors show a complete switch in their ability to activate Smad1 and Smad2. Thus, ALK-5 with the ALK-3 IB1 activates Smad1, rather than Smad2, and induces BMP-like responses, while ALK-3 with the ALK-5 IB1 activates Smad2, rather than Smad1, and induces TGF-β-like responses (Chen et al., 1998).

In this chapter I show that the similarities in the effects of ALK-2* and Smads 1 and 5 do not, however, include the ability to counteract dorsalising signals. Smad1, in contrast to ALK-2*, cannot counteract the early effects of ALK-4* (or activin) at all, while Smad5 produces only a very weak interference. The dominant ventralising effects of ALK-2* are therefore unlikely to be mediated by Smad signalling, a conclusion consistent with the observation that these ventralising effects are not mediated by the β4-β5 loop, which is likely to act through Smads (Chen et al., 1998).

The dominant ventralising effect of ALK-2* over ALK-4* is immediate (Armes and Smith, 1997), as ALK-4* is unable to induce any dorsal responses, at any stage, when co-expressed with ALK-2* in Xenopus animal caps. This suggests that ALK-2* targets ALK-4* or components of its downstream signalling pathway(s) directly to elicit its ventralising effect. Consistent with this, I show in this chapter that dorsal gene activation by Smad2, which, with Smad4, is thought to act downstream of ALK-4* (Lagna et al., 1996; Zhang et al., 1997), is unaffected by ALK-2*. This suggests that ALK-2* blocks ALK-4* dorsal gene induction upstream of Smad signalling, maybe at the ALK-4* receptor itself.

It is not known which region of ALK-2* is responsible for the ventralising effect, but it is shown in this chapter that susceptibility to ventralisation
resides in ALK-4* sequences that are not within the β4-β5 loop, because dorsal gene induction mediated by a chimeric receptor with an ALK-2* backbone cannot be inhibited by co-expressed ALK-2*.

RESULTS

The ability of ALK-2* to interfere with ALK-4*-mediated gene induction cannot be recapitulated by transfer of the IB1 region of ALK-2* into the ALK-4* backbone

It has previously been demonstrated that the ALK-2* and ALK-4* receptors do not mediate equivalent responses in the *Xenopus* animal cap assay (Armes and Smith, 1997). ALK-4*, like activin, induces expression of the midline and anterior markers *Pintallavis* and *goosecoid* and at intermediate concentrations it activates transcription of the more ventral marker *Xbra*. ALK-2* induces expression only of *Xbra*. These markers are induced by activin, in *Xenopus* animal caps, in an immediate-early fashion and they are easily detectable by the early gastrula stage or before, only 5 hours after the mid-blastula transition (Cho et al., 1991; Smith et al., 1991; Dirksen and Jamrich, 1992). Expression of these genes is, thus, an ideal assay for primary signalling events. Studying the expression of these three genes has made it possible to distinguish between signals deriving from ALK-2*, ALK-4* and ALK-2*/ALK-4* chimeric receptors (Armes and Smith, 1997; Armes et al., 1998). This assay has been used in this chapter and Chapter 4 in an effort to discover more about the signalling specificities of ALK-2* and ALK-4*.

ALK-2* is capable of interfering with the ability of ALK-4* to induce expression of *goosecoid* and *Pintallavis* (Armes and Smith, 1997). This is unlikely to result purely from competition for common signalling components; low doses of ALK-2* will interfere very efficiently with ALK-4*-like responses, and kinase-inactive ALK-2* constructs interfere only at much higher doses, producing a qualitatively different type of interference (Armes and Smith, 1997). As transfer of the β4-β5 loop (IB1) from ALK-4* into an ALK-2* backbone resulted in the induction of *Pintallavis* and *goosecoid*, and the reciprocal exchange of IB1 from ALK-2* into an ALK-4*
Figure 3.3. Chimeric receptors do not interfere with dorsal gene activation by ALK-4*. Animal caps were dissected at stage 8 from embryos co-injected with 1 ng of ALK-4* and 1 ng of either ALK-2* or of other chimeric receptors. Caps were harvested at early gastrula stage 10 and processed for RNAase protection using probes specific for goosecoid, Pintallavis and Xbra. EF-1α was used as a loading control.
backbone resulted in a loss of expression of these dorsal markers, it seemed reasonable to ask whether the IB1 from ALK-2* would confer this seemingly active inhibitory property to ALK-4*. Surprisingly this did not seem to be the case: the chimeras C3 and C5 (which contain the ALK-2* IB1 in the ALK-2* backbone, see Fig. 3.2) and C10, C12, C14 and C15 (which contain the ALK-2* IB1 in the ALK-4* backbone) all failed to demonstrate effective suppression of ALK-4* induction of goosecoid and Pintallavis in Xenopus animal caps (Fig. 3.3). Co-injection of these constructs lowered goosecoid and increased Xbra levels slightly, but effective extinction of dorsal markers did not occur and the observed effects were reminiscent of partial suppression mediated by co-injection of kinase inactive ALK-2* (Armes and Smith, 1997). From this I conclude that the active inhibitory action of ALK-2* derives predominantly from signals elsewhere in the ALK-2* receptor.

**The ability of ALK-2* to interfere with dorsal gene induction requires the ALK-4 receptor backbone**

The ability of ALK-2 to interfere with dorsal gene induction by ALK-4 requires sequences that are not contained within the ALK-2* β4-β5 loop, because transfer of this region to ALK-4* does not confer interference activity. If other regions of ALK-2* are responsible for interference with dorsal gene induction, however, this raises the question of how the C2 chimeric receptor activates dorsal genes. With the exception of the ALK-4* β4-β5 loop (which comprises just 7 amino acids), C2 is identical to ALK-2* (Fig. 3.2). Why does it not inhibit its own dorsalising activity? One possibility is that susceptibility to inhibition requires ALK-4* sequences that are not present in C2. This was tested by co-expressing C2 with ALK-2* (experiment done in collaboration with Niall Armes). Figure 3.4A (lanes 1,2) shows that ALK-2* is unable to inhibit induction of goosecoid and Pintallavis by C2, indicating that ALK-4* sequences are indeed needed to respond to the inhibitory effects of ALK-2*. This result also indicates that inhibitory effects of ALK-2* do not involve competition for molecules downstream of type I receptors; if they did, ALK-2* should inhibit dorsal gene induction by C2.
Figure 3.4. ALK-4 sequences confer susceptibility to inhibition of dorsal gene activation by ALK-2*. (A) ALK-2* inhibits dorsal gene activation by ALK-4*, but not by C2 (which is based on an ALK-2*) backbone. (B) ALK-2* inhibits dorsal gene activation by ALK-4*, C9, C11 and C13 (all of which contain an ALK-4* backbone). Animal caps were dissected at stage 8 from embryos injected with RNA (500 pg) encoding ALK-4*, ALK-2*, C2, C9, C11 or C13, either alone or in the indicated combinations. Caps were cultured until early gastrula stage 10 and then analysed by RNAase protection using probes specific for goosecoid and Pintallavis. EF-1α was used as a loading control. These experiments were done in collaboration with Niall Armes.
To confirm that susceptibility to inhibition requires sequences present in ALK-4*, ALK-2* was co-expressed with C9, C11 and C13, all of which are capable of activating expression of Pintallavis and to some extent goosecoid, and all of which are based on an ALK-4* backbone (Fig. 3.2). Fig. 3.4B shows that ALK-2* is capable of inhibiting dorsal gene induction by these constructs.

**Over-expression of Smad1 or Smad5 does not interfere with induction of dorsal genes by ALK-4***

In the introduction to this chapter I mentioned that the IB1 region of the type I receptor is thought to feed directly into the Smad signalling pathway (Chen et al., 1998). If this is so, then since the dominant ventralising effects of ALK-2* are not specified by the IB1 region, over-expression of ventralising Smad family members like Smad1 and Smad5 should also fail to counteract the effects of ALK-4*. Consistent with this suggestion, Figure 3.5A shows that Smad1 is completely ineffective at suppressing the ability of ALK-4* to induce Pintallavis and goosecoid at stage 10.5. Smad1 is also unable to suppress the ability of activin to induce expression of goosecoid and Pintallavis (Fig. 3.5B), although a recent report suggests that Smad1 is capable of reducing the induction of goosecoid by activin by stage 11 (Liu et al., 1996).

Similar experiments reveal that Smad5 is also capable of eliciting little interference with dorsal gene induction, producing only a weak inhibition which resembles that obtained with the C10 chimera (Fig. 3.5C). From this, I conclude that the rapid ventralising effects of ALK-2* are not mediated solely through Smad signalling.

**ALK-2* does not interfere with Smad2/Smad4-mediated induction of dorsal genes in Xenopus animal caps**

ALK-2* suppresses ALK-4* dorsal gene induction in a dominant and direct fashion (Armes and Smith, 1997), suggesting that ALK-2* targets ALK-4*, or
Figure 3.5. Smad1 does not interfere with dorsal gene activation by ALK-4* or activin. Smad5 elicits slight interference, comparable to that obtained with chimera C10. (A) Smad1, unlike ALK-2*, does not interfere with dorsal gene induction by ALK-4*. (B) Smad1, unlike ALK-2*, does not interfere with dorsal gene induction by activin. (C) Smad5 causes only slight interference with dorsal gene induction by ALK-4*, comparable to that caused by C10. Animal caps were dissected at stage 8 from embryos injected with RNA encoding ALK-4* (500 pg), ALK-2* (500 pg) Smad1 (2 ng), Smad5 (2 ng) or chimera C10 (500 pg), either alone or in the indicated combinations. Caps in lanes 3, 5 and 7 of panel (B) were treated with 8 units/ml activin. All animal caps were cultured until early gastrula stage 10 and then analysed by RNAase protection using probes specific for goosecoid, Pintallavis and Xbra. EF-1α was used as a loading control. All lanes shown in each panel are from the same gel, but their order has been changed for clarity of presentation. Experiments shown in panel (B) and (C) were done in collaboration Niall Armes.
components of its downstream signalling pathway(s), directly to elicit this immediate effect. Given that the IB1 of type I receptors might channel directly into the Smad signalling pathway (Armes et al., 1998), it is possible that ALK-2* counteracts dorsal gene induction by ALK-4* by preventing Smad signalling downstream of ALK-4*. I addressed this by testing the ability of ALK-2* to interfere with dorsal gene induction by Smad2, which, like ALK-4*, acts in a concentration dependent manner to induce the expression of dorsal genes (Baker and Harland, 1996).

Figure 3.6A shows that gene induction by Smad2, is not affected by ALK-2*. In this preliminary experiment, Smad2 induced expression of Xbra and a tiny amount of Pintallavis, an expression profile that looks very similar to that of ALK-2* itself. Thus, Smad2 induced no dorsal gene expression for ALK-2* to suppress.

The common mediator Smad, Smad4, co-operates with Smad2 and increases the dorsal gene inducing capacity of Smad2 (Zhang et al., 1996, Candia, 1997 #1389). I next tested the ability of ALK-2* to interfere with dorsal gene induction in animal caps co-expressing Smad2 and Smad4. Figure 3.6B shows that Smad2 and Smad4, synergise to induce an ALK-4*-like response in Xenopus animal caps. However, in contrast to results obtained with ALK-4*, dorsal gene activation by Smad2 and Smad4 is not suppressed by ALK-2* (Fig. 3.6B), suggesting that ALK-2* blocks ALK-4*-induced dorsal gene expression upstream of Smad signalling (Fig. 3.7).

DISCUSSION

Studies on the molecular basis of type I receptor signalling specificity have shown that the dorsal gene inducing properties of ALK-4* can be transferred to ALK-2* by the transfer of a small peptide loop, the β4-β5 loop, from the kinase domain of one receptor to the other (Armes et al., 1998). The dorsal inducing effects of the chimeric ALK-2* receptor resemble those that have been reported for Smad2 (Baker and Harland, 1996), and it has been postulated that this loop interacts with the Smad signalling pathway (Armes et al., 1998). Similar conclusions have been obtained by analysis of chimeric
Figure 3.6. ALK-2* does not interfere with the induction of dorsal genes in *Xenopus* animal caps expressing Smad2 and Smad4. (A) ALK-2* has no observable effect on gene activation by Smad2. In fact ALK-2* induced gene expression looks very similar to that of Smad2. (B) ALK-2* does not interfere with dorsal gene induction by Smad2 and Smad4. Animal caps were dissected at stage 8 from embryos injected with RNA encoding ALK-4* (500 pg), ALK-2* (500 pg), Smad2 (1 ng) or Smad4 (1 ng), either alone or in the indicated combinations. All caps were cultured until early gastrula stage 10 and then analysed by RNAase protection using probes specific for *goosecoid*, *Pintallavis* and *Xbra*. EF-1α was used as a loading control.
Figure 3.7. ALK-2* counteracts the dorsalising effects of ALK-4* upstream of Smad signalling, possibly by targeting ALK-4* directly.
receptors in TGF-β responsive cell lines (Feng and Derynck, 1997), but data presented in this chapter indicates that this loop of the kinase is not sufficient to account for all the signalling properties of type I receptors.

**The β4-β5 loop (IB1) does not define all signalling properties of type I receptors**

An attempt was made to transfer the activity of the ALK-2* receptor to the ALK-4* backbone. Chimera C10 carries only the β4-β5 loop of ALK-2* in the ALK-4* backbone and, like ALK-2*, is capable of inducing Xbra (Armes et al., 1998). However, whereas ALK-2* counteracts the dorsalising effects of ALK-4*, C10, and the other ALK-4* backbone chimeras containing the β4-β5 loop of ALK-2*, did not interfere efficiently with ALK-4* induction of goosecoid, and they had no effect at all on induction of Pintallavis. A likely explanation for this is that the signal specified by the IB1 region is exchanged correctly, but that additional signals transmitted via different regions of the receptor are required for the inhibitory phenomenon.

It does seem unlikely that all the effects of the type I receptors are specified solely by a loop of seven amino acids which is thought to feed into the Smad pathway (Feng and Derynck, 1997; Armes et al., 1998; Chen et al., 1998). Consistent with this, Smad1 and Smad5, like C10, have no significant effect on the ability of ALK-4* or activin to induce goosecoid at stage 10.5. This result is consistent with the observation that the ability of Smad2 to induce dorsal-specific gene expression is not compromised by co-expression of Smad1; rather, dorsal- and ventral-specific genes are expressed simultaneously (Graff et al., 1996). It appears to contrast with a suggestion that Smad1 decreases expression of goosecoid in response to activin (Liu et al., 1996), but in that paper suppression of goosecoid was incomplete, and the authors assayed the effects of Smad1 at a slightly later stage. Smad8 has recently been implicated as a downstream mediator of ALK-2* (Chen et al., 1997b), and it will be interesting to test whether it is capable of suppressing the ALK-4* dorsal response.
It is worth noting that I consistently observe induction of \textit{Xbra}, and usually \textit{Pintallavis}, in response to Smad1. In contrast Graff et al. have shown, by RT PCR, that Smad1 does not have the capacity to induce \textit{Xbra} (Graff et al., 1996). It is hard to rationalise this difference as, in my hands, Smad1 is a potent inducer of \textit{Xbra}.

**ALK-2\textsuperscript{*} blocks dorsal gene induction upstream of Smad signalling**

ALK-2\textsuperscript{*} is incapable of suppressing dorsal gene induction in animal caps expressing Smad2 and Smad4. This suggests that ALK-2\textsuperscript{*} blocks dorsal gene induction upstream of Smad signalling, quite possibly by targeting ALK-4\textsuperscript{*} directly (Fig. 3.7).

**Susceptibility to inhibition by ALK-2\textsuperscript{*} requires sequences present in ALK-4 that are not within the \(\beta_4-\beta_5\) loop**

The observation that ALK-2\textsuperscript{*} cannot inhibit dorsal gene induction by C2 indicates that ALK-2\textsuperscript{*} cannot function by competing for a common component of the Smad signalling pathway, such as Smad4 (see Candia, 1997). This is consistent with previous work indicating that the inhibitory effects of ALK-2\textsuperscript{*} are dominant (Armès and Smith, 1997) and it also indicates that susceptibility to inhibition by ALK-2\textsuperscript{*} requires sequences present in ALK-4.

**Type I receptor Smad-independent signalling pathways**

The data presented in this chapter suggests that ALK-2 and ALK-4 are capable of signalling, and receiving signals, through IB1- and Smad-independent pathways. One such pathway could involve TAB1 (Shibuya et al., 1996), a protein which regulates the activity of the serine-threonine kinase TAK1 (Yamaguchi et al., 1995; Moriguchi et al., 1996). \textit{Xenopus} TAK1 (xTAK1) can induce ventral mesoderm in animal cap explants. Furthermore expression of a kinase dead version of xTAK1 reduces mesoderm induction by Smad1, Smad5 and BMP-4 (Shibuya et al., 1998). Thus, xTAK1 and xTAB1 may function in the BMP signal transduction pathway in \textit{Xenopus} embryos in co-operation with Smad signalling. In addition, the isopropyl isomerase
FKBP12 (Wang et al., 1996a) and the farnesyl transferase $\alpha$-subunit have been shown to associate with the intracellular domains of type I receptors (Kawabata et al., 1995; Ventura et al., 1996), although any role for them in downstream signalling events is uncertain.
Chapter 4

ALK-4* specifies at least two different downstream signals

INTRODUCTION
As described in Chapters 1 and 3, the two activin type I receptors, ALK-2 and ALK-4, have distinct effects on gene expression in the *Xenopus* animal cap assay (Armes and Smith, 1997). Use of constitutively active versions of the receptors, ALK-2* and ALK-4*, shows that ALK-4* can reproduce the effects of activin treatment including the dose-dependent induction of progressively more dorso-anterior mesodermal and endodermal markers, whilst ALK-2* induces only ventral mesodermal markers and counteracts the effects of ALK-4* (Armes and Smith, 1997).

A 7 amino acid loop from ALK-4*, the IB1 (Fig. 4.1), is sufficient, when transferred into the ALK-2* backbone, to confer all the early dorsal gene inducing properties of ALK-4* to ALK-2* (Armes et al., 1998). However not all chimeric ALK-2* or ALK-4* backbone receptors that contain the ALK-4* IB1 have the ability to induce the same range of mesodermal markers as ALK-4*. Notably, neither the chimeric receptor created by transfer of the ALK-2* GS domain and its flanking sequences into ALK-4* (C9; Fig. 4.1), nor that created by transfer of the ALK-2* IB3 activation loop into ALK-4* (C11; Fig. 4.1) are capable of effectively inducing *goosecoid*, although both induce *Xbra* and *Pintallavis* (Armes et al., 1998).

In this chapter I have investigated C9 and C11 in more detail in an effort to determine why, although they contain the ALK-4* IB1, they are unable to induce significant levels of *goosecoid*. In particular, one possible explanation for the difference observed between ALK-4* and these chimeric receptors is that signalling from C9 and C11 is impaired in some way such that they simply send a weaker version of the ALK-4* signal. If this were so, one would predict that the effects of low levels of ALK-4* would resemble those of high concentrations of C9 or C11. However, the results of this and other experiments presented in this chapter suggest that C9 and C11 signalling is
Figure 4.1. Chimeric receptors. A schematic representation of ALK-2*, ALK-4* and the ALK-4* backbone chimeric receptors C9, C11 and C13, created by transfer of candidate specificity regions from ALK-2* into ALK-4*. Exchanged regions derived from ALK-2* are shown in red.
qualitatively different from that of ALK-4*, suggesting that ALK-4* may regulate at least two different signalling events, one which is retained in C9 and C11 that is required for induction of \textit{Xbra} and \textit{Pintallavis}, and a second that is required for induction of \textit{goosecoid}.

\textbf{RESULTS}

\textbf{The signalling specificities of the ALK-4* backbone chimeras, C9 and C11, are qualitatively different to that of ALK-4*.}

Transfer into ALK-4* of the ALK-2* GS domain and its flanking sequences (C9; Fig. 4.1), or of the ALK-2* IB3 activation loop (C11; Fig. 4.1), results in perturbation of receptor function and loss of \textit{goosecoid} activation (Armes et al., 1998). As a first step to understanding why these two chimeras fail to induce expression of \textit{goosecoid}, despite the fact that they contain the ALK-4* IB1, I compared the mesoderm-inducing activity of C9 and C11, over a 400 fold range of RNA concentrations, with that of ALK-4*. As in Chapter 3, I assayed for expression of the mesodermal markers \textit{goosecoid}, \textit{Pintallavis} and \textit{Xbra}. Figure 4.2 shows the dose response profile of these markers in stage 10 animal caps from embryos that were injected at the one cell stage with 10 pg – 4 ng of synthetic mRNA encoding ALK-4 or C9. As expected, low doses of ALK-4* (10 pg) were sufficient to induce expression of \textit{Xbra} and as more mRNA was injected expression of \textit{Xbra} eventually declined (Fig. 4.2). The dose-response profile for \textit{goosecoid} was different, with low doses of ALK-4* inducing only low levels of \textit{goosecoid}. More \textit{goosecoid} was expressed as the amount of ALK-4* mRNA increased and there was no decrease in expression observed at the highest concentration of ALK-4* mRNA (4 ng), in contrast to \textit{Xbra}. There is evidence to suggest that down regulation of \textit{Xbra} is attributable to suppression of transcription by homeobox containing genes such as \textit{goosecoid} (Latinkic et al., 1997). This fits perfectly with the dose response profile of ALK-4*, with a gradual down-regulation of \textit{Xbra} as the level of \textit{goosecoid} expression increases. High levels of \textit{Pintallavis} were induced in response to all doses of ALK-4*, with more
Figure 4.2. Comparison of the concentration-dependent induction of Xbra, Pintallavis and goosecoid in response to C9 and ALK-4*.* Embryos were injected at the one cell stage with 10 pg, 100 pg, 500 pg, 1 ng or 4 ng of synthetic mRNA encoding either C9 or ALK-4*. Animal caps were cut at stage 8 and expression of Xbra, Pintallavis and goosecoid was assayed by RNAase protection at stage 10.5. EF-1α was used as a loading control.
Pintallavis expressed in animal caps that expressed a significant amount of goosecoid.

In contrast to ALK-4*, C9 was incapable of inducing goosecoid over the 400 fold mRNA concentration range tested (Fig. 4.2). As with ALK-4*, low doses of C9 were sufficient to induce expression of Xbra. However, in contrast to ALK-4*, the level of Xbra induced increased as more C9 mRNA was injected, and there was no decrease in Xbra expression at the highest concentration of C9 mRNA (4 ng). It seems likely that the absence of goosecoid expression may enable the persistence of Xbra expression in response to high doses of C9 mRNA. Low doses of C9 were sufficient to induce Pintallavis, the levels of which increased as more C9 mRNA was injected. However, C9 did not induce Pintallavis to such high levels as ALK-4*. Thus, nowhere on the dose response profile of goosecoid, Xbra and Pintallavis expression, does C9 resemble ALK-4*.

The dose response profile of C11, over the 400 fold RNA concentration range tested, is similar to that of C9, but does not resemble that of ALK-4* (Fig. 4.3). Significant expression of Xbra occurred in animal caps from embryos injected with 500 pg or more of C11 mRNA, and the levels of Xbra induced in response to C11 were almost 5-fold greater than was ever seen in response to ALK-4* (Fig. 4.4). In contrast to ALK-4*, goosecoid was only expressed at low levels in animal caps from embryos injected with 4 ng C11 mRNA (Fig. 4.3). Pintallavis was expressed in animal caps from embryos injected with 500 pg or more C11 mRNA, with highest levels of Pintallavis expression in the animal cap sample that also expressed goosecoid (Fig. 4.3).

As the dose response profiles of C9 and C11 resemble each other, but do not resemble that of ALK-4*, this suggests that C9 and C11 signalling is qualitatively different from that of ALK-4*. Thus, it seems likely that ALK-4* specifies at least two different signals; one that is retained in C9 and C11 that is required for the induction of Xbra and Pintallavis, and a second that is required for the strong induction of goosecoid. In addition to this, Figure 4.2 and 4.3 show that Pintallavis is expressed at higher levels in samples that also express goosecoid. This suggests that there may be two distinct rates of
Figure 4.3. Comparison of the concentration-dependent induction of Xbra, Pintallavis and goosecoid in response to C11 and ALK-4*. Embryos were injected at the one cell stage with 10 pg, 100 pg, 500 pg, 1 ng or 4 ng of synthetic mRNA encoding either C11 or ALK-4*. Animal caps were cut at stage 8 and expression of Xbra, Pintallavis and goosecoid was assayed by RNAase protection at stage 10.5. EF-1α was used as a loading control.

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Figure 4.4. C11 induces almost 5 times more Xbra than is ever seen in response to ALK-4*. Graph showing the relative levels of Xbra induced in response to C11 and ALK-4* in the RNAase protection shown in Figure 4.3.
*Pintallavis* expression that correspond to two different signals specified by ALK-4*, one which induces lower levels of *Pintallavis* expression in the absence of *goosecoid* expression, and a second that boosts *Pintallavis* transcription and induces *goosecoid* expression.

**Co-expression of C9 and C11 does not restore *goosecoid* induction.**

In contrast to C9 or C11, simultaneous transfer of the ALK-2* GS domain and its flanking sequences together with the ALK-2* IB3 activation loop into ALK-4*, creating C13 (Fig. 4.1), has little effect on the assayable receptor function, and it can induce *goosecoid* strongly (Armes et al., 1998). This suggests that there is a requirement for compatibility between sequences in the IB3 activation loop and sequences in the juxtamembrane region, in or around the GS domain, for the induction of *goosecoid*. Thus, the signal specified by ALK-4* that is required for the strong induction of *goosecoid* could depend upon compatibility between these two regions. It is possible that these regions lie close to each other in the tertiary structure of the receptor and that the activation loop is required for phosphorylation of the juxtamembrane region.

Analysis of $^{125}$I-labelled TGF-β1 crosslinked to its receptors has suggested that the TGF-β receptor signalling complex is a heterotetramer consisting of two type I receptors and two type II receptors (Yamashita et al., 1994). Given that C9 contains the ALK-2* GS domain and its flanking sequences in an ALK-4* backbone and C11 contains the ALK-2* IB3 activation loop in an ALK-4* backbone (Fig. 4.1), it seemed possible that co-expression of these chimeric receptors would enable them to co-operate, within a heterotetrameric receptor complex, to induce the expression of *goosecoid*. However, this was not the case (Fig. 4.5). C9 and C11 cannot act in trans to restore *goosecoid* expression.
Figure 4.5. Co-expression of C9 and C11 does not restore _goosecoid_ expression. Embryos were injected at the one cell stage with 1 ng of synthetic mRNA encoding ALK-4*, C9 or C11, or 1 ng of both C9 and C11 mRNA. Animal caps were dissected at stage 8 and expression of _Xbra, Pintallavis_ and _goosecoid_ was assayed by RNAase protection at stage 10.5. EF-1α was used as a loading control.
Mutation of juxtamembrane serine 172 and threonine 176 does not affect ALK-4* signalling capacity.

The above results suggest that ALK-4* specifies at least two different signals; one that is sufficient for the induction of Xbra and Pintallavis expression, and a second that is required for induction of goosecoid expression and upregulation of Pintallavis expression. There is also evidence to suggest that ALK-5, the type I receptor for TGF-β, specifies at least two different signals (Saitoh et al., 1996). TGF-β is the founder member of the TGF-β superfamily and it regulates many cellular functions, including cell proliferation and extracellular matrix formation (reviewed in Heldin et al., 1997).

Investigation of the role of the ALK-5 juxtamembrane region preceding the GS domain identified two residues within this region, serine 172 and threonine 176, that were found to be essential for signalling a TGF-β antiproliferative response, but not extracellular matrix formation, as measured by plasminogen activator inhibitor-1 (PAI-1) and fibronectin induction (Saitoh et al., 1996). Identification of such cytoplasmic regions that are important only for a limited response suggested that at least two different signals are specified through different cytoplasmic domains of ALK-5. Analysis of the ALK-4 juxtamembrane region revealed that serine 172 and threonine 176 are conserved between ALK-4 and ALK-5 (Fig. 4.6). This suggested that, as with ALK-5, these two residues may be required for a limited ALK-4-like response, raising the possibility that these residues could be necessary for the induction of goosecoid by ALK-4*. However the effects of mutating either juxtamembrane serine 172 to alanine or threonine 176 to valine, creating ALK-4*S^{172}\rightarrow A and ALK-4*T^{176}\rightarrow V, were indistinguishable from ALK-4* (Fig. 4.7).

ΔSmad4 blocks ALK-2*, C9 and C11 responses, but not ALK-4*, C2 and C4 responses

Proteins from the Smad family are known to act downstream of type I receptors (see Kretzschmar and Massague, 1998; Heldin et al., 1997). and recent evidence, from experiments with the BMP receptor ALK-3 and the
Figure 4.6 A sequence alignment of different type I receptors. Serine 172 and threonine 176 of ALK-4 are indicated. sax= saxophone tkv=thick veins
Figure 4.7. Mutation of juxtamembrane serine 172 and threonine 176 does not affect ALK-4* signalling capacity. Embryos were injected at the one cell stage with 1 ng of synthetic mRNA encoding ALK-4*, C9, ALK-4* S\textsuperscript{172}→A or ALK-4* T\textsuperscript{176}→V. Animal caps were dissected at stage 8 and expression of Xbra, Pintallavis and goosecoid was assayed by RNAase protection at stage 10.5. EF-1α was used as a loading control.
TGF-β receptor ALK-5, suggests that the IB1 from type I receptors feeds directly into the Smad signalling pathway (Chen et al., 1998). Chen et al. demonstrated that transfer of the ALK-3 IB1 to ALK-5 causes ALK-5 to lose the ability to: associate with and activate Smad2, induce the formation of Smad2-Smad4 complexes, induce translocation of Smad2 to the nucleus and induce TGF-β responses (Chen et al., 1998). Instead it gained the ability to: associate with and activate Smad1, induce the formation of Smad1-Smad4 complexes, induce translocation of Smad1 to the nucleus and induce BMP-like responses. The reciprocal pattern was observed with ALK-3 containing the ALK-5 IB1 (Chen et al., 1998).

The importance of Smad proteins in mesoderm formation has been emphasised by experiments which show that a truncated form of Smad4 (the common mediator Smad), ΔSmad4, inhibits mesoderm induction (Lagna et al., 1996; Candia et al., 1997). ΔSmad4 completely blocks induction of mesoderm by Smad1, Smad2 and Smad4, and slightly reduces the induction of ventral mesodermal markers in response to activin (Lagna et al., 1996). The limited effect of ΔSmad4 on the induction of ventral mesoderm by activin suggests that there are at least two signalling pathways that act downstream of activin, one which is sensitive to ΔSmad4 and one (or more) that is resistant to ΔSmad4.

ΔSmad4 has been shown to dimerise with Smad4 in the absence of ligand stimulation (Lagna et al., 1996), but nothing more is known about how it functions. Although little is known about the way ΔSmad4 acts, it does provide a tool with which to dissect TGF-β signalling pathways. For this reason, I decided to compare the ability of ΔSmad4 to block C9, C11, ALK-2* and ALK-4* signalling.

Embryos were injected at the one cell stage with 1 ng of synthetic mRNA encoding ALK-2*, ALK-4*, Smad1, Smad2, C9 or C11. Half of the embryos from each sample were also injected at the same time with 1 ng of synthetic mRNA encoding ΔSmad4. Animal caps were dissected at mid-blastula stage 8, cultured until gastrula stage 10 and then assayed by RNAase protection for the expression of goosecoid, Pintallavis and Xbra. As expected, induction of
Figure 4.8. ΔSmad4 blocks ALK-2*, C9 and C11 responses. Embryos were injected at the one cell stage with 1 ng of synthetic mRNA encoding ΔSmad4, ALK-2*, ALK-4*, Smad1, Smad2, C9 or C11 and half of the embryos from each sample were also injected with 1 ng of ΔSmad4. Animal caps were dissected at stage 8 and expression of Xbra, Pintallavis and goosecoid was assayed by RNAase protection at stage 10.5. EF-1α was used as a loading control.
Figure 4.9. Chimeric receptors. A schematic representation of ALK-2*, ALK-4* and the ALK-2* backbone chimeric receptors C2 and C4, created by transfer of candidate specificity regions from ALK-4* into ALK-2*. Exchanged regions derived from ALK-4* are shown in green.
Figure 4.10. ΔSmad4 does not block C2 and C4 responses. Embryos were injected at the one cell stage with 1 ng of synthetic mRNA encoding ΔSmad4, Smad2, C2 or C4 and half of the embryos from each sample were also injected with 1 ng of ΔSmad4. Animal caps were dissected at stage 8 and expression of Xbra, Pintallavis and goosecoid was assayed by RNAase protection at stage 10.5. EF-1α was used as a loading control.
these mesodermal markers in response to Smad1 and Smad2 was completely blocked by ∆Smad4 (Lagna et al., 1996; Candia et al., 1997) and the same was true for ALK-2*, C9 and C11 (Fig. 4.8; Table 4.1). ∆Smad4 had no effect, however, on mesoderm induction in response to ALK-4*. Thus C9 and C11 signalling is ∆Smad4-sensitive and ALK-4* signalling is ∆Smad4-resistant.

As ∆Smad4 had no effect on mesoderm induction in response to ALK-4*, I decided to test whether ∆Smad4 was capable of blocking dorsal gene induction by the ALK-2* backbone chimeric receptors C2 and C4 (Fig. 4.9). C2 was created by transfer into ALK-2* of the ALK-4* IB1, and C4 was created by transfer into ALK-2* of both the ALK-4* GS domain and its flanking sequences and the ALK-4* IB1 (Fig. 4.9) (Armes et al., 1998). Like ALK-4*, C2 and C4 are potent inducers of goosecoid (Fig. 4.10) (Armes et al., 1998), and, like ALK-4*, their ability to induce goosecoid is not affected by ∆Smad4 (Fig. 4.10; Table 4.1). Thus ∆Smad4 cannot block mesoderm induction by ALK-4*, C2 and C4, all of which are capable of activating the expression of dorsal-specific genes.

Table 4.1.

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<th>∆Smad4 blocks mesoderm induction</th>
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</thead>
<tbody>
<tr>
<td>Smad1</td>
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<tr>
<td>Smad2</td>
</tr>
<tr>
<td>ALK-2*</td>
</tr>
<tr>
<td>ALK-4*</td>
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<tr>
<td>C2/C4</td>
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<tr>
<td>C9/C11</td>
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Together, these experiments reveal a difference in signalling by Smad1, Smad2, ALK-2*, C9 and C11, which cannot induce goosecoid expression and are sensitive to ∆Smad4, and signalling by ALK-4*, C2 and C4, which are
Figure 4.11. Co-expression of Smad2, but not C9 or C11, with Smad4 mimics ALK-4* responses. Embryos were injected at the one cell stage with 1 ng of synthetic mRNA encoding ALK-4*, Smad4, Smad2, C9 or C11 and half of the embryos from the samples injected with Smad2, C9 and C11 were also injected with 1 ng of Smad4. Animal caps were dissected at stage 8 and expression of Xbra, Pintallavis and goosecoid was assayed by RNAase protection at stage 10.5. EF-1α was used as a loading control.
strong activators of goosecoid and are resistant to ΔSmad4. These results are discussed below.

**Co-expression of Smad2 and Smad4, but not C9 or C11 and Smad4, mimics ALK-4* responses.**

Figure 4.11 shows that expression of C9 or C11 in animal caps results in a gene expression profile similar to that of Smad2, suggesting that C9 and C11 may signal via Smad2. Co-expression of Smad2 and Smad4 dorsalises the Smad2 response, resulting in the induction of goosecoid, thereby mimicking ALK-4* responses (Fig. 4.11). As C9 and C11 induce the same responses as Smad2 in animal caps, it seemed possible that the ability of C9 and C11 to activate goosecoid might be restored by co-expression with Smad4. This was not the case, demonstrating that C9 and C11 signalling is different from that of exogenous Smad2, even though they induce similar gene expression profiles.

Over-expression of Smad4 with C9 had no effect on the gene inducing potential of C9, while overexpression of Smad4 with C11 almost completely blocked C11 responses (Fig. 4.11; see discussion). This indicates that there are differences in C9 and C11 signalling, although both share the ability to induce Xbra and Pintallvis, and both fail to activate high levels of goosecoid.

**C9 induces secondary axes.**

ALK-4* and C9 have distinct effects in the Xenopus animal cap assay; ALK-4* induces Xbra, Pintallavis and goosecoid in a dose dependent manner, while C9 can only induce Xbra and Pintallavis. Another useful functional assay is to test the ability of a given factor to induce secondary axes when injected into the ventral side of early cleavage stage embryos. Thus, I next compared the abilities of ALK-4* and C9 to induce secondary axes when injected into the ventral side of 32-cell stage embryos.

Injection of 100 pg of ALK-4* mRNA into one C4 blastomere (see Dale, 1987a for blastomere nomenclature) of the 32-cell stage embryo was successful in generating duplicated axes (Fig. 4.12C). In a typical experiment, 38% of
Figure 4.12. C9 and ALK-4* can induce secondary axes when expressed in ventral blastomeres of the *Xenopus* embryo. (A) Schematic illustration of 32-cell stage embryo showing injection into C4 blastomere. 100 pg of synthetic mRNA encoding C9 or ALK-4* was injected into one of the C4 blastomeres of 32 cell stage embryos. Embryos were allowed to develop until stage 40 before being fixed and stained for notochord using the monoclonal antibody MZ15. (A) control embryo. (B) C9 induces a partial secondary axis in 57% of embryos. Axes contained notochord and, in most instances, one or two otic vesicles with anterior tissue extending beyond the otic vesicles. (C) ALK-4* induces a partial secondary axis in 38% of embryos. Most contained notochord and all contained one or two otic vesicles with anterior tissue extending beyond the otic vesicles.
injected embryos formed secondary axes (n=26; Table 4.1). Staining with the monoclonal antibody MZ15 revealed that the secondary axes contained notochord. The secondary axes were never complete, however almost all had one or two otic vesicles with some tissue found anterior to the otic vesicles (Table 4.2).

Equivalent experiments performed with C9 gave similar results, although injection of 100 pg of ALK-4* mRNA into one C4 blastomere of the 32-cell stage embryo was more efficient at inducing secondary axes than ALK-4* (Fig. 4.12B). In a typical experiment 57% of embryos formed secondary axes (n=30; see Table 4.2). All of the secondary axes contained notochord (Table 4.2). Indeed, C9 was a much more efficient inducer of notochord than ALK-4*. Most appeared to have one or two otic vesicles with some tissue found anterior to the otic vesicles.

**Table 4.2.** C9 can induce secondary axes when expressed in ventral blastomeres of the *Xenopus* embryo (see Fig. 6.7 for experimental details).

<table>
<thead>
<tr>
<th>Injection</th>
<th>n</th>
<th>Number of embryos with secondary axis induced</th>
<th>Number of secondary axes with notochord</th>
<th>Number of secondary axes with otic vesicle(s)</th>
<th>Number of secondary axes with tissue anterior to otic vesicles</th>
</tr>
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<tbody>
<tr>
<td>C9</td>
<td>30</td>
<td>17</td>
<td>17</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>ALK-4*</td>
<td>26</td>
<td>10</td>
<td>8</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

This data shows that ALK-4* and C9 have similar effects in the axial duplication assay, while they have distinct effects in the animal cap assay. C9 is, however, a more efficient inducer of secondary axes and notochord than ALK-4*.
DISCUSSION

ALK-4* specifies at least two different downstream signals

The IB1 of type I receptors is thought to feed directly into the Smad signalling pathway as transfer of the ALK-3 IB1 to ALK-5, and vice versa, switches the ability of these receptors to associate with and activate pathway specific Smad proteins (Chen et al., 1998). The IB1 from ALK-4* is sufficient, when transferred into the ALK-2* backbone, to confer all the dorsal gene inducing properties of ALK-4* to ALK-2* (Armes et al., 1998). However, experiments presented in this chapter using the ALK-4* backbone chimeric receptors C9 and C11 (Fig. 4.1) (Armes et al., 1998) demonstrate that, although they contain the ALK-4* IB1, they behave in a qualitatively different fashion to ALK-4*. Over a 400 fold RNA concentration range, the C9 and C11 dose response profiles of Xbra, Pintallavis and goosecoid expression are similar to each other (the exception being the capacity of C11, at very high doses, to induce some goosecoid), but bear no resemblance to that of ALK-4*. This supports the notion that C9 and C11 signalling is not impaired such that they send a weaker version of the ALK-4* signal, but rather that they are qualitatively different to ALK-4*. The inability of C9 and C11 to induce significant amounts of goosecoid suggests that there are at least two signals specified by ALK-4*; one activated by C9 and C11, that is sufficient to induce Xbra and Pintallavis transcription, and another which is required for strong activation of goosecoid.

In all of the RNAase protection experiments presented in this chapter there is an upregulation in the level of Pintallavis expressed in animal caps that also express goosecoid. Thus, it seems likely that the signal transmitted by ALK-4* that is required for goosecoid activation, also causes upregulation of Pintallavis expression. This second signal may also be sufficient to induce Xbra, however evidence suggests that Xbra expression is down-regulated by homeobox containing genes such as goosecoid (Latinkic et al., 1997). Thus, any Xbra that might be expressed in response to this signal is likely to be suppressed by goosecoid.
Consistent with the suggestion that ALK-4* specifies two signals, the TGF-β type I receptor ALK-5 transmits two signals. Point mutations in the ALK-5 juxtamembrane region demonstrated that serine 172 and threonine 176 are essential for signalling a TGF-β antiproliferative response, but not extracellular matrix formation (Saitoh et al., 1996). Mutation of these residues in ALK-4*, however, had no effect on the signalling capacity of ALK-4*, indicating that other residues in the ALK-4* juxtamembrane region are determinants of ALK-4* signalling specificity.

Co-expression of C9 and C11 does not restore goosecoid activation

Goosecoid activation by ALK-4* or ALK-4* backbone chimeric receptors depends on compatibility between sequences in the IB3 activation loop and sequences in the juxtamembrane region, in or around the GS domain. Thus, for an ALK-4*-like response, these two regions must either both be from ALK-4* or they must both be from ALK-2*. It appears that the requirement for compatibility between these regions must lie within the receptor itself because C9, which contains the ALK-2* GS domain and its flanking sequences, and C11, which contains the ALK-2* IB3 activation loop, cannot co-operate within a heterotetrameric receptor complex to restore goosecoid activation.

ΔSmad4 blocks ALK-2*, C9 and C11 responses, but not ALK-4*, C2 and C4 responses

In an effort to dissect the downstream signalling pathways of ALK-4*, ALK-2*, C2, C4, C9 and C11, I performed experiments with a truncated form of Smad4, ΔSmad4, that completely blocks the induction of mesoderm by exogenous Smad1, Smad2 and Smad4 in Xenopus animal pole explants (Lagna et al., 1996). It also reduces the induction of ventral mesodermal markers in response to activin (Lagna et al., 1996). I found that ΔSmad4 exerts different effects on the two activin receptors, ALK-2 and ALK-4; it completely blocks ALK-2* responses, but has no effect on those of ALK-4*. This suggests that the slight reduction of activin-induced ventral
mesodermal marker expression by ΔSmad4, observed by Lagna et al., is a result of inhibition of ALK-2* signalling, rather than ALK-4* signalling.

In addition to blocking ALK-2* responses, ΔSmad4 blocks the responses of C9 and C11, but, as with ALK-4*, it has no effect on C2 and C4. Thus, ΔSmad4 blocks the responses of receptors that do not induce significant levels of goosecoid expression, but has no effect on those that do. These experiments reveal a difference in signalling by ALK-2*, C9 and C11 compared with that of ALK-4*, C2 and C4.

As ΔSmad4 blocks mesoderm induction by Smad1, Smad2 and Smad4, but not that of ALK-4*, C2 and C4, it might seem reasonable to consider that ALK-4*, C2 and C4 signal independently of Smad proteins. However, this is unlikely as these receptors all require the ALK-4* IB1 to induce goosecoid (Armes et al., 1998), and the IB1 of type I receptors is thought to feed directly into the Smad signalling pathway (Chen et al., 1998).

It is difficult to determine why ΔSmad4 blocks mesoderm induction by ALK-2*, C9 and C11, but not ALK-4*, C2 and C4, as it is not known how ΔSmad4 functions. This emphasises the difficulty of interpreting experiments with dominant-negative reagents when it is not known how they act. However, present knowledge suggests that ΔSmad4 blocks mesoderm formation in response to exogenous Smad1, Smad2 and Smad4 either by forming homodimers with Smad4, or by forming homodimers with Smad4 and heterodimers with Smad1 or Smad2, thereby blocking downstream responses. As such, my experiments suggest that ΔSmad4 can associate with Smad proteins that have been activated by ALK-2*, C9 or C11, but not with those that have been activated by ALK-4*, C2 or C4. This indicates that ALK-2*, C9 and C11 are likely to regulate Smad proteins differently from ALK-4*, C2 and C4. For example, receptor-regulation may alter Smad protein conformation, or it may alter the ability of Smad proteins to interact with cofactors. Such regulatory events are likely to determine whether or not ΔSmad4 can associate with receptor-activated Smad proteins and block receptor responses.
Interestingly, Smad4 is phosphorylated in response to activin treatment, but not TGF-β or BMP treatment (Lagna et al., 1996). The functional significance of this difference is not yet known, but it does highlight a difference in the regulation of Smad4 between these ligands. It is possible that ALK-4*, C2 and C4 cause phosphorylation of Smad4, but ALK-2*, C9 and C11 do not. This may be the reason why ΔSmad4 is able to block ALK-2*, C9 and C11 responses, but not ALK-4-like responses. Phosphorylation of Smad4 and ΔSmad4 in response to ALK-4* and these ALK-2*/ALK-4* chimeric receptors is currently being tested.

Co-expression of Smad2 and Smad4, but not C9 or C11 and Smad4, mimics ALK-4* responses

Co-expression of Smad2 and Smad4 in Xenopus animal pole explants dorsalises the Smad2 response resulting in the induction of goosecoid, thereby mimicking ALK-4* transcriptional responses. At saturating levels, exogenous Smad2 and Smad4 are likely to associate to form Smad2-Smad4 hetero-dimers that can enter the nucleus and induce ALK-4*-like responses, without the need for receptor regulation (Fig. 4.13B). One model to explain why exogenous Smad2 and Smad4 can bypass the need for receptor regulation is that receptor regulation may alter the ability of Smad proteins to interact with cofactors that determine whether pathway-specific Smad proteins can interact with Smad4. At saturating levels, exogenous Smad2 and Smad4 would saturate any Smad cofactors, which might otherwise inhibit Smad2-Smad4 association, enabling exogenous Smad2 and Smad4 to associate and induce downstream responses.

The mesodermal marker expression profiles of C9 and C11 resemble that of Smad2, although, unlike Smad2, co-expression of C9 or C11 with Smad4 does not enable them to activate goosecoid. Results described above show that C9 and C11 signalling is blocked by ΔSmad4, which strongly suggests that these chimeric receptors signal through Smad proteins. One possible reason why co-expression of C9 or C11 with full length Smad4 does not rescue their ability to activate goosecoid is that C9 and C11 receptor regulated Smad proteins may have lost the ability to form complexes with Smad4.
Figure 4.13. (A) Model showing ALK-4* signalling in animal caps. (B) Model showing that exogenous Smad2 and exogenous Smad4 can induce an ALK-4*-like response when co-expressed in animal caps in the absence of receptor regulation. (C) Model showing the possible response to co-expression of C9 and Smad4 in animal caps. C9-receptor activated Smad2 is unable to interact with exogenous Smad4 and induces expression of Xbra and Pintallavis. (D) Model showing the possible response to co-expression of C11 and Smad4 in animal caps. C11-receptor activated Smad2 associates with exogenous Smad4, but this complex blocks downstream responses.
and/or to enable these complexes to translocate to the nucleus. This suggests a simple model in which normal receptor activation is disrupted in C9 and C11 such that they signal independently of Smad4.

Unlike C11, C9 signalling is not reduced by saturating levels of exogenous Smad4. One possible explanation for this is that C9-receptor-activated Smads are unable to interact with exogenous Smad4. This corresponds with the idea that C9 transmits a Smad4-independent signal (Fig 4.13C). C11 signalling is completely blocked by saturating levels of exogenous Smad4. In terms of C11, it seems likely that Smad4 is acting like ΔSmad4, probably by forming hetero-oligomers with C11-receptor-activated Smads, thereby blocking downstream signalling (Fig. 4.13D). This corresponds with the model that C11 transmits a Smad4-independent signal. The abilities of ALK-4*, C9 and C11 to induce Smad2-Smad4 association and nuclear translocation are currently being tested.

The fact that exogenous Smad4 blocks C11 responses, but not C9 responses, demonstrates that there are differences in signalling by C9 and C11, although both share the ability to induce Xbra and Pintallavis, and both fail to activate high levels of goosecoid.

**Model: ALK-4* specifies at least two different downstream signals**

The limited responses of C9 and C11 suggest a model in which ALK-4* specifies at least two different signals (Fig. 4.13); one, like C9 and C11, that is sufficient for the induction of Xbra and Pintallavis, and a second that is that is required for strong activation of goosecoid and causes the upregulation of Pintallavis (Fig. 4.14A). As mentioned earlier, the second signal may also be sufficient to induce Xbra, but as goosecoid has been shown to down-regulate Xbra (Latinkic et al., 1997), it is likely that any expression of Xbra in response to this signal would be suppressed.

One possibility described above is that C9 and C11 induce expression of Xbra and Pintallavis via a Smad4-independent signalling pathway. This suggests that ALK-4* may specify a Smad4-independent signal, like C9 and C11, for
Figure 4.14 (A) Model showing ALK-4* specifies at least two signals one, like C9 and C11, that induces expression of \textit{goosecoid} and a second that induceds expression of \textit{goosecoid} and upregulates expression of \textit{Pintallavis}. (B) Model showing C9 and C11 signal via a Smad4-independent pathway, and ALK-4* signals via Smad4-dependent and Smad4-independent pathways. C9, C11 and ALK-4* signal via a Smad4-independent pathway to induce expression of \textit{Xbra} and \textit{Pintallavis}, and ALK-4* also signals through a Smad4-dependent pathway to induce expression of \textit{goosecoid} and to upregulate expression of \textit{Pintallavis}. 
the induction of Xbra and Pintallavis, and a Smad4-dependent signal for the activation of goosecoid and the upregulation of Pintallavis (Fig. 4.14B).

ALK-4*, like activin, induces prospective ectodermal tissue to express different mesoderm-specific genes in a concentration-dependent fashion (Armes and Smith, 1997). The dose-dependent effects of activin and ALK-4* can be explained by this model (Fig. 4.14A); low doses of activin or ALK-4*, like C9 and C11, induce a signalling pathway that is sufficient to activate Xbra and Pintallavis, whereas high doses of activin and ALK-4* activate a signalling pathway that is required for the expression of goosecoid and upregulates the level of Pintallavis.

It has been shown that increasing occupancy of a single receptor type with activin can cause cells to switch gene expression (Dyson and Gurdon, 1998). It is possible that low receptor occupancy, like C9 and C11, induces a signalling event that is sufficient to activate expression of Xbra and Pintallavis, while a higher receptor occupancy induces a signalling event that is required for expression of goosecoid and upregulates the level of Pintallavis.

C9 induces secondary axes

Both C9 and ALK-4* are capable of inducing partial secondary axes containing notochord when injected into the ventral side of 32-cell stage embryos. Thus, C9 and ALK-4* exert similar effects in this assay while they exert distinct effects in the Xenopus animal cap assay. This demonstrates that marginal zone tissue and animal pole tissue respond differently to these receptors.

Surprisingly, ALK-4* was a less potent inducer of secondary axes and notochord than C9. One possible explanation for this is that ALK-4* may act in a dose-dependent way, when injected into the ventral side of a 32-cell stage embryo, to induce secondary axes that contain different tissue types, in the same way that ALK-4* acts in a concentration-dependent manner to induce dorsal mesodermal markers in animal cap explants. In support of this, in my hands ALK-4* induced no obvious head structures, although it
has previously been shown that ALK-4* is capable of inducing secondary axes that contain an eye (Armes and Smith, 1997). The induction of eyes by ALK-4* may well be a concentration-dependent effect. It will be necessary to do a titration of a range of concentrations of ALK-4* mRNA to determine its full axis-inducing potential.

Co-expression of synthetic mRNA encoding *Pintallavis* and *Xbra* in *Xenopus* prospective ectodermal tissue causes formation of dorsal mesoderm, including notochord (O'Reilly et al., 1995). As C9 induces *Xbra* and *Pintallavis* in animal caps, it is not surprising that it is capable of inducing secondary axes, including notochord, when injected into the ventral side of 32-cell stage embryos. Smad2, like C9, is a potent inducer of *Xbra* and *Pintallavis*, suggesting that it may play a role in regulating notochord cell-fate.

It will be interesting to compare the nature of the tissue found anterior to the otic vesicles in ALK-4* and C9 secondary axes by looking for the expression of anterior markers by in-situ hybridisation. It will be useful to include C11 in these experiments to see if it is also capable of inducing secondary axes.
Chapter 5

Identification of sequences within -2.1 kb of the Xbra2 transcription start site that respond to both FGF and activin

INTRODUCTION

Functional Brachyury protein is required for the proper formation of mesoderm in mouse, zebrafish and Xenopus embryos. Loss of Brachyury function in these species causes loss of posterior mesoderm and impairment of notochord differentiation (Herrmann et al., 1990; Halpern et al., 1993; Schulte-Merker et al., 1994b; Conlon et al., 1996). In mouse, chick, zebrafish and Xenopus embryos, the Brachyury gene is expressed at the onset of gastrulation throughout the nascent mesoderm and as gastrulation proceeds it is expressed in posterior mesoderm and notochord (Fig 1.11) (Herrmann et al., 1990; Smith et al., 1991; Schulte-Merker et al., 1992; Kispert et al., 1995b).

Understanding how expression of Brachyury is regulated will provide insight into how mesoderm is formed and patterned in vertebrate embryos. As a first step towards understanding the transcriptional control of Xenopus Brachyury (Xbra), the 5' regulatory region of the gene was isolated (Latinkic et al., 1997). The gene cloned appears to be a pseudoallele of Xbra and has been designated Xbra2. The existence of two Brachyury genes, Xbra and Xbra2, is consistent with the pseudotetraploid nature of the Xenopus laevis genome (Kobel and Du Pasquier, 1986). The regulation of Xbra2 is similar to that of Xbra (Smith et al., 1991), suggesting that they play similar roles in mesodermal differentiation.

2.1 kb of Xbra2 5' flanking sequence drives mesoderm specific expression of reporter constructs in Xenopus embryos (Latinkic et al., 1997). Furthermore, experiments using animal cap tissue demonstrated that 2.1 kb of Xbra2 5' flanking sequence is sufficient to confer responsiveness to FGF, to components of the FGF signal transduction pathway, and to low doses of activin (Latinkic et al., 1997).
This chapter presents experiments performed in an effort to define the cis-acting elements that confer responsiveness to FGF (and activin). In order to proceed with these experiments, it was first necessary to optimise the conditions of the luciferase assay system being used. I then went on to show that a reporter construct containing 381 bp upstream of the \textit{Xbra2} transcriptional start site responds to FGF (and activin) and \textit{Xenopus} Smad2, like the endogenous gene. A reporter construct containing only 231 bp upstream of the \textit{Xbra2} transcriptional start site was unable to respond to any of these factors. Thus, elements that respond to FGF, activin and \textit{Xenopus} Smad2 reside between -381 bp and -231 bp of the \textit{Xbra2} transcriptional start site.

**RESULTS**

**Removal of vector sequences from \textit{Xbra2pGL2} reporter constructs**

During the course of experiments designed simultaneously to record the expression of endogenous \textit{Xbral} and \textit{Xbra2} promoter reporter constructs in response to FGF, I discovered, by RNAase protection analysis, an unexpected ~200 nucleotide protected fragment protected by the \textit{Xbra2} RNAase protection probe in all RNA samples from embryos injected with linearised \textit{Xbral} promoter reporter constructs (data not shown, but see Fig. 5.1). It seemed possible that this fragment might be a result of expression of the luciferase reporter gene, regardless of FGF treatment. If so, this might have caused there to be high levels of luciferase activity that did not relate to FGF treatment in luciferase reporter assays. This was a concern as it might have made it difficult to interpret data from these assays.

Further RNAase protection analyses were performed in order to elucidate the origin of the 200 bp fragment. Animal caps were dissected at stage 8 from embryos that had been injected with: linearised -2100\textit{Xbra2pGL2}; linearised -220\textit{Xbra2pGL2} (a PCR based deletion of -2100\textit{Xbra2pGL2}); -2100\textit{Xbra2pGL2} from which the vector sequence had been removed; or supercoiled pEF-1\(\alpha\) \(\beta\)-galactosidase. pEF-1\(\alpha\) \(\beta\)-galactosidase was included as a control to determine whether or not the 200 bp protected fragment is specific to animal caps from
Figure 5.1. An ~200 nucleotide protected fragment, recognised by the Xbra2 RNAase protection probe, is only present in animal cap samples from embryos injected with linearised Xbra2 luciferase reporter constructs. Animal caps were dissected at stage 8 from embryos injected with supercoiled -2100Xbra2pGL2, linearised -2100Xbra2pGL2, -2100Xbra2pGL2 from which the vector sequence had been removed or supercoiled pEF-1α β-galactosidase. At stage 12 they were assayed for the expression of endogenous Xbra2 by RNAase protection. DNAs were injected in a volume of 10 nl at a concentration of 7 pg/nl. EF-1α was the loading control.
embryos injected with Xbra2 promoter reporter constructs. The animal caps were cultured to stage 12 with or without FGF, after which all the samples were subjected to RNAase protection analysis.

Figure 5.1 shows that endogenous Xbra2 is present in those samples that were treated with FGF-2. Strikingly, the 200 bp protected fragment recognised by the RNAase protection probe is only present in samples from embryos injected with either linearised -2100 Xbra2pGL2 or linearised -220Xbra2 pGL2. The removal of vector sequence from -2100Xbra2pGL2 eliminates this band.

One explanation for this observation is that pGL2 vector sequence drives transcription of the Xbra2 deletion reporter construct even in the absence of FGF, resulting in a transcript that is recognised by the Xbra2 RNAase protection probe as a 200 bp protected fragment. The Xbra2 probe is made from a genomic fragment of Xbra2 comprising nucleotides -148 to +222 (see Fig. 5.2). This probe recognises nucleotides +1 to +222 of the endogenous gene. Xbra2 promoter constructs contain 53 bp of exon 1 (Fig. 5.2). Thus, a transcript from such a construct would be visualised as a 201 (148 + 53) nucleotide band on an RNAase protection autoradiograph. This is what was observed.

These investigations show that the 200 bp protected fragment is only detected in the presence of pGL2 vector sequence. It seems likely that sequences present in the pGL2 vector sequence cause expression of the luciferase reporter gene in FGF treated and untreated animal caps. In an effort to reduce the level of luciferase activity that does not relate to FGF induction of the Xbra2 reporter constructs, all Xbra2 luciferase reporter experiments described below were performed using reporter constructs from which the vector sequence had been removed. In spite of this precaution, levels of luciferase activity in untreated caps from embryos injected with Xbra2 luciferase reporter constructs, with or without vector sequence, varied from experiment to experiment (data not shown), so it was hard to determine whether background levels of luciferase activity were lower or not. However, as removal of the vector sequence from Xbra2pGL2 eliminates the questionable 200 bp protected fragment that is recognised by
Figure 5.2. Schematic representation of the region of Xbra2 used to make the Xbra2 RNAase protection probe. The region of Xbra2 The Xbra2 probe is made from a genomic fragment of Xbra2. This probe recognises nucleotides +1 to +222 of the endogenous gene. The Xbra2 RNAase protection probe may recognise nucleotides -148 to +53 of transcript from the Xbra2 luciferase reporter construct containing at least 148 bp of the Xbra2 5' flanking sequence.
the Xbra2 RNAase protection probe, it seems to be a reasonable precaution to have taken.

**Normalising luciferase activity**

Microinjection of plasmids directing transcription of reporter genes into *Xenopus* embryos results in highly mosaic expression of the gene product (Etkin and Pearman, 1987, Vize, 1991 #757). Mosaic expression of the luciferase reporter constructs, together with slight variations in the amount of plasmid DNA injected, could result in considerable variability from experiment to experiment, and might lead to difficulties in interpreting the results of a deletion series (Vize, 1996). In an attempt to overcome this problem, my luciferase reporter constructs were co-injected with an internal control reporter plasmid, pEF-1α β-galactosidase. The EF-1α promoter drives transcription in all cells from the onset of zygotic transcription (Krieg et al., 1989). In Xbra2 reporter experiments, therefore, animal caps from embryos injected with an Xbra2 luciferase reporter construct and with pEF-1α β-galactosidase were assayed for both luciferase and β-galactosidase activities. Any differences in reporter gene activity between samples in such experiments will be reflected by differences in β-gal activity. A ratio of the β-gal activity of each sample within an experiment can be used as a conversion factor to normalise the luciferase activity.

Figure 5.3 shows the results of a typical experiment using a luciferase based reporter construct driven by 2.1 kb of Xbra2 5′ flanking sequence, -2100Xbra2pGL2, and two other luciferase reporter constructs which truncated the Xbra2 promoter at -1.0 kb and -0.15 kb, termed -1000Xbra2pGL2 and -150Xbra2pGL2 respectively. Animal caps were dissected at stage 8 from embryos that had been co-injected with -2100Xbra2pGL2, -1000Xbra2pGL2 or -150Xbra2pGL2 together with the reference plasmid pEF-1α β-galactosidase. They were then cultured in the presence or absence of FGF. Luciferase and β-galactosidase activities were analysed in animal cap extracts at stage 12. Figure 5.3A shows the luciferase activity and Figure 5.3B shows the normalised luciferase activity measured in the animal cap extracts. Normalisation of luciferase activity has slightly altered the levels of
Figure 5.3. Normalisation of luciferase activity in Xbra2 5’ flanking sequence reporter assays. Animal caps were dissected at stage 8 from embryos that were co-injected at the one/two cell stage with pEF-1α β-galactosidase and each of the following Xbra2 promoter reporter constructs: -150Xbra2pGL2, -1000Xbra2pGL2 and -2100Xbra2pGL2. Half were treated with FGF and the others were left untreated. Firefly luciferase activity and β-galactosidase activity were measured in stage 12 animal caps. Luciferase activity in untreated animal caps is shown in blue and that of FGF treated animal caps is shown in red. Luciferase activity was measured in arbitrary units. Graph (A) shows luciferase activity and Graph (B) shows normalised luciferase activity.
**Figure 5.4.** Graph showing time course of normalised luciferase activity in extracts made from animal caps dissected from embryos that were co-injected at the one/two cell stage with the reporter constructs -866Xbra2pGL2 and pEF-1α β-galactosidase. Animal caps were dissected at stage 8 and half of each sample was treated with FGF. Luciferase and β-galactosidase activity was measured in animal cap extracts at the time points indicated. Normalised luciferase activity in animal caps treated with FGF is shown in red and that of untreated caps is shown in blue.
luciferase activity observed (Fig. 5.3). It does not alter the variability observed between parallel experiments (data not shown), but normalisation of luciferase activity is an important internal control to be included in all experiments of this type. All subsequent experiments present normalised luciferase activity.

**Time course analysis of -866Xbra2pGL2 activation in response to FGF**

A time course analysis of -866Xbra2pGL2 (a PCR-based deletion of -2100Xbra2pGL2 that responds in the same way to FGF treatment as -2100Xbra2pGL2) activation in response to FGF was performed to determine the optimal stage at which to harvest animal caps for luciferase assays. Animal caps were dissected at stage 8 from embryos injected with vectorless -866Xbra2pGL2 and pEF-1α β-galactosidase and cultured with or without FGF. Luciferase and β-galactosidase activities were analysed in animal cap extracts at stages 10.5, 12 and 14. Figure 5.4 shows a significant increase in the level of luciferase activity between stage 10.5 and stage 12 in animal caps that were treated with FGF. There was very little change in the levels of luciferase activity in untreated animal caps over that time. For crude deletion analysis of the Xbra2 5' flanking sequence, stage 12, therefore, appears to be the best time to assay luciferase activity in animal cap extracts. At this stage levels of luciferase activity are high enough to distinguish between the levels of luciferase activity of one deletion construct compared with another.

**Sequence within 381 bp of the transcriptional start site is sufficient to elicit a response to FGF (and activin)**

To identify elements in the Xbra2 promoter responsible for mediating the response to FGF and activin a 5' deletion series of -2100Xbra2pGL2 was generated by PCR or restriction digestion (see Materials and Methods). Each of the following constructs: -2100Xbra2pGL2, -1000Xbra2pGL2, -866Xbra2pGL2, -700Xbra2pGL2, -381Xbra2pGL2, -231Xbra2pGL2 and -150Xbra2pGL2 was co-injected with the reference plasmid pEF-1α β-
Figure 5.5. Xbra2 luciferase reporter constructs containing 381 bp of Xbra2 5’ flanking sequence have the capacity to respond to FGF whereas those containing only 231 bp, or less, of 5’ flanking sequence cannot. However, the level of reporter activation varies from experiment to experiment. (A) Graph showing normalised luciferase activity in extracts made from stage 12 animal caps from embryos co-injected with pEF-1α β-galactosidase and the following Xbra2 reporter constructs: -2100Xbra2pGL2, -1000Xbra2pGL2 or -150Xbra2pGL2. Animal caps were dissected at stage 8. Half were treated with FGF and the other half were left untreated. (B) Same as in (A). (C) Same as in (A) except using the following Xbra2 luciferase reporter constructs: -2100Xbra2pGL2, -866Xbra2pGL2, -381Xbra2pGL2 and -231Xbra2pGL2. (D) Same as in (C) except -700Xbra2pGL2 was also included. Luciferase activity in untreated animal caps is shown in blue and that of FGF treated animal caps is shown in red. Normalised luciferase activity was measured in arbitrary units.
Figure 5.6. FGF and activin activate expression of a reporter gene construct containing 2.1 kb of Xbra2 of 5' flanking sequence. Sequences upstream of -381 in the Xbra2 5' flanking sequence are dispensable for response of Xbra2 to FGF and activin. Embryos were injected with the indicated constructs and reference plasmid, pEF-1α β-galactosidase and, in some cases, with 1 pg of activin mRNA. (The activin experiments were performed by Branko Latinkic, see (Latinkic et al., 1997)). Normalised firefly luciferase activity from stage 12.5 animal caps is expressed as fold activation over untreated caps. (n) Number of experiments.
galactosidase into *Xenopus* embryos at the one or two cell stage. Animal caps were dissected at blastula stages and were incubated in medium containing FGF or were left untreated. Reporter gene activities were analysed in animal cap extracts at gastrula stage 12. The degree of induction by FGF varied from experiment to experiment (Fig. 5.5). Graph 5.5A shows a strong induction of -2100Xbra2pGL2 in response to FGF whereas there is no activation of the -1000Xbra2pGL2 and -150Xbra2pGL2 reporter constructs. In contrast, graph 5.5B shows that -2100Xbra2pGL2 is activated to a much lesser extent and -1000Xbra2pGL2 is strongly activated in response to FGF. Graphs 5.5C and 5.5D show a similarly varied response to FGF.

This variability makes it difficult to interpret the results of such experiments. However, by analysing data from sixty three experiments it was possible to determine which of my Xbra2 deletion constructs have the capacity to respond to FGF. Figure 5.6 shows the collected data from all of these experiments. It shows that removal of 5' sequences down to -381 bp did not prevent induction by FGF, whereas a reporter construct containing only 231 bp of Xbra2 5' flanking sequence was unable to elicit a significant response. (The same is true for low doses of activin in experiments done by Branko Latinkic (Latinkic et al., 1997)). Examination of Figure 5.6 suggests that the response to FGF or activin in constructs containing between 866 bp and 2100 bp of flanking sequence is higher than in those that contain only 381 bp. However, variation in levels of induction observed between experiments has made it difficult to study this issue in a quantitative way and has also hindered our efforts to locate FGF and activin response elements more precisely.

**Xenopus Smad2 activates a luciferase reporter construct containing 381 bp of Xbra2 5' flanking sequence**

Since the -381Xbra2pGL2 was the smallest luciferase reporter construct that responded to activin, I next addressed whether *Xenopus* Smad2, which has been implicated in the activin signalling pathway (Massague et al., 1997), is sufficient to drive expression of -381Xbra2pGL2. This was tested by co-injecting RNA encoding full-length Smad2, activin or, as a control, globin.
**Figure 5.7.** *Xenopus* Smad2 activates a luciferase reporter construct containing 381 bp of *Xbra2* 5′ flanking sequence. Animal caps were dissected at stage 8 from embryos that were co-injected with mRNA encoding Smad2 (1 ng), activin (1 pg) or globin (1 ng), the plasmid -381Xbra2pGL2 or -231Xbra2pGL2 and the reference plasmid pEF-1α β-galactosidase. Normalised firefly luciferase activity from stage 12 animal caps is expressed as fold activation over untreated caps.
together with -381Xbra2pGL2 or -231Xbra2pGL2 and the reference plasmid pEF-1α β-galactosidase into Xenopus embryos at the one or two cell stage. Animal caps were dissected at mid-blastula stages. Reporter gene activities were analysed in animal cap extracts at gastrula stage 12. There was no significant luciferase activity in animal caps expressing the control globin mRNA (Fig. 5.7). However Smad2, like activin, caused strong induction of -381Xbra2pGL2 in animal caps. Neither factor activated -231Xbra2pGL2. This result is discussed below.

**DISCUSSION**

**Sequences within -381 bp of the Xbra2 transcription start site respond to both FGF and activin**

In this chapter I demonstrate that sequences within 381 bp of the Xbra2 transcription initiation site confer FGF and activin responsiveness (activin experiments performed by Branko Latinkic (Latinkic et al., 1997)). Reporter constructs containing only 231 bp of Xbra2 5' flanking sequence are unable to respond to any of these factors. Efforts to define more precisely distinct elements that respond to FGF and activin failed due to variability from experiment to experiment. This variability is likely to be due to several factors, including mosaic expression of the reporter construct (Vize et al., 1991), heterogeneity of different embryo batches, and perhaps the absence of some elements required for full activity of the Xbra2 promoter. Nevertheless, by repeating these deletion analysis experiments sixty three times it has been possible to determine which deletion constructs have the capacity to respond to FGF and activin. I therefore conclude that elements that respond to FGF and activin reside between -381 bp and -231 bp of the Xbra2 transcription start site. The response of the -381 bp construct appears to be weaker than that of the larger -866 bp, -1000 bp and -2100 bp constructs (Fig. 5.6), suggesting that elements between -866 and -381 are required for enhancement of the transcriptional response.

It is intriguing that elements that respond to both FGF and activin lie between -381 bp and -231 bp. As both factors are targeting the same region,
this might suggest that activation of the -381 bp reporter construct is a secondary response to the induction of mesoderm in animal caps by FGF or activin. However, preliminary experiments show that FGF activation of a Xbra2 promoter reporter construct in animal caps is not blocked by the protein synthesis inhibitor cycloheximide. Thus the effect of FGF appears to be direct.

**Sequences within -381 bp of the Xbra2 transcriptional start site respond to Smad2**

Of the Smad family members, Smad2 has been implicated in activin signalling (Baker and Harland, 1996; Graff et al., 1996; Lagna et al., 1996). Fig. 5.7 shows that activin activation of the -381 bp Xbra2 reporter construct can be reproduced by *Xenopus* Smad2. A recent report has shown that the N terminal domain of the *Drosophila* family member MAD can itself bind DNA (Kim et al., 1997). However, there are no similarities between the MAD binding site in the *vestigial* promoter, to which MAD binds, and the sequence that lies between -381 bp and -231 bp of the transcription initiation site of Xbra2.

Activin response regions have been identified for the *Xenopus* genes goosecoid (Watabe et al., 1995), Mix.2 (Huang et al., 1995; Vize, 1996) and XFD-1/XFKH1 (Kaufmann et al., 1996, Howell, 1997 #1386). None share any significant sequence similarity with each other or with that of the Xbra2 promoter. Of all these genes, most is known about the transcriptional activation of Mix2 in response to activin, which is associated with the formation of the activin response factor (ARF) (Chen et al., 1996a). The ARF is a protein complex with specific DNA binding activity that recognises the 50 bp ARE in the Mix2 promoter (Chen et al., 1996a). It comprises a novel winged-helix transcription factor, Forkhead-like activin signal transducer-1 (FAST-1), Smad2 and Smad4 (Chen et al., 1996a; Chen et al., 1997a; Liu et al., 1997). It seems possible that transcriptional activation of these other activin responsive genes may occur through the formation of different Smad containing transcriptional complexes in response to activin stimulation, which show different DNA-binding specificities.
The *Xbra2* promoter sequence bears no homology to the regulatory region of its mouse homologue, *Brachyury* (*T*), even though the mouse sequence is sufficient to direct expression in the primitive streak (Clements et al., 1996). It will be interesting to understand the apparent difference in the mechanism of regulation of *Xbra* and *T*, as it may provide some insight into the evolution of regulatory pathways. There is also little similarity between the *Xbra2* promoter sequence and that of *Ciona intestinalis Brachyury* (Corbo et al., 1997a), although there is a putative Suppressor of Hairless site (TTCCCAAGG) between nucleotides -165 and -158, which may play a role in the regulation of *Ciona intestinalis Brachyury* expression (Corbo et al., 1997a).
Chapter 6

A ternary complex factor like Elk-1 is unlikely to be involved in the transcriptional activation of Xbra2

INTRODUCTION

In Xenopus, normal mesoderm formation depends upon signalling through the fibroblast growth factor (FGF) tyrosine kinase receptor (Amaya et al., 1991; Amaya et al., 1993; Cornell and Kimelman, 1994). FGF has the capacity to respecify ectodermal animal cap cells to form ventral mesoderm (Kimelman and Kirschner, 1987; Slack et al., 1987; Isaacs et al., 1992), and it has been shown that activation of MAP kinase (ERK kinase) is critical for all aspects of FGF induced ventral mesoderm formation (Gotoh et al., 1995; LaBonne et al., 1995; Umbhauer et al., 1995).

FGF signals through the Ras/ERK MAPK pathway to activate Xbra2 5’ flanking sequence reporter constructs in Xenopus animal caps (Latinkic et al., 1997). In mammalian cells, the Ras/ERK MAPK pathway provides a common route by which signals from different growth factor receptors converge at a major regulatory element of the promoters of c-fos and other immediate early genes, called the serum response element (SRE). Inspection of the Xbra2 5’ regulatory sequence has revealed an SRE-like element (Fig 6.1), suggesting that this element may be important in FGF activation of Xbra2.

The c-fos SRE binds a ternary complex comprising serum response factor (SRF) and a member of the ternary complex factor (TCF) family of Ets domain proteins (Fig. 6.2A) (see Treisman, 1995). The SRE is a sequence including a CArG box [CC(AT)₆GG] and a weak Ets-like binding site (consensus Ets binding site CAGGAT) (for references see Latinkic et al., 1996). SRF, which is a member of the MADS-box family of transcription factors (for a review see Shore and Sharrocks, 1995), binds as a dimer to the c-fos CArG box (Treisman, 1987). While bound upon this site, SRF interacts physically with a TCF recruiting it to bind at the Ets-like binding site, thereby forming a
Figure 6.1. 1.2 kb of the 5' flanking sequence of Xbra2. Transcription initiation site is indicated by +1 and is also the first nucleotide in boldface type. 6 Ets-like binding sites are underlined and one CARG-like binding site is underlined twice. This sequence has been submitted to the EMBL database, accession number AJ001528.
ternary complex (Fig. 6.2B) (see Treisman, 1995). Genomic footprinting indicates that an SRF-TCF ternary complex binds the c-fos SRE constitutively in vivo (Herrera et al., 1989). TCFs can bind independently to high affinity binding sites characteristic of Ets-domain proteins and can act alone as transcriptional activators, however TCFs do not bind to the c-fos Ets-like site on their own (see Treisman, 1994).

The major targets of the Ras/ERK MAPK pathway in the c-fos promoter are the TCF proteins Elk-1 or SAP-1. These ternary complex factor proteins have three conserved functional domains A, B and C (Fig. 6.2A). Region A corresponds to the Ets DNA binding domain and region B is required for interaction with SRF (Price, 1995; Treisman, 1995; Treisman, 1994). The C-terminal region is a conserved transcriptional activation domain with multiple S/T-P motifs, which are potential MAPK phosphorylation sites (Dalton and Treisman, 1992). This domain is a good substrate for ERKs in vitro and, moreover, activation of the ERK MAPK pathway by growth factor stimulation in vivo causes phosphorylation at these S/T-P motifs and thereby potentiates transcriptional activation (Fig. 6.2A) (Gille et al., 1992; Janknecht et al., 1993; Marais et al., 1993; Treisman, 1994; Price et al., 1995). TCFs act to connect the c-fos promoter to the Ras/ERK MAPK pathway: SRE mutations that prevent ternary complex formation block c-fos activation via this pathway, and they can be complemented by expression of appropriate altered-specificity mutants of the TCF, Elk-1 (Hill et al., 1994).

SRF can also regulate transcription independently of TCFs (Hill et al., 1994). Evidence has shown that SRF activity is regulated, independently of the Ras/ERK MAPK pathway, by signalling pathways involving the small GTPases Rho, Rac and Cdc42 (Hill et al., 1995).

The Drosophila homologue of SRF, pruned, has provided the first example of SRF function in development. Pruned regulates cytoplasmic outgrowth during terminal branch formation of the tracheal (respiratory) system in Drosophila (Guillemin et al., 1996). Experiments using constitutively active and dominant negative forms of the mammalian TCF, Elk-1, suggest that pruned functions with an as yet unidentified TCF. A dominant negative
Figure 6.2. (A) Primary structure of SRF and the TCFs (Treisman, 1995). MADS-box homology and functional domains of SRF are shown. Functional domains and three signature motifs (A, B, C) of TCFs are shown. (B) The Grappling Hook model for ternary complex formation (Treisman and Ammerer, 1992). According to the model contacts essential for ternary complex formation are made only between SRF and the TCF B box.
form of Elk-1, Elk-1 Δ307, prevents terminal branching of the *Drosophila* tracheal system, thereby mimicking the phenotype of loss-of-function mutants for *pruned* (Guillemin et al., 1996). In contrast, constitutively active forms of Elk-1 and SRF, termed Elk-1 VP16 and SRF-VP16, both drive formation of extra cytoplasmic processes and terminal branches in the *Drosophila* tracheal system (Guillemin et al., 1996). Thus, *pruned* appears to function like mammalian SRF in an inducible transcription complex with a TCF. Furthermore, the implication of a TCF suggests the involvement of Ras and MAP kinase in upstream signalling (Guillemin et al., 1996).

The 866 bp region upstream of the transcriptional start site of *Xbra2* contains 6 Ets-like binding sites (Fig. 6.1). One of these Ets-like sites lies just upstream of a CArG-like binding site (Fig. 6.1), and together these two elements may be sufficient to support ternary complex formation. This suggested that activation of *Xbra2* in response to FGF may occur in a similar way to the activation of *c-fos* by the Ras/ERK MAPK pathway, through the activation of a ternary complex bound to an SRE-like element (Fig. 6.3). In support of this model, SRF has been shown to bind to the *Xbra2* CArG-like site (Branko Latinkic, personal communication).

Experiments presented in this chapter investigate whether a TCF is a nuclear target of the FGF signalling pathway in the transcriptional activation of *Xbra2*. Initial experiments using an interfering form of the ternary complex factor Elk-1, looking at the expression of endogenous *Xbra2* in animal pole explants, supported the idea that a ternary complex, including a TCF and an SRF-like factor, is involved in the transcriptional activation of *Xbra2* in response to FGF. However, further investigation using constitutively active forms of Elk-1 and SRF, looking at both the expression of endogenous *Xbra2* and the activation of *Xbra2* 5′ flanking sequence reporter constructs, revealed that this is unlikely. Data presented in this chapter also provides preliminary evidence of a role for SRF, or an SRF-like factor, as a negative regulator of mesoderm formation in *Xenopus* embryos.
Figure 6.3. (A) Activation of \textit{c-fos} via activation of a ternary complex, bound to an SRE, in response to FGF signalling through the Ras/ERK MAPK pathway (B) Model for activation of \textit{Xbra2} via the activation of a ternary complex, bound to an SRE-like element, in response to FGF signalling via the Ras/ERK MAPK pathway.
RESULTS

A dominant negative ternary complex factor reduces FGF and activin induction of endogenous Xbra2

As a first step to determine whether a TCF is required for the transcriptional activation of Xbra2 in response to FGF signalling, I used a dominant negative form of the TCF, Elk-1, called Elk-1 Δ307 (mentioned in the introduction to this chapter), which is a C-terminal truncation of Elk-1 that removes its regulated transcriptional activation domain, so that the protein can still interact with SRF and bind DNA, but fails to activate transcription (Hill et al.; Fig. 6.4). Thus, Elk-1 Δ307 acts as a dominant negative form of Elk-1. To test for the involvement of a TCF-SRF ternary complex in the transcriptional activation of Xbra2 I used a mutant form of Elk-1 Δ307, called Elk-1 ΔB Δ307, which encodes a protein with a deletion in the SRF interaction domain (the B box) that makes it unable to interact physically with SRF to form a ternary complex (Fig. 6.4).

Embryos were injected, at the one cell stage, with mRNA encoding Elk-1 Δ307, Elk-1 ΔB Δ307 or globin and animal caps were dissected at stage 8. Half of the explants from each sample were cultured with FGF and half were cultured alone. Animal caps were harvested at stage 10.5 and were assayed for expression of Xbra2 by RNAase protection analysis. If a TCF, such as Elk-1, is a nuclear target of the FGF signalling pathway in the transcriptional activation of Xbra2, one might predict that expression of Elk-1 Δ307 would inhibit the induction of Xbra2 by FGF. Moreover, if expression of Xbra2, in response to FGF, required activation of a TCF-SRF ternary complex, that induction of Xbra2 by FGF would not be inhibited in animal caps expressing Elk-1 ΔB Δ307, which cannot form a ternary complex with SRF. This is exactly what was seen (Fig. 6.5A): Elk-1 Δ307, but not Elk-1 ΔB Δ307, reduced the amount of Xbra2 induced by FGF (Table 6.1).

These results show that Elk-1 Δ307 reduces the expression of Xbra2 in response to FGF signalling and this activity is dependent on Elk-1 Δ307 having an intact SRF interaction domain. To determine whether this effect
Figure 6.4. Primary structure of Elk-1 and four derivatives showing the positions of functional domains A, B and C of ternary complex factors (Hill et al., 1993; Hill et al., 1994).
Figure 6.5. (A) Elk-1 Δ307, but not Elk-1 ΔB Δ307, reduces the level of Xbra2 induced in response to FGF. Animal caps were dissected from blastula stage embryos that had been injected at the one cell stage with 500 pg of synthetic mRNA encoding globin, Elk-1 Δ307 or Elk-1 ΔB Δ307. Half of each set of animal caps were cultured with FGF (50 ng/ml) and the other half were untreated. At early gastrula stage 10, samples were analysed by RNAase protection using a probe specific for Xbra2. EF-1α was used as a loading control. (B) Elk-1 Δ307, but not Elk-1 ΔB Δ307, reduces the level of Xbra2 induced in response to activin. Same as in (A) except half of the animal caps from each sample were cultured with activin (8 units/ml) rather than FGF.
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**Table 6.1.** Summary of the effects of the Elk-1 and SRF derivatives on the expression of endogenous *Xbra2* and the *Xbra2* promoter reporter constructs -866*Xbra2pGL2* and -381*Xbra2pGL2*. E = endogenous *Xbra2*. R = reporter construct. - = blocks expression. + = activates expression. 0 = no effect. ND = not done.
of Elk-1 Δ307 is specific to FGF treatment of animal caps, an equivalent experiment was performed in which animal caps were treated with activin rather than FGF. As with FGF, Elk-1 Δ307, but not Elk-1 ΔB Δ307, reduced the amount of Xbra2 induced by activin (Fig. 6.5B; Table 6.1). The effect of Elk-1 Δ307 on the expression of Xbra2 in response to activin is less dramatic than with FGF, suggesting that this effect could be indirect. The maintenance of Xbra expression is thought to occur through an autoregulatory loop in which Xbra induces expression of eFGF which in turn induces expression of Xbra (Schulte-Merker and Smith, 1995). Maintenance of Xbra expression requires a functional FGF signal transduction pathway, thus Elk-1 Δ307 may reduce the maintenance of activin-induced Xbra2 expression by blocking FGF signalling.

An activated ternary complex factor reduces FGF and activin induction of Xbra2

The above results show that Elk-1 Δ307 reduces the expression of Xbra2 in response to FGF and activin signalling and that this effect is dependent on Elk-1 Δ307 having an intact SRF interaction domain. This indicates that a TCF, like Elk-1, may be a nuclear target of FGF signalling in the transcriptional activation of Xbra2. Another experiment to address this possibility is to see if an activated form of Elk-1 is capable of inducing Xbra2 in animal pole explants. The constructs used for this were Elk-1 VP16 (mentioned in the introduction to this chapter), a constitutively active form of Elk-1 in which the regulated transcriptional activation domain of Elk-1 is replaced with the Herpes simplex virus VP16 transcriptional activation domain (Hill et al., 1994), and a mutant form of Elk-1 VP16 called Elk-1 ΔB VP16 which encodes a protein with a deletion in the B box that makes it unable to interact physically with SRF to form a ternary complex (Fig. 6.4). If a TCF, such as Elk-1, is a nuclear target of the FGF signalling pathway in the transcriptional activation of Xbra2, one would predict that ectopic expression of Elk-1 VP16 in animal pole explants would induce expression of Xbra2, in the same way as FGF or activated components of its signal transduction cascade. Moreover, if FGF induction of Xbra2 required activation of a TCF-
Figure 6.6. (A) Elk-1 VP16, but not Elk-1 ΔB VP16, reduces the level of *Xbra2* induced in response to FGF. Animal caps were dissected from blastula stage embryos that had been injected at the one cell stage with 500 pg of synthetic mRNA encoding globin, Elk-1 VP16 or Elk-1 ΔB VP16. Half of each set of animal caps were cultured with FGF (50 ng/ml) and the other half were untreated. At early gastrula stage 10, samples were analysed by RNAase protection using a probe specific for *Xbra2*. EF-1α was used as a loading control. (B) Elk-1 VP16, but not Elk-1 ΔB VP16, reduces the level of *Xbra2* induced in response to activin. Same as in (A) except half of the animal caps from each sample were cultured with activin (8 units/ml) rather than FGF.
SRF ternary complex, one would predict that Xbra2 would not be induced in animal caps expressing Elk-1 ΔB VP16, which cannot form a ternary complex with SRF. In fact, neither Elk-1 VP16 nor Elk-1 ΔB VP16 induced expression of Xbra2 in animal caps (Fig. 6.6; Table 6.1), which suggests, in contrast to results presented in Fig. 6.5, that a TCF is not involved in the transcriptional activation of Xbra2.

Surprisingly, Elk-1 VP16, like Elk-1 Δ307, reduced the expression of Xbra2 in response to FGF and activin whereas Elk-1 ΔB VP16, like Elk-1 ΔB Δ307, had no effect (Fig. 6.6; Table 6.1). As with Elk-1 Δ307, the effect of Elk-1 VP16 on the expression of Xbra2 in response to activin is less dramatic than is seen with FGF. This suggests that Elk-1 VP16, like Elk-1 Δ307, is interfering with the autoregulatory loop for maintenance of Xbra2 expression, rather than directly with the activation of Xbra2 expression in response to activin treatment. It is remarkable that Elk-1 Δ307 and Elk-1 VP16 have identical effects on the expression of Xbra2 in response to FGF and activin. These results cast doubt on the idea that a TCF is involved in the transcriptional activation of Xbra2.

An activated form of SRF blocks FGF and activin induction of Xbra2 and prevents the extension of animal caps in response to FGF and activin

Elk-1 VP16 and Elk-1 Δ307 require an intact SRF interaction domain (B box) to reduce induction of Xbra2 by FGF and activin, suggesting that they must interact with SRF, or an SRF-like factor, to elicit this effect. If so, an activated form of SRF should elicit the same effects as Elk-1 VP16 and Elk-1 Δ307 on the expression of Xbra2 in response to FGF and activin. To investigate this I made use of a constitutively active form of SRF, SRF-VP16 (mentioned in the introduction to this chapter), in which a VP16 transcriptional activation domain is fused to the carboxyl terminus of SRF (Dalton and Treisman, 1992). Like Elk-1 VP16, SRF-VP16 did not induce expression of Xbra2, but while Elk-1 VP16 merely reduced expression of Xbra2 induced by FGF and activin, SRF-VP16 completely blocked this expression (Fig. 6.8; Table 6.1).
Figure 6.7. Primary structure of SRF and a constitutively active derivative containing the VP16 transcriptional activation domain. MADS-box homology and functional domains of SRF are shown (Dalton and Treisman, 1992).
Figure 6.8. (A) SRF-VP16 blocks the expression of Xbra2 induced in response to FGF. Animal caps were dissected from blastula stage embryos that were injected at the one cell stage with 500 pg of synthetic mRNA encoding globin or SRF-VP16. Half of each set of animal caps were cultured with FGF (50 ng/ml) and the remaining half were untreated. At early gastrula stage 10, samples were analysed by RNAase protection using a probe specific for Xbra2. EF-1α was used as a loading control. (B) SRF-VP16 blocks the expression of Xbra2 induced in response to FGF. Same as in (A) except half of the animal caps from each sample were cultured with activin (8 units/ml) rather than FGF.)
Figure 6.9. SRF-VP16 inhibits elongation typical of animal caps cultured with FGF and activin. (A) Animal caps dissected at blastula stage 8 from uninjected embryos. (B) As in (A) except the animal caps were cultured with FGF (50 ng/ml). (C) As in (A) except the animal caps were cultured with activin (8 units/ml). (D) Animal caps dissected at blastula stage 8 from embryos that were injected at the 1 cell stage with synthetic mRNA encoding SRF-VP16 (1 ng). Animal caps were photographed at stage 20.
Elk-1 VP16 and SRF-VP16 both inhibit *Xbra2* expression in response to FGF and activin. However, the effects of these two constructs on the morphological movements of animal cap explants that are induced by FGF and activin are different. Animal caps expressing SRF-VP16 that are treated with FGF or activin do not undergo the morphological movements that are typical of FGF and activin treatment (Fig. 6.9), whereas Elk-1 VP16 has no effect on the morphological movements of animal caps treated with FGF and activin (data not shown). In this respect, therefore, SRF-VP16 exerts different effects on *Xenopus* animal pole explants from Elk-1 VP16.

The results above suggest that the effects of the Elk-1 derivatives on expression of *Xbra2* in response to FGF and activin depend upon interaction with an SRF-like factor. As the effects of SRF-VP16 differ from those of the Elk-1 derivatives, it appears that SRF-VP16 is involved in some TCF-independent functions.

**Sequence within 866 bp, but not 381 bp, of the transcriptional start site of *Xbra2* is sufficient to elicit a response to Elk-1 VP16 and Elk-1 ΔB VP16**

As the results obtained looking at the effects of the Elk-1 derivatives on the expression of endogenous *Xbra2* were unexpected and difficult to interpret, I investigated their effects further by using the *Xbra2* promoter. I tested the ability of Elk-1 VP16 and Elk-1 ΔB VP16 to activate two *Xbra2* 5' flanking sequence, luciferase reporter constructs, -866*Xbra2*pGL2 and -381*Xbra2*pGL2. Both of these constructs elicit a response to FGF and activin, and -381*Xbra2*pGL2 is the smallest *Xbra2* reporter construct that can respond to these factors. The sequence 381 bp upstream of the transcriptional start site of *Xbra2* contains one CArG-like binding site and two Ets-like binding sites, and, in addition to this, the sequence 866 bp upstream of the transcriptional start site of *Xbra2* contains four more Ets-like binding sites (Fig. 6.1).

Animal caps were cut from blastula stage embryos that had been co-injected at the one or two cell stage with synthetic mRNA encoding globin, activin, Elk-1 VP16 or Elk-1 ΔB VP16, -866*Xbra2*pGL2 or -381*Xbra2*pGL2 and the
Figure 6.10. (A) Elk-1 VP16 and Elk-1 ΔB VP16 activate expression of a reporter construct containing 866 bp of Xbra2 5' flanking sequence. Graph showing normalised luciferase activity in gastrula stage 12 animal caps from embryos co-injected, at the one cell stage, with pEF-1α β-galactosidase, -866Xbra2pGL2 and 500 pg of synthetic mRNA encoding Elk-1 VP16, Elk-1 ΔB VP16, globin or globin and 1 pg activin. Animal caps were dissected at stage 8. One half of the animal caps from embryos injected with globin were cultured with FGF (50 ng/ml) and the other half were untreated. (B) Elk-1 VP16 and Elk-1 ΔB VP16 do not activate expression of a reporter construct containing 381 bp of Xbra2 5' flanking sequence. Same as in (A) except -381Xbra2pGL2 was used instead of -866Xbra2pGL2 also there is no FGF treatment of animal caps injected with globin. Normalised luciferase activity is expressed as fold activation over untreated globin expressing animal caps.
reference plasmid pEF-1α β-galactosidase. Half of the animal caps from embryos injected with globin mRNA were treated with FGF. Reporter gene activities were analysed in animal cap extracts at gastrula stage 12. There was no significant luciferase activity in untreated animal caps expressing the control globin mRNA. However Elk-1 VP16 and Elk-1 ΔB VP16, like activin and FGF, caused strong induction of -866Xbra2pGL2 (Fig. 6.10A; Table 6.1).

These results show that the effects of Elk-1 VP16 on -866Xbra2pGL2 and endogenous Xbra2 are different. Firstly, Elk-1 VP16 activates -866Xbra2pGL2, but cannot activate endogenous Xbra2. Secondly, Elk-1 VP16 does not require an intact SRF interaction domain (B box) to activate -866Xbra2pGL2, whereas Elk-1 VP16 requires an intact B box to reduce the level of endogenous Xbra2 induced in response to FGF and activin.

Unlike -866Xbra2pGL2, -381Xbra2pGL2 was not activated by Elk-1 VP16 or Elk-1 ΔB VP16 (Fig. 6.10B; Table 6.1). As this construct is the smallest construct that can respond to FGF and activin, this demonstrates that a TCF, like Elk-1, is not the nuclear target of the FGF signalling pathway that is required for the transcriptional activation of Xbra2 in response to FGF. If it were, Elk-1 VP16 should activate this construct like FGF and activated components of the FGF signal transduction cascade (Latinkic et al., 1997). These experiments show that sequence that lies between 866 and 381 bp of the transcriptional start site of Xbra2 is required to elicit a response to Elk-1 VP16 and Elk-1 ΔB VP16.

An activated form of SRF blocks the activation of two luciferase reporter constructs, containing 866 bp or 381 bp of Xbra2 5' flanking sequence, by FGF and activin

The different effects elicited by Elk-1 VP16 on endogenous Xbra2 and Xbra2 promoter reporter constructs were not seen with SRF-VP16. SRF-VP16 prevents activation of -866Xbra2pGL2 and -381Xbra2pGL2 in animal caps by FGF and activin (Fig. 6.11). Thus, SRF-VP16 has an equivalent effect on endogenous Xbra2 and these Xbra2 5' flanking sequence reporter constructs (Table 6.1).
Figure 6.11. (A) SRF-VP16 blocks the activation of -866Xbra2pGL2 in response to FGF and activin. Graph showing normalised luciferase activity in gastrula stage 12 animal caps from embryos co-injected, at the one cell stage, with pEF-1α β-galactosidase, -866Xbra2pGL2 and 500 pg of synthetic mRNA encoding SRF-VP16, globin or globin and 1 pg activin. Animal caps were dissected at stage 8. One half of the animal caps from embryos injected with globin were cultured with FGF (50 ng/ml) and the other half were untreated. (B) SRF-VP16 blocks the activation of -381Xbra2pGL2 in response to FGF and activin. Same as in (A) except -381Xbra2pGL2 was used instead of -866Xbra2pGL2. Luciferase activity in untreated animal caps is shown in green, that of FGF treated animal caps is shown in yellow and that of activin expressing animal caps is shown in red. Normalised luciferase activity is expressed as fold activation over untreated globin expressing animal caps.
An SRF-engrailed fusion protein causes animal cap extension and activates a luciferase reporter construct containing 381 bp of Xbra2 5' flanking sequence

At the time I was doing experiments with SRF-VP16, Tim Mohun was working with another SRF fusion protein, SRF-engrailed (SRF-En^), which comprises the MADS box of SRF fused to the repressor domain of the Drosophila engrailed protein. This domain of engrailed is an active repressor of transcription that impairs the ability of a wide variety of transcription activators to interact with the basal transcription machinery (Han and Manley, 1993). This domain is functional when fused to heterologous DNA-binding domains, and chimeric proteins of this sort can act in cis to repress the activity of adjacent enhancer elements (Jaynes and O'Farrell, 1991; Badiani et al., 1994; Conlon et al., 1996).

Like Tim Mohun, I found that animal caps from embryos that were injected with synthetic mRNA encoding SRF-En^ extended and formed protrusions (Fig. 6.12). Interestingly, animal cap extension caused by SRF-En^ appears to be different to that caused by FGF or activin. Figure 6.9A shows animal caps treated with FGF extend, forming “dumbbell” shaped animal caps and Figure 6.9C shows the elongation of animal caps treated with activin. Neither FGF or activin treated animal caps form protrusions like those seen in animal caps from embryos injected with SRF-En^ (Fig. 6.12). The morphological movement induced by SRF-En^ is unlikely to be caused by the engrailed repressor domain itself, because expression of synthetic mRNA encoding the control NKX 2.5 engrailed fusion protein or the engrailed repressor domain alone in animal cap explants did not elicit this effect.

SRF-En^ induces expression of endogenous Xbra in animal cap explants (Tim Mohun, personal communication), so I decided to test whether SRF-En^ was capable of activating -381Xbra2pGL2, the smallest Xbra2 reporter construct that is capable of responding to FGF and activin. Figure 6.13 shows that SRF-En^, like FGF, is a potent activator of -381Xbra2pGL2. Thus, a dominant interfering form of SRF, SRF-En^, induces animal cap extension,
Figure 6.12. SRF-En<sup>8</sup> causes animal caps to extend and form protrusions. (A) Animal caps dissected at blastula stage 8 from uninjected embryos. (B), (C) and (D) Animal caps dissected at blastula stage 8 from embryos that were injected at the 1 cell stage with 500 pg synthetic mRNA encoding (B) SRF-En<sup>8</sup>, (C) NKX 2.5-En<sup>8</sup>, and (D) Engrailed repressor domain. Animal caps were photographed at stage 20.
Figure 6.13. SRF-En\textsuperscript{R} activates expression of a reporter construct containing 381 bp of Xbra2 5' flanking sequence. Graph showing normalised luciferase activity in gastrula stage 12 animal caps from embryos co-injected, at the one cell stage, with pEF-1\alpha β-galactosidase, -381Xbra2pGL2 and 500 pg of synthetic mRNA encoding globin, SRF-En\textsuperscript{R}, NKX 2.5-En\textsuperscript{R} or engrailed repressor domain. Animal caps were dissected at stage 8. One half of the animal caps from embryos injected with globin were cultured with FGF (50 ng/ml) and the other half were untreated. Normalised luciferase activity is expressed as fold activation over untreated globin expressing animal caps.
expression of Xbra and activation of -381Xbra2pGL2, while a constitutively active form of SRF, SRF-VP16, blocks the extension of animal caps and expression of Xbra2 in response to FGF and activin, and blocks activation of -381Xbra2pGL2 in response to the same factors (Table 6.1).

**DISCUSSION**

In the experiments described in this chapter I used derivatives of the TCF, Elk-1, and SRF to investigate a possible role for these factors in the transcriptional activation of Xbra2. The results presented suggest that a TCF, like Elk-1, either alone or in a ternary complex with SRF, is not a nuclear target of the FGF signalling pathway required for the transcriptional activation of Xbra2 in response to FGF. In addition to this, I provide preliminary evidence that an SRF-like factor functions as a negative regulator of mesoderm formation in Xenopus embryos.

**Elk-1 Δ307 and Elk-1 VP16, but not Elk-1 ΔB Δ307 and Elk-1 ΔB VP16, reduce the level of Xbra2 induced by FGF or activin**

Initial experiments supported a role for a TCF as a nuclear target of FGF signalling in the transcriptional activation of Xbra2. Elk-1 Δ307 reduces FGF, and to a lesser extent, activin induction of Xbra2. The level of activin induction of Xbra2 may be reduced as a result of Elk-1 Δ307 interfering with FGF signalling, which is required for the maintenance of Xbra expression (Schulte-Merker and Smith, 1995). The effects of Elk-1 Δ307 require an intact SRF interaction domain, which suggests that it must interact with SRF, or an SRF-like factor, to reduce the expression of Xbra2 in response to FGF and activin. This is consistent with the idea that a ternary complex comprising a TCF and SRF-like factor forms on the SRE-like element of the Xbra2 promoter (Fig. 6.3B), and, when activated by FGF, induces transcription of Xbra2 (Fig. 6.3B). For this model to be true, an activated form of Elk-1, Elk-1 VP16, should be sufficient to activate transcription of Xbra2, in the same way as FGF and activated components of the FGF signalling pathway (Latinkic et al., 1997). As Elk-1 VP16 cannot induce expression of Xbra2, it seems unlikely
that a TCF is a nuclear target of the FGF signalling pathway in the transcriptional activation of Xbra2.

Like Elk-1 Δ307, Elk-1 VP16 also reduces the level of Xbra2 induced in response to FGF and activin, in a B-box dependent manner. It is suprising that dominant negative and constitutively active forms of Elk-1 cause the same effects in this assay, and it is hard to interpret how these Elk-1 derivatives are functioning. As interference in both cases is dependent on them having an intact SRF interaction domain, one possible explanation is that Elk-1Δ307 and Elk-1 VP16 interfere indirectly with the induction of Xbra2 in response to FGF and activin by competing to interact with a MADS-box protein that is also required for transcription of Xbra2, thereby reducing the amount of Xbra2 that is expressed.

**Xbra2 promoter sequences between -866 and -381 bp of the transcriptional start site respond to Elk-1 VP16 and Elk-1 ΔB VP16**

Neither Elk-1 VP16 nor Elk-1 ΔB VP16 are capable of activating a reporter construct containing 381 bp upstream of the Xbra2 transcriptional start site, the minimum sequence of Xbra2 that is sufficient to elicit a response to FGF and activin. This, together with the fact that Elk-1 VP16 does not induce expression of endogenous Xbra2, strongly suggests that a TCF is not a nuclear target of the FGF signalling pathway in the transcriptional activation of Xbra2.

Elk-1 VP16 and Elk-1 ΔB VP16 are capable of activating a reporter construct containing 866 bp upstream of the Xbra2 transcriptional start site. Thus, elements that respond to Elk-1 VP16 and Elk-1 ΔB VP16 must lie between -866 and -381 bp of the Xbra2 transcription start site, which contains 4 Ets-like binding sites (Fig. 6.1). This may fit with a role for a TCF as an enhancer of the transcriptional response to mesoderm inducing factors, because results presented in the previous chapter suggest that -866Xbra2pGL2 elicits a stronger response to FGF and activin than -381Xbra2pGL2. However the *in vivo* relevance of this is unclear as Elk-1 VP16 and Elk-1 ΔB VP16 exert different effects on endogenous Xbra2 and -866Xbra2pGL2.
Why do Elk-1 VP16 and Elk-1 ΔB VP16 exert different effects on endogenous and exogenous \textit{Xbra2}? It is possible that the -866\textit{Xbra2}pGL2 reporter construct is missing certain regulatory sequences that are required for the proper regulation of \textit{Xbra2}, thereby enabling the reporter construct, but not the endogenous gene, to be activated by Elk-1 VP16 and Elk-1 ΔB VP16. Another possibility is that the microinjected reporter DNA is more accessible than the endogenous gene for these Elk-1 derivatives to bind to, perhaps due to differential nuclear localisation of the injected DNA versus the endogenous gene, or differences in chromatin structure. Several studies on transfected genes have shown that both their nucleoprotein structure and regulatory properties differ from those of chromosomal genes and they can be abnormally accessible to transcription factors (see Alberts et al., 1998; Archer et al, 1992).

A role for an SRF-like factor as a negative regulator of mesoderm formation in \textit{Xenopus} embryos

In the assays used in this chapter the effects of SRF-VP16 are largely different to those of Elk-1 VP16 and Elk-1 Δ307. Thus, it appears that in these experiments SRF-VP16 functions independently of a TCF. SRF-VP16 is sufficient to block the expression of \textit{Xbra2}, the activation of two \textit{Xbra2} reporter constructs, -866\textit{Xbra2}pGL2 and -381\textit{Xbra2}pGL2, and the extension of animal caps in response to FGF and activin. These effects are unlikely to be direct as SRF is fused to a transcriptional activation domain. It seems more likely that SRF-VP16 activates transcription of another factor that in turn blocks the effects of FGF and activin. It has recently been shown that \textit{Xegr-1}, a zinc finger protein-encoding gene with similar spatial and temporal expression characteristics to \textit{Xbra}, is transcribed in animal caps expressing SRF-VP16 (Panitz et al. 1998). It is conceivable, therefore, that \textit{Xegr-1}, expressed in response to SRF-VP16, directly blocks \textit{Xbra2} activation. Consistent with the idea that SRF-VP16 acts indirectly, mutation of the CArG-like site in the \textit{Xbra2} promoter has no effect on the pattern of \textit{Xbra2} promoter-driven reporter gene expression in whole embryos (Walter Lerchner, personal communication). Mutation of a critical region of the SRF
MADS-box that is required for DNA binding will confirm whether or not the effects of SRF-VP16 are dependent upon it binding DNA.

Expression of a dominant interfering SRF construct, SRF-En\(^R\), in animal cap explants induces the expression of \(Xbra\) (Tim Mohun personal communication) and activates -381\(Xbra2\)pGL2, the smallest \(Xbra2\) reporter construct that responds to FGF and activin. As with SRF-VP16, it seems unlikely that these activation events caused by SRF-En\(^R\) are direct, as this fusion protein contains the repressor domain of the \(Drosophila\) engrailed protein. It may be that SRF-En\(^R\) acts indirectly by repressing the transcription of another factor that is required to negatively regulate mesoderm formation. This can be tested in the same way as mentioned for SRF-VP16. Together these data provide preliminary evidence for the involvement of an SRF-like factor in the negative regulation of mesoderm formation in \(Xenopus\) embryos.

In addition to the induction of \(Xbra\), it will be interesting to see what other mesodermal markers are induced in SRF-En\(^R\) expressing animal caps. Since SRF-VP16 blocks animal cap extension by FGF and activin, I anticipate that SRF-En\(^R\) is capable of inducing a wide range of mesodermal markers that are induced by FGF and activin. Indeed SRF-En\(^R\) has the capacity to induce animal caps to extend and form protrusions, but in a manner different to that caused by FGF or activin. Histological analysis will be necessary to determine what kind of tissues are induced in animal caps expressing SRF-En\(^R\).

Consistent with a role in mesoderm formation, \(Xenopus\) SRF is expressed in the unfertilised egg right through until swimming tadpole stages (Mohun et al., 1991), although, as yet, the spatial expression of \(Xenopus\) SRF is not known. It remains to be tested whether the effects of the SRF-En\(^R\) construct are specific to SRF. This construct consists of the MADS-box of SRF fused to the repressor domain of \(Drosophila\) engrailed (Fig. 6.12). Experiments are in progress to determine whether the MADS-box from other members of the MADS-box family of transcription factors are capable of eliciting the same
effects as SRF-En^R when fused to the repressor domain of the *Drosophila* engrailed protein.

In addition to elucidating the role of the SRF MADS-box in SRF-En^R function, it will be important to determine the minimum sequence of the *Xbra2* promoter that is required for SRF-En^R to drive expression of a reporter gene. Together these two lines of investigation should provide insight into the role of an SRF-like factor in the formation of mesoderm in *Xenopus* embryos.
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