Patterning the *Xenopus* embryo: the role of Xom, a novel homeobox gene

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I dedicate this thesis to my parents in gratitude for the love, overwhelming support, encouragement and kindness that they have given me.

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Abstract

The body plan of the *Xenopus* embryo is established by a series of inductive events. The identity of the signalling molecules that mediate these events are known, however little is known as to the transcriptional response the cell makes to a particular signal. In an attempt to clone transcription factors that were induced as a response to various signals, two approaches were used. The first involved subtraction of cDNA libraries. A cDNA library made from animal caps cultured in media was used to remove commonly occurring sequences from a cDNA library made from animal caps cultured in the presence of FGF. This enabled genes to be identified that were specifically induced by FGF. Unfortunately this procedure was hampered by cross-hybridisation of vector sequences between the two pools of cDNA. The other approach involved the cloning of homeobox-containing genes from the early embryo.

By using a degenerate primer recognising sequences in the homeobox, a gene was isolated that is expressed at high levels in the *Xenopus* gastrula. At the early gastrula stage expression of this gene, *Xom*, is excluded from the organiser. This expression pattern is very similar to the expression pattern of BMP-4. Activation of *Xom* transcription is inhibited if embryos are dissociated during blastula stages, but addition of BMP-4 to the medium rescues expression. This rescue occurs even in the absence of protein synthesis. Procedures which disturb BMP signalling block the expression of *Xom*.

Animal caps that have been loaded with Xom and then treated with activin, do not undergo the extensive gastrulation-like movements seen in control animal caps treated with activin. Injection of Xom RNA into the dorsal cells of the four cell embryo results in the loss of anterior structures and the inhibition of notochord formation. Both assays resemble the ventralising effects seen when BMP-4 is injected into the *Xenopus* embryo.

This suggests that *Xom* is not only an immediate early response of BMP-4, it may also mediate some of the effects of BMP-4.
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Chapter 1

1. Introduction

The establishment of the vertebrate body plan is critically dependent upon the induction of mesoderm (Sive, 1993; Smith, 1989). The way in which mesoderm is induced and patterned is, therefore, an area of intense study. To date, the embryo of the amphibian *Xenopus laevis* has 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 with which to study the mechanisms governing patterning. The embryo develops outside the mother and can be cultured in a simple salts solution, allowing easy access to all stages of development. The embryo is also large and easy to dissect into pieces that can be assayed for both their developmental and inductive potential.

The greatest disadvantage of the amphibian embryo is that genetic techniques cannot be employed. The long generation time of 18 months, its pseudotetraploidy (see Kobel and Du Pasquier, 1986), and the lack of any *in vivo* recombination for making transgenic animals preclude the use of genetic manipulations similar to techniques developed for *Drosophila* (Nüsslein-Volhard et al., 1984), zebrafish (Dreiver et al., 1994), and mice (Beddington, 1992; Robertson et al., 1992).

These disadvantages notwithstanding, the development of *Xenopus* has been very well characterised from oogenesis to the later stages of differentiation.

1.1. The Maternal contribution to the development of the *Xenopus* embryo

The generation of asymmetry in the *Xenopus* embryo occurs well before fertilisation. The formation of the primary axis in *Xenopus* development, the animal-vegetal axis, can be traced back to the long period of oogenesis the egg undergoes. During this time, the developing oocyte stores RNAs and proteins which it will use during the first few hours
of fertilised life until zygotic transcription and translation begins (the mid-blastula transition; MBT) (Newport and Kirschner, 1982).

When the egg is laid it is radially symmetrical but polarised along the animal-vegetal axis. The most obvious aspect of which is the animal hemisphere, which is rich in cytoplasm, is heavily pigmented and the vegetal pole, which contains a large amount of yolk, is lightly pigmented. The differing characteristics of both hemispheres are apparent at the subcellular level with differences in the localisation of various RNAs and in the organisation of the cytoskeleton. This is apparent in the animal hemisphere which shows disorganisation in the arrangement of at least one component of the cytoskeleton, the keratin filaments, whereas the vegetal hemisphere shows a regular geodesic array of filaments (Klymkowsky et al., 1987). The differences in the arrangement of the cytoskeleton may be responsible for the differential translocation of other components into one or the other hemisphere.

Another element of this asymmetry is the localisation of mRNAs to the animal or vegetal hemisphere. Several mRNAs have been identified that are localised to just one of the hemispheres. Several mitochondrial genes have been found to be localised to the animal hemisphere (An-1-3 (Weeks and Melton, 1987a)). In the vegetal hemisphere Vg-1 (Weeks and Melton, 1987b), a TGF-β type molecule, Xcat-2 (Mosquera et al., 1993), a mRNA that is found associated to the cytoskeleton and Xwnt-11 (Ku and Melton, 1992), a member of the wnt/wingless family are all localised to the vegetal cortex.

The mechanism of Vg-1 localisation has been the most closely studied. Vg-1 is uniformly distributed in the newly formed oocyte but becomes localised towards the end of oogenesis. This localisation appears to be mediated through a 340 nucleotide sequence element present in the RNA (Mowry and Melton, 1992). Localisation occurs by two separate processes, translocation and anchoring, each process being dependent on a cytoskeletal component (Yisraeli et al., 1990). Translocation to the vegetal hemisphere is dependent on microtubule polymerisation and is blocked by inhibitors of this process.
such as nocodazole and colchicine. Anchoring to the vegetal cortex requires microfilaments, as shown by the sensitivity of the process to cytochalasin B, a drug which specifically blocks the organisation of microfilaments. This anchoring appears to be mediated by a 69 KDa protein which binds the 340-nt element to the microfilaments of the vegetal cortex (Elisha et al., 1995; Schwartz et al., 1992). On oocyte maturation Vg-1 is released from the cortex and moves deeper into the vegetal cytoplasm (Weeks and Melton, 1987b).

This vegetal localisation of Vg-1 is important when the events that immediately follow fertilisation are considered. By overlaying the asymmetric Vg-1 localisation with one more symmetry-breaking event, the necessary information for the establishment of the dorsal-ventral axis is, in principle, established.

1.2. Fertilisation

The entry of the sperm provides the egg with a set of co-ordinates for the positioning of the dorsal-ventral axis. The sperm entry point defines the axis for cortical rotation, where the cortex of the embryo rotates in relation to the inner cytoplasmic mass of the embryo (Vincent and Gerhart, 1986). This rotation causes the equatorial regions of the embryo to have either the animal cortex over vegetal cytoplasm (the ventral side) or the vegetal cortex overlying the animal cytoplasm (dorsal side). These asymmetries cause the region opposite the sperm entry point to be specified as dorsal (see figure 1.2).

The exact mechanism of cortical rotation and the impetus that sperm entry provides is unclear, although the role of the cytoskeleton is known to be essential. It is known that cortical rotation relies on a parallel array of microtubules in the cortex of the vegetal hemisphere (Elinson and Rowning, 1988). The polarity of this network is such that the plus ends of the microtubules point in the direction of rotation and towards the future dorsal side, away from the point of sperm entry (Houliston and Elinson, 1991). Microtubules also radiate out from the sperm aster in the animal hemisphere and ramify
throughout the cytoplasm of the egg. Where these cytoplasmic microtubules meet the microtubule network in the vegetal cortex, they become re-oriented and contribute to this array.

The cortical network serves as a track along which rotation is directed. The molecular motor, kinesin, provides the driving force for the cortical microtubule network to slide against the membrane of the endoplasmic reticulum which juxtaposes the cortex (Houliston and Elinson, 1991).

The mechanism by which the entry of the sperm directs the orientation of the microtubule network, and therefore cortical rotation, is unclear. The sperm aster is known to provide a nucleation point for the microtubule networks in the egg, and may bias the orientation of the cortical network. However, the aster is not necessary for the normal development of the embryo; activated embryos or embryos that have been fertilised with aster-less sperm, develop normally (Elinson and Palecek, 1993; Sardet et al., 1994).

The importance of cortical rotation can be seen if the vegetal pole is irradiated with UV light before cortical rotation has occurred (Malacinski et al., 1975). This has the effect of destroying the machinery of rotation. Such embryos do not develop any dorsal tissues such as notochord and neuro-ectoderm (Cooke and Smith, 1987). However, normal development can be restored simply by tipping the embryo after UV irradiation, so that cortical rotation is re-imposed (Scharf and Gerhart, 1980). The effect of "over polymerisation" of microtubules can be seen when embryos are treated with D$_2$O (deuterium oxide). This stabilises the microtubules and allows precocious polymerisation. Embryos treated in this way are hyperdorsalised (Scharf et al., 1989).

Cortical rotation causes the animal cytoplasm to undergo rearrangements which are due to the shifting of the vegetal cytoplasm and polymerisation of microtubules originating from the animal sperm aster. These movements form a characteristic swirl of cytoplasm in the prospective dorsal marginal region of the embryo if the cytoplasm is viewed using fluorescent labelling of one of its components, vitellogenin (Danilchik and
Denegre, 1991). The swirls are also sensitive to treatments which cause axial re-specification of the embryo (Brown et al., 1993; Denegre and Danilchik, 1993). Whether these swirls are formed as a consequence of any other changes in the cytoplasmic character of that area, or are in fact instrumental in determining the dorsal side of the embryo, is unclear.

Irradiation of the vegetal surface of oocytes with ultra-violet light at prophase I (before oocyte maturation) will lead to an embryo that does not develop any dorsal structures, as is observed after UV irradiation of fertilised eggs (Holwill et al., 1987). However, unlike the irradiation of fertilised eggs, axial development cannot be rescued by tipping the embryo (Elinson and Pasceri, 1989). The proposal is that a maternal dorsal determinant is present before the events of cortical rotation and that it is activated by cortical rotation. Further data localises this dorsal determinant to the cortical cytoplasm (Holowacz and Elinson, 1993).

Vg-1 is a good candidate for the dorsal determinant. Vg-1 is localised to the vegetal cortex before oocyte maturation and then is released into the deeper cytoplasm. Perhaps the difference in early and late UV irradiation is reflected in this change in localisation where UV irradiation before oocyte maturation, and therefore while Vg-1 is associated with the cortex, may destroy the more exposed RNA.

Vg-1 is poorly, if at all, secreted (Dale et al., 1989; Tannahill and Melton, 1989). In common with all TGF-β family members, Vg-1 can only be secreted if it is correctly processed. Processing cleaves the N-terminal pro region of the molecule from the mature "signalling" region, allowing the protein to be secreted and therefore become active. Synthetic RNA derived from Vg-1, if injected into the Xenopus embryo, has little effect on development and no secreted protein can be detected. However, if the pro region of Vg-1 is replaced with that of another TGF-β such as BMP-2 (Thomsen and Melton, 1993), BMP-4 (Dale et al., 1993) or activin (Kessler and Melton, 1995) active Vg-1 is
produced and secreted. These chimeric molecules have a common feature; they will all induce dorsal types of mesoderm.

The difficulty in making active Vg-1 may be relevant to the process of cortical rotation. It is possible, for example, that the role of cortical rotation for dorsal development is to process Vg-1. Perhaps by bringing the unprocessed Vg-1 protein present in the vegetal cortex into contact with a processing enzyme present in the animal cytoplasm, dorsal development can occur.

1.3. Mesoderm Induction

The induction of mesoderm is the first point at which the asymmetries generated by cortical rotation are apparent. The differences in the types of mesoderm that are induced (see below), make mesoderm induction a primary patterning process.

Mesoderm induction begins at the 32 cell stage of development (Jones and Woodland, 1987), in the equatorial region of the embryo, at the junction between the animal and vegetal hemispheres. Nieuwkoop (see Smith, 1989 for references) demonstrated that whilst isolated animal pole cells formed ectoderm when cultured in a simple salts solution and isolated vegetal explants developed into endoderm, conjugates of the two embryonic regions gave rise to mesoderm. When the experiments were later repeated in the Xenopus embryo using more modern lineage labelling techniques, it was apparent that the induced mesoderm derived exclusively from the animal pole (Dale et al., 1985; Smith et al., 1985).

Experiments with micropore filters have argued for a diffusible signal being responsible for mesoderm induction. Here, an animal explant is separated from the vegetal explant by a micropore filter, allowing diffusion of small proteins but not allowing cell contact (Grunz and Tacke, 1986; Slack, 1991). In this type of conjugation mesoderm is still induced. The identity of molecules that might mediate this process will be discussed later.
Nieuwkoop was also able to show that the type of mesoderm induced by vegetal blastomeres depended on the region of the vegetal pole from where the blastomeres were explanted. Conjugates that have been made with animal caps and dorsal vegetal blastomeres result in the formation of dorsal mesodermal cell types such as notochord. Ventro-lateral vegetal blastomeres will induce mesoderm of a ventral character, typically blood, mesenchyme and mesothlium (Boterenbrood and Nieuwkoop, 1973). These experiments originally performed in axolotyls have been repeated in Xenopus, with similar results (Dale et al., 1985).

The regional properties of the vegetal hemisphere are established as a result of cortical rotation. Indeed, in embryos where cortical rotation has been inhibited the ability of different parts of the vegetal pole to induce different types of mesoderm is lost (Gimlich and Gerhart, 1984). Axial development can be rescued in these embryos by transplanting a dorsal vegetal cell from a normal, untreated donor into the vegetal region of an irradiated host (Gimlich, 1986). The dorsal vegetal blastomere, termed the Nieuwkoop centre, will also induce a second dorsal axis, if a ventro-vegetal blastomere from a host is replaced with a dorsal vegetal blastomere from donor. In both cases, the Nieuwkoop centre does not contribute to any axial structures. Instead it populates the most anterior endoderm (Gimlich and Gerhart, 1984). Both induction of the second axis and restoration of axial development are also temporally regulated. The dorsal vegetal blastomere must be taken before the onset of zygotic transcription; after this point the blastomere loses its competence both to induce mesoderm (Nieuwkoop, 1973) or rescue a UV treated embryo (Gimlich, 1986). The Nieuwkoop centre does not contribute to the organiser itself. Rather it induces the formation of dorsal organiser tissue (Gimlich, 1986).

In the next section I will describe gastrulation. Gastrulation can be considered to be the point at which the different types of mesoderm that have been induced are apparent.
1.4. Gastrulation

Gastrulation is the process by which the mesoderm, until this time a ring around the equator of the embryo, is internalised and becomes sandwiched between the ectoderm and the endoderm. A further consequence of gastrulation is the alteration of the shape of the embryo, from a radially symmetrical sphere with no anterior-posterior patterning to a three layered cylinder possessing obvious rostral-caudal differences. This process is depicted in Figure 1.1.

The cell movements of gastrulation, though the cell movements during gastrulation are very complicated and involve several different types of cell behaviour. They have been well described by the work of Ray Keller (Keller et al., 1989; Keller and Tibbetts, 1989; Keller, 1985; Keller et al., 1985).

Gastrulation is presumed to start with the appearance of the dorsal lip, a small crescent of pigmentation produced by the concentration of pigment granules at the apices of a specialised group of cells, the bottle cells. In fact, by the appearance of the lip, gastrulation has already started, with epiboly type movements in the animal cap (the prospective ectoderm).

Epiboly begins soon after the mid-blastula transition. By a process of intercalation, the deep layers of the cap integrate into one layer, resulting in an animal cap containing just two layers, the superficial layer and the deep layer (Keller, 1980). Although the cells of the two layers do not mix, the superficial cells divide, flatten and lengthen so that the area of the animal cap is increased (Keller, 1991). As the animal cap moves more vegetally, the vegetal cells disappear inside the embryo by processes described below.

As mentioned, gastrulation is heralded by the appearance of a crescent of pigment on the dorsal side of the embryo. The bottle cells, which are responsible for the pigmentation, cause an invagination in the surface of the embryo in order to direct cell
Figure 1.1.
Gastrulation in the *Xenopus* embryo (facing page)

Shown are cartoons illustrating a lateral view (a-d) or a mid saggital view (e-h) undergoing the tissue rearrangements described in the text. Dorsal is to the left. The illustrations are arranged in chronological order, with embryos at the late blastula (a, e), early gastrula (b, f), late gastrula (c, g) and mid-neurala (d, h) being depicted. Landmarks are the animal pole (AP); the archenteron (A); the blastocoel (BLC); the bottle cells (BC); the blastopore (arrow-head); the involuting marginal zone (IMZ); the non-involuting mesoderm (NIMZ); and the vegetal pole (VP). Movements are marked by arrows. Prospective tissues shown are epidermis (light blue); neural tissue (dark blue); dorsal NIMZ (blue-green); notochord (red); head mesoderm (orange).

As the apices of the bottle cells constrict, the mesoderm of the head is brought to lie next to the blastocoel roof (f). More posterior mesoderm then follows the head mesoderm, as the IMZ continues to converge (c, g). The dorsal aspect of the embryo is elongated by the extensive movements of the future midline of the embryo (Taken from Keller, 1991)
Figure 1.1
Gastrulation in the *Xenopus* embryo
movement internally. Indeed the bottle cells are responsible for the invagination that brings the head mesoderm to lie between the ectoderm and endoderm. The rest of the mesoderm is then internalised by epiboly and convergence.

Although the process of epiboly provides some of the motive force to drive internalisation of the mesoderm, most of the force is provided by the convergence of cells of the deep mesodermal layer. These cells intercalate in a manner analogous to that seen during epiboly of the animal cap. However, unlike the animal cap, where the intercalation occurs in radial manner, the intercalation in the deep mesodermal layer occurs in the medio-lateral plane (Keller and Tibbetts, 1989; Keller et al., 1992). The net effect of this is to increase the animal-vegetal "height" of the mesoderm with a decrease in the circumference of the ring of mesoderm.

Gastrulation starts on the dorsal side first and then spreads ventrally with the most ventral tissue being the last to involute. This difference in the timing of gastrulation between the dorsal and ventral side is not enough to generate the obvious differences that exist between them. This responsibility falls on the cells of the presumptive notochord. The behaviour of these cells potentiate the difference between dorsal and ventral regions of the embryo. Although convergence behaviour is exhibited by all cells of the marginal zone, the cells of the future midline exhibit a modified version. The interdigitation of these cells rather than being random, is directed to the midline of the embryo. This process, known as extension (Keller et al., 1985), has the effect of converting a wide sheet of cells into a thin rod.

The integration of epiboly, invagination directed by the bottle cells, convergence and extension are the key pieces of gastrulation. The actual movements involved in gastrulation are described in the legend to Figure 1.1.
1.5. Dorsalisation

Mesoderm induction establishes two domains in the equator of the embryo (see above). Ventral mesoderm comprises most (about three-quarters) of the tissue. Dorsal mesoderm makes up the remainder of the equator.

A comparison of the fate map of the 32 cell stage *Xenopus* embryo with the specification map of the embryo (Dale and Slack, 1987a; Dale and Slack, 1987b) introduces a paradox which provides the most compelling argument for a subsequent patterning signal. Whereas the fate map indicates that most of the muscle in the *Xenopus* embryo arises from the ventral side of the embryo, the specification map, which addresses the autonomous differentiation of pieces of the embryo, reveals that an isolated ventral half embryo makes very little muscle. The results suggest that the ventral mesoderm is further patterned after its induction and that this patterning leads to the development of more dorsal and lateral types of mesoderm (Slack and Forman, 1980).

The idea of the dorsal mesoderm being a source of patterning signals stemmed from experiments undertaken by Hilda Mangold and Hans Spemann in 1924 (Spemann, 1938). In a now classic piece of embryology Spemann and Mangold were able to induce a complete duplication of the axis of the amphibian embryo by transplanting dorsal mesoderm from an early gastrula to the ventral side of a host. By using two differently pigmented species of the newt *Triturus*, they were able to show that not only does the transplanted tissue contribute to the notochord, it will also induce cells to take on a neural character. Smith and Slack (Smith and Slack, 1983) repeated these observations in *Xenopus* and extended the findings. By using more modern lineage labelling techniques, they were able to show that the transplanted dorsal marginal zone contributes mainly to the notochord. However it will also induce neighbouring cells to adopt a more dorsal fate (muscle) and will induce neural tissue. The dorsal mesoderm, also known as the Spemann Organiser, is implicated in the further patterning of the ventral mesoderm, to produce dorso-lateral tissue such as muscle.
Conjugates made from explants further confirm this idea. If a dorsal marginal zone and ventral marginal zone are combined, muscle can be induced in the ventral marginal zone (Dale and Slack, 1987b; Slack and Forman, 1980). By using a transfilter assay where dorsal and ventral gastrula marginal zones are separated by a porous membrane, some information about the nature of this signal can be obtained. The experiments suggest that if the dorsalising signal is diffusible then it is large (over 100 KDa) as the induction of muscle could only occur if the pore size is large (Lettice and Slack, 1993). It must be noted that the pore size is such that the cell-cell contact can also occur. Indeed, the efficiency of dorsalisation of the ventral marginal zones in the transfilter assay was substantially less than the direct combinations.

1.6. The Three Signal Model

The embryological experiments described above have been used to construct a model of *Xenopus* development, the three signal model (Dale *et al.*, 1985; Smith and Slack, 1983). This is shown in Figure 1.2.

In this model, cortical rotation sets in place the necessary asymmetries to place the first and second signals. These signals induce the two domains of mesoderm, either a dorsal type or a ventral type mesoderm. The dorsal mesoderm then patterns the ventral domain (the third signal) to establish the spectrum of mesodermal types found in the later embryo. In the next section I describe candidates for the signals predicted by this model, however, it is necessary to invoke a fourth signal, counteracting dorsalisation, in the model predicted from studies on the function and expression patterns of the candidate signalling molecules that are present in the *Xenopus* embryo.

1.7. Inducing and Patterning Factors

The identity of the factors that mediate the inductive interactions described above eluded researchers for a long time. However, by using a modification of Nieuwkoop's
The Three Signal Model

The 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 (A), the ventro-vegetal blastomeres (VV) and the dorso-vegetal blastomeres (DV). The vegetal blastomeres induce mesoderm of a corresponding character in the overlying animal cells. The dorsal mesoderm or the organiser (O) then patterns the ventral mesoderm (VM) during gastrulation. The organiser is also responsible for the induction of neural tissue.
conjugation experiment, the discovery and purification of mesoderm inducing factors was made possible. In his experiments, Nieuwkoop was able to observe mesoderm induction in the ectodermal part of conjugates of presumptive ectoderm and presumptive endoderm. This observation was extended to a putative mesoderm inducing factor; if it could induce mesoderm, the factor would be able to replace the vegetal pole signal. At its simplest, the animal cap assay (Slack et al., 1987; Smith, 1987) employs blastula staged animal caps that are placed in medium containing the factor to be assayed. Whereas a blastula staged animal cap will round up and eventually form epidermis, an animal cap induced to form mesoderm will elongate as it undergoes extensive gastrulation-like movements (Symes and Smith, 1987). The amount of elongation is dependent on the types of mesoderm induced. Ventral mesoderm will not elongate dramatically, whereas the elongation elicited by a cap induced to form dorsal mesoderm is easy to score.

Using this assay, two groups of mesoderm-inducing molecule have been identified, members of the transforming growth factor beta (TGF-β) family and members of the fibroblast growth factor (FGF) family.

In the next section I will describe, in turn, the current data on these two families, and also on patterning molecules such as noggin and members of the wingless/wnt family.

1.7.1. FGF-like molecules

The fibroblast growth factor family has been shown to be a potent mitogen and differentiation factor in a variety of systems (Basilico and Moscatelli, 1992; Burgess and Maciag, 1989). The FGF family comprises of at least 8 members termed FGF-1 through to FGF-8 and there is considerable overlap in function between these members. The prototype for this group is FGF-2, or basic FGF as it was previously named.
The importance of the FGF family in *Xenopus* development was first demonstrated with the finding that FGF-2 could induce mesoderm in isolated animal caps (Kimelman and Kirschner, 1987; Slack *et al.*, 1987). The type of mesoderm induced was of a ventral character, contrasting with activin which induced more dorsal mesoderm (see below). This has led to the notion that an FGF signal specifies the "ground state" mesoderm which is then patterned by the organiser. This idea has only recently been challenged.

Though most of the early work on FGF was performed using FGF-2, it has always been unclear whether FGF-2 is responsible for mesoderm induction *in vivo*. FGF-2 RNA is present maternally (Kimelman *et al.*, 1988; Kimelman *et al.*, 1987) and is expressed at the highest levels in the animal hemisphere (Song and Slack, 1994). Although this places FGF-2 at the right time to induce mesoderm, it is in the wrong place. Taken with data showing that FGF-2 lacks a traditional signal sequence necessary for secretion, this casts doubt on the role of FGF-2. Indeed, injections of RNA encoding native FGF-2 show a very weak mesoderm inducing activity in the animal cap assay. For efficient activity, a signal sequence must be added (Thompson and Slack, 1992), suggesting that if FGF-2 was an endogenous inducer of mesoderm it would need to be secreted. In fact two other FGF family members, FGF-4 (k-FGF) and embryonic FGF (a homologue of either FGF-4 or FGF-6) are present in the embryo. Both these proteins have signal sequences and both have been shown to be able to induce mesoderm in the animal cap assay.

This data does not preclude a role for FGF-2 in mesoderm induction. It is known that FGF-2 can be secreted in a manner that does not require a signal sequence and it is possible that active FGF-2 is produced in this way in the *Xenopus* embryo.

The importance of FGF in mesoderm induction can be judged using experiments that block various components of the FGF signal transduction pathway. FGF signals through a transmembrane receptor that possesses an intracellular tyrosine kinase domain, and this in turn signals through the ras/raf pathway (Marshall, 1995). This is shown in
Figure 1.3
The FGF signalling pathway

Shown is a cartoon representing the probable pathway that the binding of a ligand for the FGF receptor activates. Ligand binding requires the presence of heparin sulphate. Ligand binding stimulates the activity of the tyrosine kinase receptor which then cause ras to exchange GDP for GTP and thus activating it. This in turn phosphorylates the serine/threonine kinase, raf, which in turn causes the phosphorylation of a MAP Kinase Kinase, leading to the phosphorylation of MAP Kinase. The nature of the nuclear response to FGF stimulation in the *Xenopus* embryo is, however, unclear.
Figure 1.3. Over-expression of truncated versions of the FGF receptor, which lack the intracellular tyrosine kinase domain, block FGF signalling. This leads to embryos that develop no ventro-posterior mesoderm (Amaya et al., 1991; Amaya et al., 1993). Dominant negative constructs of the other components of the signalling pathway, such as ras (Whitman and Melton, 1992), raf (MacNicol et al., 1993) and MAP kinase (LaBonne et al., 1995; Umbhauer et al., 1995), reveal that these can block induction by FGF, indicating that they are in the same pathway.

A surprising finding was that inhibition of the FGF signalling pathway also led to an inhibition of some aspects of activin-mediated mesoderm induction (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). Some genes, such as Xbra, Xnot, Mix-1 and MyoD, required the presence of FGF for expression while others, such as goosecoid, Xlim-1 and Xwnt-8 seemed to be insensitive to inhibition of FGF signalling.

Another aspect of activin signalling that the dominant negative FGF receptor appears to mediate is the ability of an activin treated animal cap to extend (Cornell and Kimelman, 1994). This effect can be observed at the single cell level. An untreated animal cap cell will adhere but not spread on a fibronectin coated dish, but a cell treated with activin will spread on the coated dish (Howard, 1992; Smith et al., 1990b). An animal cap cell taken from an embryo injected with the dominant negative receptor and then treated with activin does not spread on a fibronectin coated dish. This brings in the question of adhesion. Does FGF signalling modulate the adhesive properties of mesoderm? In support of this idea is the recent finding that various cellular adhesion molecules such as NCAM, N-cadherin and L1 are also ligands for the FGF receptor (Mason, 1994b). This will be re-examined later in this chapter.

Data obtained using truncated activin receptor constructs have led to a reappraisal of the role of FGF in mesoderm induction. The truncated activin receptor blocks the formation of all mesoderm in the embryo (Hemmati-Brivanlou and Melton, 1992). However, it does not block the induction of mesoderm in animal caps by FGF (Hemmati-
Brivanlou and Melton, 1992). The suggestion is that FGF is not the in vivo mesoderm inducing agent.

Cornell and Kimelman (Cornell et al., 1995) argue that rather than being a mesoderm inducing factor per se, a certain ratio of FGF signalling and activin/Vg-1 signalling specifies mesoderm. In the embryo, FGF and an activin type signal synergise to give mesoderm. However, as described above, FGF alone is able to induce mesoderm in an animal cap. Perhaps the level of FGF-2 protein applied to the animal cap is sufficient to relieve the cap of its requirement for activin/Vg-1 signalling, maybe by forcing a synergistic interaction with an endogenous TGF-β type molecule in the animal cap. Alternatively, maybe the action of exogenous FGF-2 is not the role of the maternal FGF-2 in the animal cap but reflects the action of a zygotic gene (such as eFGF; see later). In support of this is, as mentioned already, data suggesting that injected RNA encoding native FGF-2 does not act as a mesoderm inducing factor in the animal cap assay (Thompson and Slack, 1992).

A corollary is that the vegetal pole never expresses mesodermal markers due to the lack of FGF. Indeed by simply adding FGF to vegetal pole explants Cornell and Kimelman were able to induce mesoderm, presumably by synergising with endogenous Vg-1. This experiment could be used to determine if native FGF-2 can act differently in the two hemispheres, namely, whether native FGF-2 is now able to induce mesoderm in the vegetal pole.

1.7.2. TGF-β like molecules

The roles of members of the TGF-β superfamily during Xenopus development are complex and varied. Their function in Xenopus patterning can be split into three general categories: mesoderm induction, dorsalisation and ventralisation. Each of these is specified by a different member of this superfamily.
Members of the TGF-β superfamily are synthesised as large precursor molecules of about 370 amino acids. These are then cleaved at a basic site with the consensus sequence R-X-X-R, thus liberating an amino terminal pro region, and a carboxyl terminal mature region (Massague, 1987). The processing of the prototype of this family, TGF-β1, is thought to be mediated by furin, a protein responsible for the processing of many other pro-proteins (Dubois et al., 1995).

The homology between different family members is found in the C terminal mature region of the molecule and contains 7-9 cysteines arranged in a stereotypical manner. These cysteines have been studied in activin A, another TGF-β family member, and are thought to mediate both intramolecular disulphide bridges to stabilise the structure of a monomer as well as intermolecular disulphide bonds to form homo and hetero-dimers (Mason, 1994a).

The response of TGF-β family members is elicited via a transmembrane receptor complex consisting of 4 subunits each possessing a serine/threonine kinase intracellular domain. Two classes of receptor can be distinguished: type I and type II. Each plays a separate role in the transduction of the TGF-β signal. The type I receptor is thought to be responsible for relaying a signal into the cell, and central to this is a series of glycine-serine repeats, the so-called GS box. The type II is responsible for the initial binding of the ligand. The type I receptor and the type II form a complex, probably as a tetramer (Burgess and Maciag, 1989; Wrana et al., 1992), where the type II receptor-ligand complex is recognised by the type I receptor and a stable complex is formed. The type II receptor then phosphorylates the type I in the GS box (Weis-Garcia and Massagué, 1996). This then allows propagation of the signal presumably by the kinase domain of the type I phosphorylating an intermediate that is associated with the phosphorylated GS domain. A representation of the signalling of TGF-β-like molecules is shown in Figure 1.4.
**Figure 1.4**

**The co-operative interactions of the type I and II TGF-β receptor**

The type II receptor is able to bind to ligand. The bound ligand/type II complex is recognised, with high affinity, by the type I receptor. The type I receptor is then phosphorylated in its GS domain by the type II and is then able to transduce a signal. It must be noted that although the representation above depicts TGF-β receptors binding as ligand, it is more probable that binding is mediated through a tetramer, each receptor pair binding a monomer of the dimeric TGF-β molecule.
The downstream components of the TGF-β signalling cascade are as yet unknown. Although some have been characterised, in the absence of other members it is impossible to place any of these in a pathway. At the present time, it appears that a farnesyl-transferase interacts directly with the GS box of the type I receptor (Kawabata et al., 1995). This may cause the tethering of yet another protein to the membrane, in a manner analogous to the activation of ras (Marshall, 1993). Though the identity of this protein is unclear, genetic screens in *C. elegans* (Savage et al., 1996) and in *Drosophila* (Raftery et al., 1995) have identified likely candidates. The Mad (*mothers against dpp*) gene encodes a presumptive cytoplasmic factor (Sekelsky et al., 1995). This gene has no known homology to domains in other genes, so its sequence does not offer any clues about the possible mode of action. Another gene thought to be involved in the dpp pathway is schnurri (Arora et al., 1995; Greider et al., 1995; Staehling-Hampton et al., 1995). This encodes a large zinc finger gene thought to encode a transcription factor. Again, it is unclear what the exact role for this transcription factor is, and whether it is activated during the dpp response or merely forms part of a basal complex that is required for dpp signalling.

The possibility that the specificity of the different TGF-β members lies in the interactions of proteins to the GS box is intriguing, and it is possible that the different interactions lead to the activation of the different Mad proteins, indeed several *Xenopus* Mad proteins are known and each appears to possess differing functions (G. Thomsen, personal communication)

1.7.2.1. Activin and mesoderm induction

The involvement of activin in mesoderm induction has been introduced above. Activin was isolated as the active fraction in the supernatant from a *Xenopus* cell line, XTC. This supernatant was shown to be a potent mesoderm inducer (Smith, 1987). By using the animal cap assay Smith and colleagues were able test each fraction from a HPLC column
to and test its mesoderm inducing capabilities by simply observing the morphology of the caps (Smith et al., 1990a).

Surprisingly, the type of mesoderm that activin was able to induce was exquisitely sensitive to the amount of activin used (Green et al., 1990). This threshold response of caps is most clearly seen if the tissue is dispersed during treatment and later reaggregated (Green et al., 1992; Green and Smith, 1990). In this way changes in activin concentration of just 1.5-fold elicited dramatic changes in the expression of molecular makers and tissue differentiation, so that increasing concentrations first activate Xhox 3, a marker of ventral mesoderm, then muscle-specific actin and finally goosecoid, a marker of the most dorso-anterior mesoderm. However these precise thresholds only form if cell-cell contact is maintained (Green et al., 1994; Symes et al., 1994; Wilson and Melton, 1994). A broad, mixed early response may be sharpened by local cellular interactions to define the exact fate of different territories in the marginal zone.

Further evidence for activin functioning as a morphogen gradient comes from experiments by Gurdon and colleagues (Gurdon et al., 1994; Gurdon et al., 1995). In this work animal caps were wrapped around a bead soaked in activin. These explants were then fixed after 5 hours and expression of either Xbra or goosecoid was assayed by in situ hybridisation. The results, consistent with the dispersed cap experiments (Green et al., 1992) show that goosecoid is expressed close to the bead and Xbra is expressed at a further distance away.

These experiments suggest that activin can induce mesoderm and then direct mesodermal patterning and indeed an activin-like activity has been detected in early cleavage stage embryos (Asashima et al., 1991), indicating that this protein maybe supplied as a maternal factor. Although activin RNA is not present in the oocyte, activin subunits are known to be expressed in the follicle cells that surround the egg during oogenesis (Dohrmann et al., 1993). It is possible that maternal activin protein is made in the follicle cells and then transported into the oocyte.
Additional evidence for the role of activin in Xenopus development became available from studies using activin receptors. By truncating the intracellular kinase domain of the type II receptor, it is possible to interfere with the normal signalling mediated through that receptor complex. Presumably over expression of the truncated receptor complex sequesters available ligand from the endogenous functional type II receptor, and consequently from forming an active receptor complex. Over expression of this construct in Xenopus embryos causes the development of an embryo with little or no mesoderm (Hemmati-Brivanlou and Melton, 1992).

Though the arguments supporting the role of activin in mesoderm induction are compelling, there is evidence that argues against its function in mesoderm induction. Over-expression of follistatin, a specific inhibitor of activin has, unlike the truncated activin receptor, no effect on the development of the whole Xenopus embryo (Schulte-Merker et al., 1994a), though both will induce neural tissue in isolated animal caps (Hemmati-Brivanlou et al., 1994; Thompson and Slack, 1992).

Instead, activin may mimic the effect of another TGF-β family member, Vg-1. Vg-1 has all the expression characteristics necessary to fulfil a role in mesoderm induction; it is expressed maternally and localised to the vegetal hemisphere. An active version of the protein, produced by splicing the pro region of the BMP-2, 4 or activin molecule to the mature region of Vg-1, is an efficient inducer of mesoderm (Dale et al., 1992; Kessler and Melton, 1995; Thomsen and Melton, 1993). Substantiating this claim is the fact that the truncated activin receptor used in the "dominant negative" experiment also binds and blocks Vg-1. Follistatin, which as mentioned above has no effect on mesoderm induction, does not block Vg-1 (Kessler and Melton, 1995; Schulte-Merker et al., 1994a).
1.7.2.2. TGF-β type molecules as dorsalising signals

The fact that activin can induce different types of mesoderm in a dose dependent manner does not imply that activin acts as a dorsalising signal. Indeed there is evidence that argues against this. A prediction from embryological experiments using isolated ventral halves of the embryo is that the dorsalising signal is not maternally provided; rather it seems to be active at gastrulation (Dale and Slack, 1987b). If activin is indeed the dorsalising signal, it should direct muscle development in an isolated ventral marginal zone if it is expressed zygotically. This can be achieved using a DNA construct. This places activin under the control of the ubiquitously expressed cytoskeletal actin promoter. When this is injected, transcription from this promoter only begins at the mid-blastula transition (MBT). If the ventral marginal zone of an injected embryo is assayed, one fails to find any evidence of dorsalisation (Jones et al., 1995; Lettice and Slack, 1993; Smith et al., 1993). Though this assay rules out activin as a dorsalising signal, it has revealed other candidates. These include the secreted proteins noggin and chordin (see later) and more members of the TGF-β superfamily, the nodal related proteins.

The Xenopus nodal-related proteins are, as the name implies, close homologues of the mouse nodal gene. Nodal was cloned from a retroviral insertion in mice that led to the disintegration of the primitive streak and abolished the formation of mesoderm (Conlon et al., 1991; Conlon et al., 1994). Nodal is a member of the TGF-β superfamily and is expressed around the node, the mouse equivalent of the Spemann organiser (Zhou et al., 1993). Xenopus nodal related 1 and 2 (Xnr-1 and Xnr-2) were cloned by screening a dorsal lip library with the mouse nodal probe (Jones et al., 1995). Xnr-3 was cloned in an expression screen looking for molecules that will rescue axial development in a UV treated embryo (Smith et al., 1995).

The expression patterns of Xnr-1 and Xnr-2 are similar. Xnr-1 is expressed in an arc on the dorsal side of the embryo. Xnr-2 is expressed around the margin of the embryo, with higher levels on the dorsal side of the embryo. Both genes have similar functions.
although Xnr-2 appears to be more potent. Xnr-2 will induce differing types of mesoderm in animal caps depending on the differing amount of RNA injected. In a manner similar to that shown for activin, increasing concentrations of Xnr-2 induce progressively more and more dorsal tissues. Moreover, the expression of either Xnr-1 or Xnr-2 from a DNA construct will induce the formation of dorso-lateral mesoderm in isolated ventral marginal zones (Jones et al., 1995).

The fact that Xnr-1 and Xnr-2 will induce mesoderm and dorsalise a ventral marginal zone is unusual. Noggin and chordin (see later), will both dorsalise ventral mesoderm. However, in isolated animal caps they induce neural tissue. As I will discuss in the next section, the properties of noggin and chordin can be ascribed to an inhibition of a ventralising signal. This is probably not the case for the nodal-related genes. Instead these may act in an independent pathway directly specifying dorsal development.

1.7.2.3. BMP-4 and ventralisation

As I have hinted above, some aspects of dorsalisation can be accounted for by an inhibition of a ventralising force. Such a force is likely to be provided by yet another TGF-β member, bone morphogenetic protein-4 (BMP-4). Although embryological evidence for such a force is very weak, data has now accumulated to such an extent that the molecular data supporting a ventralising signal is strong.

The first hint that BMP-4 may play a role in development came from work on the decapentaplegic (dpp) mutant in Drosophila melanogaster. The dpp gene shows remarkable homology to BMP-2 and BMP-4 (Padgett et al., 1987) and indeed BMP-4 can rescue the dpp mutation (Padgett et al., 1993). Though dpp has many functions during development, one function is to pattern the ectoderm of the fly (Ferguson and Anderson, 1992a). Transcripts of dpp are detected in the most dorsal 40% of the embryo in the aminoserosa and dorsal ectoderm and in mutants for dpp, these tissues are missing (Irish
and Gelbart, 1987). DPP can pattern fly ectoderm in a dose dependent manner (Ferguson and Anderson, 1992a).

The expression of BMP-4 already gives some clues to what role it may play during early Xenopus development. BMP-4 is expression is excluded from the organiser region of the embryo and the prospective neural plate (Fainsod et al., 1995; Schmidt et al., 1995). This expression pattern suggests that BMP-4 may antagonise the formation of dorsal tissue. Indeed, ectopic expression of BMP-4 causes ventralisation of the Xenopus embryo (Dale et al., 1992; Jones et al., 1992).

Interestingly, a difference does exist in the action of injected BMP-4 RNA and the exogenously applied protein. The injection of BMP-4 RNA leads to the formation of ventral mesoderm in isolated animal caps (Dale et al., 1992; Jones et al., 1992). However, the protein itself cannot induce mesoderm if used in the same way as, for example, activin. It will, however, ventralise the caps response to high concentrations of activin. The apparent discrepancies in these results may point to the need for BMP-4 to be co-synthesised in the embryo with another molecule so that it acts, not as a homodimer, but as a heterodimer. In fact recent data (Naoto Ueno, unpublished report) suggests that exogenously applied protein made up of a dimer of BMP-4 and BMP-7 is sufficient to induce ventral mesoderm in an animal cap explant.

Recent experiments have stressed the importance of BMP-4 in patterning the Xenopus embryo. In a similar way to the activin receptor experiments, ectopic expression of a truncated BMP receptor that is known to block BMP-2, -4 and -7, but not Vg-1, activin or TGF-β1 results in an embryo that is dorsalised (Graff et al., 1994; Maeno et al., 1994; Schmidt et al., 1995; Suzuki et al., 1994).

The truncated BMP receptor causes two switches in developmental fate. Injection of the truncated receptor promotes the formation of dorsal types of meoderm, as judged by the ability to cause notochord development in the ventral marginal zone (Graff et al., 1994) or to induce a second axis if injected into a ventral blastomere of the 4 cell embryo.
Suzuki et al., 1994). The other effect of the truncated receptor is to divert the fate of isolated animal cells from epidermis to neural tissue.

The involvement of BMP-4 in epidermal differentiation had been shown using two separate experiments. Cho and co-workers injected BMP-4 and -7 constructs that were mutated in the regions necessary for processing (Hawley et al., 1995). These inhibited the processing of the native BMPs. In such cases, they were able to cause animal cap tissue to express neural markers. In an elegant series of experiments Hemmati-Brivanalou (Wilson and Hemmati-Brivanlou, 1995) showed that a BMP signal was responsible for directing epidermal development in the animal cap. He made use of the observation that the prolonged dissociation of animal caps resulted in the formation of neural tissue (Sato and Sargent, 1989). By simply adding BMP-4 to the medium the epidermal pathway could be re-imposed. The hypothesis is that dissociation results in neural development due to the dilution of BMP-4.

These results have caused workers to re-appraise the three signal model (Sive, 1993). A ventralising signal, which was not predicted from embryological experiments, has been placed in a revised model of Xenopus development. This model is presented later. Although this section has made much mention of the action of BMP-4, a close relative, BMP-2 possesses almost identical functions (Clements et al., 1995). However, unlike BMP-4 which is expressed at gastrulation stages, high levels of BMP-2 are detected maternally. It is not present during gastrulation stages.

It is also clear that dorsal development and neural induction occurs by inhibition of BMP signalling. In lieu of a truncated BMP receptor the embryo has its own molecules to inhibit BMP-4. These molecules are discussed below.

1.7.3. Noggin and Chordin

The existence of molecules thought to antagonise BMP-4 signalling was hypothesised from analysis of Drosophila mutants. The gene product of the short gastrulation locus
(sog) can be shown to antagonise the dpp mutant (Ferguson and Anderson, 1992b). The gene encodes a 1038 amino acid protein (François et al., 1994) which is thought to be inserted into the membrane, so that it has a very small cytoplasmic tail and a large extracellular domain. The protein is made up from four cysteine rich repeats that show some homology to the cysteine rich domains of thrombospondin and α-procollagen.

Mutants of sog lose neurogenic regions, with an increase in dorsal ectoderm (Ferguson and Anderson, 1992b; Wharton et al., 1993).

The Xenopus homologue of sog, chordin (Sasai et al., 1994), also encodes a large protein, though in Xenopus this is secreted. It was initially cloned as a downstream response of the organiser gene goosecoid (see later), and its function closely mirrors that of goosecoid. The effects of chordin is also very similar to those seen for the truncated BMP-4 receptor; chordin will cause a partial secondary axis if over-expressed in ventral blastomeres, and will change the fate of isolated ectoderm to become neural tissue (Sasai et al., 1995). Chordin is expressed in the areas of the embryo where the function of BMP-4 is expected to be inhibited, that is in the organiser region of the embryo at gastrula stages and in the prospective neural plate. The function of both sog and chordin are interchangeable (François and Bier, 1995; Holley et al., 1995), so that chordin will rescue the sog mutation and sog will induce a secondary axis.

A proposed function for chordin therefore is to directly associate with BMP-4 and sequester it, thus providing some modulation of BMP-4 activity. The question now arises of whether the interaction between chordin and BMP-4 can also be modulated? One possible answer may lie in the activity of a metalloprotease, BMP-1. BMP-1 is a close homologue of the Drosophila gene, tolloid, the product of which is known to enhance the activity of DPP (Ferguson and Anderson, 1992b). The Xenopus homologue will also cause ventralisation (Les Dale, unpublished observations) perhaps potentiating the sensitivity of the embryo to ventralising influences. It is possible that BMP-1 destabilises the binding of BMP-4 to chordin by cleaving sites in chordin. In support of this is recent
data which identifies BMP-1 as being responsible for the cleavage of procollagen during bone growth (Kessler et al., 1996). Chordin, as mentioned above, shares homologous domains with procollagen.

The *Xenopus* gene noggin has no known homologues in *Drosophila*. It was initially cloned in an expression screen as an activity that rescued axial development in UV ventralised embryos (Smith and Harland, 1992). The expression pattern of noggin is also consistent with a proposed function in promoting axial development. Noggin is present as a maternal RNA, but its expression increases around the time of the midblastula transition, and at gastrula stages transcripts are localised to the dorsal marginal zone. Later noggin is expressed in the notochord and prechordal mesoderm. Noggin protein will induce neural tissue in isolated animal caps, and will induce dorso-lateral development in ventral marginal zones (Lamb et al., 1993). The maternal expression of noggin may correspond to a role in the Nieuwkoop centre, that is to induce the organiser tissue. In fact, the most efficient rescue of axial development in UV treated embryos arises from injections of noggin RNA into a vegetal cell of the 32 cell embryo, roughly the area of the Nieuwkoop centre.

The evidence that noggin antagonises BMP-4 comes from unpublished observations reported by Jeffrey Wrana. By injecting noggin RNA into *Drosophila* he was able to create a phenotype very similar to the dpp loss of function mutation. However, injections of noggin into embryos that carried a mutation activating the thick veins BMP receptor did not effect the ventralised phenotype of the mutant. This indicated that noggin acts in the BMP-4 pathway upstream to the receptor.

The inhibition of BMP-4 is not sufficient to allow all dorsal development. This is clearly exemplified in the induction of secondary axes. Both chordin and noggin induce incomplete secondary axes, with anterior levels of the induced axes rarely extending past the hindbrain. Full axial development can only be achieved when RNA made from genes of the wingless/wnt family are injected into ventral blastomeres.
1.7.4. Wnt family

The wnt family of signalling molecules contains a number of members each mediating different decisions, at different times of embryonic development. The prototype for this family is wnt-1 and its Drosophila homologue, wingless (Rijsewijk et al., 1987). Wnt-1 is a extra-cellular glycoprotein. It is unclear whether it can function as a secreted molecule or as a membrane bound molecule, although in either case wnt-1, and wnt genes in general, are unlikely to act over more that a few cell diameters.

In Drosophila development, wingless is known to have a variety of functions (Kessler et al., 1996), the best characterised being the mediation of segment polarity. A genetic pathway has also been elucidated and putative downstream genes of wingless are known. The wingless signal appears to be mediated through the product of the dishevelled gene, where it represses the activity of the Drosophila homologue of Glycogen Synthase Kinase 3β (GSK-3β), Zeste White 3 (zw3). This repression releases a block on the activity of armadillo, the homologue of plakoglobin and β-catenin, genes involved in gap junction assembly. This is shown in figure 1.5.

The function of these genes as downstream targets of wnt signalling appears to be conserved in Xenopus. In Xenopus, wnt-1 over expression on the ventral side of the embryo leads to the duplication of a complete secondary axis (Chakrabarti et al., 1992; McMahon and Moon, 1989; Sokol et al., 1991). Perturbations in the activity of the other members of the wingless signalling cascade lead to the same phenotype. Injection of a kinase dead GSK-3 that has the net effect of activating the wnt signalling pathway will also promote dorsal development and will duplicate a complete double axis (Kawabata et al., 1995; Sekelsky et al., 1995). Over-expression of both the Xenopus homologue of dishevelled (Raftery et al., 1995) and of β-catenin (Greider et al., 1995; Heasman et al., 1994), the Xenopus homologues of armadillo induce the formation of a complete
Figure 1.5

The wingless/wnt signalling pathway

Taken from Siegfried and Perrimon, 1994. Wingless or a wnt protein binds an, as yet, unidentified receptor and its signal is transduced through Dishevilled. This leads to the inactivation of Glycogen Synthase Kinase 3β. One function of active GSK-3β is to inactivate the β-catenin protein. Wnt signalling prevents this, so an accumulation of β-catenin occurs. The downstream effects of this are unclear.
secondary axis. Indeed antibodies thought to activate β-catenin cause duplication of a
secondary axis (Arora et al., 1995).

Injected wnt RNA is translated soon after it is introduced into the Xenopus
embryo. In this respect RNA injections mimic the maternal function for a wnt gene. In
fact at least one wnt gene, Xwnt-11, is known to be expressed maternally. Xwnt-11 is
localised to the vegetal cortex of the unfertilised egg and its expression profile is very
similar to that of Vg-1 (Ku and Melton, 1992), leading to the suggestion that the localised
activation of Xwnt-11 may direct normal dorsal development. However, whereas RNA
injections promote dorsal development, it is clear that expression after the mid-blastula
transition results in the opposite effect. Another wnt gene, Xwnt-8, will induce a
secondary axis when injected as a RNA (Christian et al., 1991; Smith and Harland,
1991). When a construct is injected where Xwnt-8 transcription is controlled using a
cytoskeletal actin promoter the resulting embryo, rather than being hyperdorsal, is
ventralised (Christian and Moon, 1993). Xwnt-8 is expressed in a manner consistent with
this function. It is not expressed maternally, and expression is detected in the ventral and
lateral margin of the embryo (Christian et al., 1991).

The intracellular effects of the wnt genes are unknown. Wnt-1 is an oncogene
associated with the development of a mouse mammary tumour, so the possibility that wnt
genes in general are associated with cell division is attractive. Equally attractive is the
modulation of the intracellular signalling of other pathways particularly those of the IP3
pathway. This is best exemplified by the correlation between the activity of the wnt genes
in Xenopus and the activity of the lithium ion. As described above wnt genes have two
activities, one resulting in dorsalisation when a wnt is present maternally, the other to
ventralisation when the wnt is supplied zygotically. By treating the embryo with lithium
before MBT the embryo becomes hyperdorsalised (Laemmli, 1970). However if lithium
treatment occurs after MBT, the embryo becomes ventralised (Raftery et al., 1995). It is
known that lithium blocks the inositol triphosphate pathway (Berridge et al., 1989) and
indeed the effects of lithium can be blocked by injecting an intermediate of the inositol
triphosphate pathway (Busa and Gimlich, 1989; Masalski et al., 1992).

The co- incidental effects of lithium and wnt genes suggest that wnt genes
attenuate pathways which activate the inositol triphosphate pathway.

1.7.5. Summary

In this section I will attempt to summarise the data presented above and to highlight the
contributions that these signalling molecules make to the development of the Xenopus
embryo. By using the three signal model as a guide (see figure 1.2) a revised model for
Xenopus development, based on the data from the signalling molecules will be presented.

Cortical rotation sets up the asymmetric activation of a gene that specifies dorsal
development. This asymmetrically activated gene is probably Vg-1 due to the attributes
mentioned above, however, as wnt genes are known to induce the most dorsal types of
mesoderm, equally likely is the asymmetric activation of Xwnt-11. These vegetally
localised genes are able to synergise with FGF-2 in the animal hemisphere to induce
dorsal mesoderm in the domain of overlap. Ventral mesoderm may result from the action
of BMP-2 or BMP-4 at these stages. Just as Vg-1 and FGF-2 induce dorsal mesoderm,
perhaps BMP-2/4 and FGF-2 act to cause the formation of ventral mesoderm.

The organiser region of the embryo is known to consist of different domains and
this may reflect a need for both Vg-1 and Xwnt-11 in the induction of dorsal mesoderm. It
is known that the vegetal edge of the organiser gives rise to head mesoderm and that the
animal edge will form components of the midline (Stewart and Gerhart, 1990). It is
conceivable that Xwnt-11 gives rise to the most anterior mesoderm, a function suggested
by its activity when the RNA is mis-expressed on the ventral side of the embryo. Vg-1
may be responsible for the longer range signalling to the more animal regions to direct
the formation of more dorso-posterior cell types.

The organiser patterns the domain of ventral mesoderm using two mechanisms. The first is a direct induction of dorso-lateral cell types by the Xnr genes (Jones et al., 1995). The second results from the inhibition of the ventralising force provided by BMP-4 exerted as a result of the action of noggin and chordin (Sasai et al., 1994; Smith et al., 1993). The reason that the embryo uses two classes of dorsalisation is unclear. A difference does exist between the action of noggin and of Xnr-2. Mike Jones in the Laboratory of Developmental Biology has found that whereas noggin is diffusible and may act over a long range, Xnr-2 signals function over a very short range.
1.8. Transcriptional response to induction and patterning

In the above section I have described how the embryo is able to specify territories by using signalling molecules. In this section I will describe the mechanism by which the embryo is able to interpret that information by describing the transcription factors that are induced in response to growth factor stimulus. As this thesis does not contain any information on the molecular function of transcription factors only a very brief description follows.

Transcription factors are able to bind DNA sequences associated with a particular gene, either activating or repressing its transcription. A transcription factor is able to either modify pre-existing transcription (basal transcription) in which case the DNA sequence bound is an enhancer or it is able to initiate de novo transcription, in which case the sequences bound are referred to as a promoter. The modification of transcription is brought about by means of protein-protein interactions (Roeder, 1991) and in the case of transcriptional activation this is mediated through stereotypical sequences, such as acidic rich domains (Lin and Green, 1991) or proline rich domains (Mermod et al., 1989). Repression on the other hand can function "passively" by inhibiting the binding of an activator to DNA or by limiting its function (e.g. (Han et al., 1989; Keleher et al., 1988)), or it can work "actively" in a manner analogous but opposite to activation, using a mechanism that has not yet been established (Gray et al., 1994; Jaynes and O'Farrell, 1991; Levine and Manley, 1989). It should be noted that an activator and a repressor can both bind the same DNA sequence adding a further level of control to the transcription of genes (Johnson and Krasnow, 1992). In terms of Xenopus development, these activities add a second tier of regulation in the process of induction and patterning, allowing the refinement and integration of the information that a cell receives.

The DNA binding activity of various transcription factors is mediated through several different types of domain, each possessing a characteristic structural motif. These include the helix-turn-helix, the POU domain, the zinc finger, the basic leucine zipper,
the basic helix-loop-helix and the homeobox (Harrison, 1991). Of these, the homeobox gene family plays the most fundamental role in development. As the work presented in this thesis is on the characterisation of a member of this gene family, the next section introduces the homeobox family.

1.8.1. Homeobox genes

The homeobox encodes a 60 amino acid polypeptide known as the homeodomain and it is this domain that represents the DNA binding activity of the respective transcription factors. The homeodomain has been remarkably well-conserved through evolution, with representatives found not only in metazoa but also in fungi (Kües et al., 1992; Shepard et al., 1984) and in plants (Bellmann and Werr, 1992; Vollbrecht et al., 1991). Figure 1.6 presents the consensus homeodomain from a survey of 364 homeodomains (from Bürglin, 1993). The amino acids are listed in decreasing order under a particular position in the homeodomain. The amino acids shown in red are those that are found in less than 15% of homeodomains.

The three dimensional structure of the homeodomain indicates that three alpha helices are adopted. Helix I is separated by a loop from helix II. Helix II and helix III form a helix-turn helix motif. This motif has been described for many prokaryotic transcription factors (Pabo and Sauer, 1992), and it is clear that the motif found in the homeodomain is based on this. However, helix III is extended slightly by two turns of a more flexible helix, termed helix IV, which contains several basic residues (Qian et al., 1989). The constituent amino acids of each helix is shown as boxes above the consensus sequence in figure 1.7.

Already mentioned is the fact that the homeodomain mediates DNA binding. The resolution of the crystal structure of homeodomains complexed to DNA sequences (see Gehring et al., 1994 for references), together with genetic and biochemical studies (Furukubo-Tokunaga et al., 1992; Hanes and Brent, 1991; Schier and Gehring, 1992;
Figure 1.6

The consensus amino acid sequence of the homeodomain

From Bürglin (1992). Amino acids are placed such that the most commonly occurring amino acid in a particular position is at the top of the list. Those amino acids in red are found in 15% of the 364 sequences surveyed. Amino acids occurring less than five times at a particular position are not included.
Treisman et al., 1992) have identified those residues that are important for the binding to DNA. Most of the residues critical for sequence-specific DNA binding are found in helix III/IV. This is not surprising when one considers the docking of the homeodomain to the DNA. This helix is positioned in the major groove of the DNA, helix I and II are aligned in an anti-parallel arrangement above the DNA. A representation of this arrangement is shown in Figure 1.7A. Residues in the region N-terminal helix I also contribute to the DNA binding. This make contacts in the bases in the minor groove of the DNA molecule. Most homeobox genes recognise DNA sequences containing a core motif of 5′-TAAT-3′. This recognition can be altered by altering the sequences in the third helix of the homeodomain (for example see (Dear et al., 1993)). Figure 1.7B shows the residues important for DNA binding in the consensus homeodomain.

Perhaps the most compelling example of the degree of conservation and function amongst the homeobox gene family is that of the Hox family (Kenyon, 1994). The Hox genes encode Antennapedia-class homeodomain proteins that specify the positional information along the anterior-posterior axis of many organisms (see (Krumlauf, 1994; Lawerence and Morata, 1994; McGinnis and Krumlauf, 1992; Salser and Kenyon, 1994) for reviews), and in most species, ranging from the Leech (Shankland et al., 1991) to humans, the organisation of the genes is conserved in a cluster in the genome. The order in which they are expressed along the A-P axis is colinear with their position along the genome. The sequence of the homeodomain is also remarkably conserved, for example only 1 amino acid out of 60 is altered between the Drosophila anntenapaedia gene and the human homologue HOX -A7. The Hox genes do not appear to specify specific body structures, in flies they specify denticle belts in the embryo and flies and antennae in the adult (Lawerence and Morata, 1994). Similarly in vertebrates they not only specify vertebrae along the central body column but also are vital in the patterning of the limb and digit formation (Krumlauf, 1994). The mechanism by which Hox genes are able to specify spatial information is unclear.
Figure 1.7

A. Tertiary structure adopted by the homeodomain

Taken from Gehring et al., 1994. Shown is the resolved structure of the homeodomain of Antennaepedia. The recognition helix (Helix III/IV) is located in the major groove. Helix I and II contact the are perpendicular to this.

B. Residues important in the sequence specific recognition of DNA by the homeodomain

From Bürglin, 1993. Schematic representation of the contacts made by the homeodomain on the DNA and represents a summary of the structures of the homeodomains of Antp and of MATa2 and is fairly applicable to other homeodomains. Residues represented in bold make base specific contacts. Those residues marked with an arrow contact the phosphate backbone.
The ability to provide a cell with spatial information or cellular identity is not limited to the Hox genes. Throughout development, members of various homeobox gene subfamilies have proven to play a vital role in providing spatial information and cellular identity to a region of the embryo. In the next section, I describe some of the transcription factors that are thought to play a role in Xenopus development. Most are homeobox genes and provide yet more examples for the patterning role this family seems to play in the development of a great many species.

1.8.2. Transcription Factors in early Xenopus development

The exact role that the battery of transcription factors in the early Xenopus embryo play is, as yet, unclear. This section is intended to be a description of the function of some of the transcription factors that are present and the possible signals that the transcription factor may be responding to.

The transcription factors that are involved in early Xenopus development can be placed into one of three groups based on their expression pattern at stage 10.5. Those that are expressed in the dorsal marginal zone, those that are expressed throughout the mesoderm, and those that are expressed in the ventral and lateral marginal zones. This is shown in figure 1.8. There is some variation in the exact expression pattern amongst these groups, but the broad categorisation remains valid.

1.8.3. Organiser-specific transcription factors

The importance of the organiser in directing axial development has concentrated efforts to find transcription factors that may mediate the patterning role of the organiser. In fact many genes are known to be expressed in the organiser, however, they have slightly differing functions. This difference may relate to their mode of activation. To exemplify the differing properties of these genes, the function of the genes goosecoid
Organiser specific genes
- goosecoid
- pintallavis
- Xlim-1
- siamois
- Xnot

Genes expressed in entire marginal zone
- Xbra
- XSnail

Ventral-Lateral genes
- Xpo
- XMyoD
- Xom
- Xvent
- Xgbx-1

Figure 1.9
Domains of expression of transcription factors in the *Xenopus* gastrula

This classification of the expression pattern of various transcription factors is based on the localisation of transcripts at gastrula stages of *Xenopus* development.
\( Xlim-1 \) (Taira et al., 1992), \( siamois \) (Lemaire et al., 1995) and \( Xnot \) (von Dassow et al., 1993) are described in this section.

Like the organiser itself, the first three genes listed all possess the ability to induce a secondary axis if mis-expressed, in some form, on the ventral side of the embryo. Although \( Xnot \) cannot induce a secondary axis, it does appear to play a role in axial development.

1.8.3.1. Goosecoid

The gene \( goosecoid \) was originally cloned as part of a screen for homeobox genes expressed in the dorsal marginal zone. It is expressed at the onset of zygotic transcription (Blumberg et al., 1991) in the region of the organiser (Cho et al., 1991). Consistent with this localisation is the observation that treatments perturbing the organiser also perturb the expression of \( goosecoid \). Lithium treatment, which causes the entire mesoderm to act as an organiser (Kao and Ellinson, 1988), results in the expression of \( goosecoid \) throughout the mesoderm (Cho et al., 1991). Conversely an embryo that has been ventralised by ultra-violet irradiation of the vegetal pole, lacks \( goosecoid \) transcripts. The expression of \( goosecoid \) is an immediate early response to activin signalling; that is to say \( goosecoid \) transcription is stimulated by activin in the absence of protein synthesis (Cho et al., 1991). \( Goosecoid \) is not induced by FGF. \( Goosecoid \) will also mimic the organiser in duplicating axial development if ectopically expressed in the ventral regions of the embryo (Cho et al., 1991; Niehrs et al., 1993), however this duplication is with variable frequency and unlike the full secondary axis induced by an organiser graft, the \( goosecoid \) duplicated axis never extends beyond the auditory vesicle (Niehrs et al., 1993). The axial duplication is very similar to the duplicated axis seen by mis-expressing noggin, chordin and the truncated BMP receptor on the ventral side of the embryo (see above). This suggests that the function of \( goosecoid \) may be to repress or counteract the function of a
ventralising agent such as BMP-4. However, inhibition of BMP signalling mediated by the BMP receptor does not induce \textit{goosecoid} ectopically, suggesting that the initiation of \textit{goosecoid} transcription occurs through an alternative route.

One mechanism that \textit{goosecoid} may employ to inhibit BMP-4 function is by the transcription of chordin. Chordin expression (described earlier) closely mirrors that of \textit{goosecoid} and other data, in particular the requirement for protein synthesis for chordin expression, suggests that its transcription may be a target for the \textit{goosecoid} protein (Sasai \textit{et al.}, 1994).

\subsection*{1.8.3.2. Xlim-1}

\textit{Xlim-1} is most highly expressed in the organiser region of the embryo at gastrula stages of development (Taira \textit{et al.}, 1992) and is an immediate early response to activin. \textit{Xlim-1} not only contains a homeobox motif, it also possesses two LIM domains. Although the function of the LIM domain is unclear, it is thought to mediate certain protein-protein interactions. In other LIM-containing transcription factors, this domain has been shown to prevent DNA binding. It is possible that the LIM domain of \textit{Xlim-1} also has the same function. In support of this is the observation that the native gene is essentially non-functional unless certain residues in the LIM motif, thought to mediate the binding of zinc or iron, are changed. This mutant \textit{Xlim-1} was now able to show the range of activities consistent with the fact that is expressed in the organiser (Taira \textit{et al.}, 1994). The mutant \textit{Xlim-1} is able to induce neural tissue in not only explanted animal caps but also in adjacent cells. Here neural marker are induced to form in the untreated cap part of a tissue recombination between mutant expressing animal caps and untreated caps. In a similar recombinant between mutant \textit{Xlim-1} expressing caps and caps injected with \textit{Xbra} (which will normally form ventral mesoderm), a signal emanating from the mutant \textit{Xlim} cap causes the formation of muscle in the adjacent cap. However, \textit{Xlim-1} is unable to duplicate a full axis if the mutant construct is mis-expressed on the ventral side.
of the embryo. Like goosecoid, the induced secondary axis has an anterior limit at the auditory vesicles. Data investigating the requirement for zygotic protein synthesis (Sokol, 1994) for the induction of various genes in explanted marginal zones reveals a difference in the induction of Xlim-1 to that of goosecoid. Whereas goosecoid does not require protein synthesis for its expression in the marginal zone, Xlim-1 does. It is unclear whether this reflects an epistatic relationship between goosecoid and Xlim-1 or whether the two are activated in the organiser by parallel pathways.

The importance of either gene has been addressed in the context of mouse development. Embryos that are genetically null for goosecoid do not have an early patterning defect, instead targeted mutation results in defects in the craniofacial region of the mouse (Rivera-Pérez et al., 1995; Yamada et al., 1995). This region shows goosecoid transcripts during its later phase of expression. The Xlim-1 homologue Lim1 when mutated, shows a spectacular phenotype. Embryos that are homozygous for the disruption lose all structures anterior to the hindbrain (Shawlot and Behringer, 1995). Embryos lack a fore- and mid-brain due to an absence of an organised node, head process and prechordal mesoderm at 7.5 days of development. These results indicate that whereas goosecoid is dispensable in the development of the mouse node, Lim1 is not, and may define the head organising centre of the embryo. Another possible explanation is that the function of goosecoid may be compensated for by another homeobox gene. The importance of Lim1 to the mouse however cannot be disputed.

The experiments in Xenopus described above (Taira et al., 1994) lead to several interesting questions. The first is the identity of the in vivo signal that relieves the inhibition by the LIM domains allowing Xlim-1 to function in vivo. The second is the nature of the signal induced by mutant Xlim-1 over-expression. Taira et al. (Taira et al., 1994) suggest that this signal is able to act over several cell diameters. It is not noggin or follistatin, as neither is induced by over-expression of the mutant Xlim in animal caps. Chordin induction may be a possibility, however it must be noted that the gene product is
a large glycoprotein and therefore unlikely to act over a large range. Finally, the similarity between goosecoid, mutant Xlim-1 and BMP-4 inhibition at these early stages is intriguing; in all three cases only a partial secondary axis is induced by ventral mis-expression. The function of another homeobox gene, siamois (Lemaire et al., 1995) indicates another pathway leading to the development of the organiser.

1.8.3.3. Siamois

The homeobox gene siamois was cloned as part of an expression screen for genes which would cause axial duplication when ventrally mis-expressed (Lemaire et al., 1995). Unlike goosecoid and Xlim-1, siamois mis-expression causes the induction of a complete secondary axis. Siamois expression commences with the onset of zygotic transcription and although it is localised to the dorsal part of the embryo, it is expressed higher in cells located more vegetally to the marginal zone. Lemaire et al. (Lemaire et al., 1995) hypothesise that siamois may play a role in determining the Nieuwkoop centre, that is, in inducing overlying mesoderm to become organiser tissue. However, it is difficult to reconcile this with data suggesting that the activity of the Nieuwkoop centre in inducing the organiser is very weak at zygotic stages (Gimlich, 1986).

More intriguing is unpublished data that suggests that siamois requires the wnt signalling pathway for its expression (F. Fagotto and B. M. Gumbiner; unpublished observations). This may reflect the role that these signals play in vivo (see Figure 1.6).

1.8.3.4. Xnot

The characteristics of the gene Xnot (von Dassow et al., 1993) set it aside from the rest of the genes described in this section. Unlike the other organiser specific genes described, Xnot expression requires the presence of FGF. Xnot was originally cloned in a screen for homeobox genes and occurs not only in frogs but also fish (Talbot et al., 1995) and chick (Stein and Kessel, 1995). Xnot is found as a maternally expressed RNA, but
transcripts accumulate just before gastrulation. The localisation of Xnot is also interesting. After a period of ubiquitous expression, Xnot transcripts are down-regulated in the regions of the embryo that will not give rise to the mesoderm. The next phase of down-regulation localises transcripts to the dorsal marginal zone of the embryo. The Xnot repression in the ventral and lateral parts of the mesoderm is sensitive to cycloheximide, indicating that it requires protein synthesis. Xnot is an immediate early response to both FGF and to activin (von Dassow et al., 1993). Indeed, blocking the FGF signalling pathway using a dominant negative approach (Cornell and Kimelman, 1994) reveals that Xnot transcription requires FGF signalling.

The nature of the signal that excludes the expression of Xnot from the ventral and lateral mesoderm is unknown. However, it is known that BMP-4 mis-expression will down-regulate Xnot expression (von Dassow et al., 1993). As the two are expressed reciprocally, the temptation to place the negative regulatory role of Xnot upon BMP-4 is great.

Xnot appears to mark the notochord progenitors, and it is in these cells that Xnot plays a vital role. From over-expression and mis-expression studies, a pseudo-allele of Xnot, Xnot-2, will enlarge the notochord if the RNA is injected into the dorsal blastomeres of the embryo. It will also, at a lower frequency direct the formation of an ectopic patch of notochord if injected into the ventral side of the embryo (Gont et al., 1996). However, the most compelling evidence for the role of Xnot come from the studies performed in zebrafish. The fish mutation floating head lacks a notochord. This mutation is due to a frame shift mutation in the open reading frame of the zebrafish homologue of Xnot (Talbot et al., 1995)
1.8.4. Mesodermally expressed genes

Of the genes that are expressed in equator of the embryo, the *Xenopus* homologue of the mouse brachyury gene, *Xbra* is the most studied. For that reason this section will concentrate on describing work performed using this gene.

The *Brachyury* or *T* gene encodes a sequence specific DNA binding protein. It was first identified as a dominant mutation in mice with heterozygotes developing truncated posterior axis. Homozygotes develop little or no posterior mesoderm and lack a differentiated notochord (see (Beddington et al., 1992) for review). Homologues have been identified in many other vertebrate species (Herrmann et al., 1990; Kispert et al., 1995; Schulte-Merker et al., 1992; Smith et al., 1991), indeed mutants for the zebrafish homologue, *notail* give a similar phenotype to the mouse *T* mutant (Schulte-Merker et al., 1994b). The conservation of the sequences of various *Brachyury* homologues is largely confined to the N-terminal region. This region, the so-called T-domain, mediates the DNA binding activity of *Brachyury* (Kispert, 1995; Kispert and Herrmann, 1993). This domain does not contain any homologies to other DNA-binding domains, but forms the prototype to a larger family of transcription factors, the T-box family (Bollag et al., 1994).

The conservation, however, is not limited to sequence. The *Brachyury* genes are expressed in an essentially identical expression pattern in mouse, chick, zebrafish and in *Xenopus*. It is expressed most highly in the tissues that are most affected by the mutation, namely the notochord and the posterior mesoderm. It is also expressed transiently in the whole of the prospective mesoderm.

*Xbra* transcripts can be found in the unfertilised *Xenopus* egg, however the onset of zygotic transcription causes a massive up-regulation of transcription. Localised expression first appears in the prospective dorsal mesoderm of the embryo at late blastula stages (Ruiz i Altaba and Jessell, 1992), but by early gastrula stages expression is found throughout the marginal zone of the embryo (Smith et al., 1991). As gastrulation
proceeds, Xbra expression is down-regulated throughout the involuted mesoderm with the exception of the cells of the prospective notochord. Xbra transcripts persist until tailbud stages with expression remaining in the notochord and in the posterior mesoderm (Gont et al., 1993). The expression of Xbra indicates that it may be controlled by mesoderm inducing factors. Indeed, the expression of Xbra can be induced in the animal cap by activin, FGF, BMP-4 and Vg-1 (Graff et al., 1994; Schulte-Merker et al., 1994a; Smith et al., 1991). For activin and FGF this induction can occur in an immediate early fashion (Graff et al., 1994; Schulte-Merker et al., 1994a; Smith et al., 1991). Inhibition of either pathway using a dominant negative receptor approach blocks Xbra expression (Amaya et al., 1991; Hemmati-Brivanlou and Melton, 1992). A truncated BMP receptor does not block the expression of Xbra (Schmidt et al., 1995; Suzuki et al., 1994) indicating that the BMP pathway is not necessary for the expression of Xbra. The ectopic expression of activated components of the FGF signalling pathway (see figure 1.3) will also induce the expression of Xbra (Gotoh et al., 1995; LaBonne et al., 1995; MacNicol et al., 1993; Umbhauer et al., 1995; Whitman and Melton, 1992). Further evidence for the role of intercellular signalling in inducing the expression of Xbra comes from dispersed embryos. Dispersal inhibits the expression of Xbra, although other mesodermal genes such as goosecoid and Xwnt-8 remain unaffected (Lemaire and Gurdon, 1994).

The analysis of the mouse and fish mutations indicates that Brachyury is required for mesodermal formation. Although the lack of genetics in the Xenopus embryo preclude such a loss-of-function experiment, the converse gain-of-function experiment can be easily performed. The injection of Xbra RNA can drive the formation of mesoderm in the presumptive ectoderm (Cunliffe and Smith, 1992). This mesoderm expresses typical ventral markers such as Xhox 3. Higher concentrations of Xbra RNA cause the formation of more lateral cell types such as muscle. This is similar to the effects of FGF upon dispersed animal caps; low concentrations cause the formation of ventral mesoderm,
higher concentration give rise to dorso-lateral mesoderm, typified by muscle
differentiation (Green et al., 1992).

\textit{Xbra} is expressed in the notochord, and both the mouse and fish mutants lack this
structure. However, in \textit{Xenopus}, \textit{Xbra} is unable to induce the formation of notochord.
This implies that \textit{Xbra} is not sufficient to drive the formation of notochord, and that
another factor is required. Co-expression studies using the \textit{Xenopus HNF-3\beta} homologue
\textit{Pintallavis} does result in the formation of notochord. Concentrations at which \textit{Xbra}
would normally form ventral mesoderm, co-expression with low concentrations of
\textit{Pintallavis} induces muscle and with higher concentrations, notochord (O'Reilly et al.,
1995). It is interesting to note that a mouse embryo that is genetically null for \textit{HNF-3\beta}
does not form notochord (Ang and Rossant, 1994; Weinstein et al., 1994), suggesting that
both \textit{brachyury} and \textit{HNF-3\beta} are necessary for the formation of the mouse notochord.

Work on the mechanism of action of \textit{Brachyury} has been mainly performed with
the mouse and with \textit{Xenopus} homologues. One clue comes from the observation that
\textit{Xbra} requires the FGF signalling pathway to induce mesoderm, that is, co-injections of
RNA encoding a truncated FGF receptor and \textit{Xbra} block mesoderm formation in
explanted animal caps (Schulte-Merker and Smith, 1995). An explanation for this comes
from data suggesting an auto-inductive loop between \textit{Xbra} and a member of the FGF
family \textit{eFGF}. \textit{Xbra} is able to induce the expression of \textit{eFGF} (Schulte-Merker and Smith,
1995) and this expression seems to occur in an immediate early fashion (M. Tada, M.
O'Reilly and J. C. Smith; personal communication). The reverse is also true, \textit{eFGF} can
induce the expression of \textit{Xbra}. It is interesting to note that the phenotype of the \textit{Xenopus}
embryos that over-express a truncated FGF receptor resembles the mouse and zebrafish
\textit{Brachyury} homologues.

The reason for this extracellular step in the regulation of \textit{Xbra} is unclear. Perhaps
this mechanism ensures a group of cells rather than a single cell follows a particular fate.
Another possible reason comes from studies performed in the mouse. By using ES cells
derived from homozygotes mutant for \textit{brachyury}, Wilson et al. (Wilson \textit{et al}., 1995; Wilson \textit{et al}., 1993) found that the primary reason for the defects associated with the mutation was due to cells being unable to migrate out of the primitive streak. Indeed analysis of embryos injected with an interfering form of \textit{Xbra} (F. L. Conlon and J. C. Smith; submitted) such that it blocks endogenous \textit{Xbra} function, suggests that the primary cause for the phenotype in \textit{Xenopus} is also due to cell movement. One mechanism by which this may occur is by using the FGF receptor. As mentioned earlier the FGF receptor does not only recognise FGF, various cellular adhesion molecules can also act as ligands (Mason, 1994b). Perhaps the role of the extracellular step is not only to form an auto-inductive loop between \textit{Xbra} and \textit{eFGF}, the secretion of \textit{eFGF} may also antagonise the binding of cellular adhesion molecules with an FGF receptor, thus changing the adhesive nature of \textit{brachyury} expressing cells. Although this is predicted from the data of Wilson et al. (Wilson \textit{et al}., 1995), there is no evidence that such an auto-inductive loop between an FGF and \textit{brachyury} actually exists in mouse.

\textbf{1.8.5. Ventrally-expressed transcription factors}

This class of genes is expressed in the ventral and lateral regions of the embryo. As this thesis describes the induction and activity of a member of this class, this section will be brief. The transcription factors in this class include the \textit{Xenopus} homologue of \textit{MyoD} (Harvey, 1990), a zinc finger gene, \textit{Xpo} (Sato and Sargent, 1991) and the homeobox gene \textit{Xvent} (Gawantka \textit{et al}., 1995). All of these seem to be regulated in different ways and two of them (\textit{XMyoD} and \textit{Xvent}) appear to have different functions in the \textit{Xenopus} embryo.

The \textit{Xenopus} homologue of the \textit{MyoD} gene is a member of the basic helix-loop-helix family of transcription factors and has been shown in a variety of systems to be important for the differentiation of muscle (Weintraub \textit{et al}., 1989). It is expressed maternally, but significant expression only occurs after the mid-blastula transition. At late
blastula stages expression is seen as a ring around the equator. It is then excluded from the dorsal-most tissue, leaving transcripts in the ventral and lateral mesoderm.

Both activin and FGF will induce the expression of MyoD (Harvey, 1990; Hopwood et al., 1989; Rupp and Weintraub, 1991). However, MyoD does not affect the patterning of the mesoderm. Over expression of the gene results in the differentiation of muscle in isolated animal caps (Hopwood and Gurdon, 1990), by a process that seems to involve the nuclear import of the protein (Rupp et al., 1994). It is noted that MyoD is expressed in the muscle progenitors. It is probable that for myogenesis, a variety of factors are required to activate MyoD in only the muscle progenitors.

The characteristics of a homeobox gene, Xvent, are consistent with its expression in the most ventral regions of the embryo. Transcription of Xvent commences at around the late blastula-early gastrula stage of development. It is expressed in the ventral half of the embryo, but is not just confined to the mesoderm, transcripts are also present in the animal hemisphere. Later expression is observed in the posterior part of the embryo (Gawantka et al., 1995). The study of the induction of Xvent expression has so far concentrated on the fact that BMP-4 injections induce its expression in the dorsal marginal zone, however it is probable that its characteristics are similar to the ones reported for Xom in this thesis.

The function of Xvent is consistent with its expression pattern. The mis-expression of RNA in presumptive dorsal blastomeres blocks the formation of the most dorsal tissue. Embryos that have received such dorsal injections show anterior truncations similar to those seen for Xom (see Figure 7.1) and rarely form notochord. It is intriguing that the homeodomain of Xvent, similar to the homeodomain of Xom (this thesis) and to other ventralising homeoboxes (Schmidt et al., 1996) is part of an unusual subclass marked by a threonine at position 47 of the homeodomain thought to change the DNA binding specificity of this family (Dear et al., 1993).The consequence of this is unclear.
1.9. Aims of this Thesis

This project was started in an attempt to isolate a gene that was activated by FGF mesoderm induction without being responsive to activin. Such a marker would be valuable in placing FGF in a hierarchy of signalling events that specify different mesodermal cell types.

Two approaches to isolating such a gene was adopted. The first was to enrich for transcripts of FGF-inducible genes by using subtractive hybridisation. The second utilised the homology between members of the homeobox gene family to clone more members of this family.

Using the latter approach, I have cloned a new homeobox-containing gene, that is expressed in ventral and lateral regions of the embryo. This thesis describes the sequence, expression pattern, induction and function of this gene named Xom. Xom is not purely FGF responsive, indeed in dissociated embryos, Xom transcription is not affected by FGF. However, the characteristics of this gene have afforded insights into the action of the signalling molecule BMP-4.
Chapter 2

2. Materials and Methods

This chapter describes methods that have been commonly used throughout the course of my study. Other procedures are described in detail in the corresponding chapters. The compositions of all solutions are given in Appendix A.

2.1. Embryos and Embryo manipulations

2.1.1. Obtaining Xenopus embryos

Xenopus embryos were obtained by artificial fertilisation as described by Smith and Slack (Smith and Slack, 1983). Briefly these were obtained from adult females that had been injected 12 hours previously with 800 units of human gonadotrophin (HCG). The eggs were expelled by gentle peristalsis of the mother's ventrolateral surface and transferred to a 90 mm petri dish. They were then fertilised by rubbing them with dissected testes from a sacrificed male. Five minutes later the eggs were flooded with 10% Normal Amphibian Medium. The efficiency of fertilisation was initially assessed by observing the numbers of embryos that had rotated so that the animal hemisphere was at the top of the embryo. Embryos were then de-jellied using 2% cysteine hydrochloride at pH 7.8-8.1 and allowed to develop. The embryos were staged according to The Normal Table of Xenopus Development (Nieuwkoop and Faber, 1967).

2.1.2. Microinjections of Xenopus embryos

Dejellied embryos were transferred to 35 mm petri dished lined with 1.2% agarose into 75% NAM supplemented with 4% ficoll. Embryos at the one- to four-cell stage were injected using an air-driven injection system (Inject+matic). Typically, volumes of 10 nl were delivered into the embryo 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 easily determined.

Injections into embryos at the 32 cell stage of development were performed using a Narishige nitrogen driven injector. This allowed the reproducible delivery of small volumes of liquid usually about 4 nl.

Occasionally it was necessary to inject specific blastomeres. To select for dorsal ventral differences, a four-cell embryo showing a marked difference in pigment was used. The light half of the embryo was taken as dorsal. 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.

2.1.3. Embryo manipulations

Animal caps were dissected from embryos at stage 8 (mid-blastula). The vitelline membrane surrounding the embryo was removed manually using sharpened number 5 watchmaker's 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, the animal caps were cut in the same way but were placed in 75% NAM supplemented with 0.1% bovine serum albumin (BSA).

The growth factors used are listed below;

**BMP-4** Recombinant human bone morphogenetic protein 4 (BMP-4; batch number 3178-163/3811-16) was a gift from Genetics Institute Inc. (Cambridge, Massachusetts).

**FGF-2** Xenopus FGF-2 was prepared and purified by Jeremy Green using an expression plasmid provided by David Kimelman and Marc Kirschner.

**Activin A** 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).

A unit of mesoderm inducing activity is defined as the smallest amount of factor present in 1 ml of medium needed to induce mesoderm (Cooke et al., 1987).

An alternative to the animal cap assay was to study dissociated embryos. Dissociated embryos were obtained by rinsing an intact 4-cell stage embryo several times in sterile distilled water and then placing the embryo into calcium-magnesium free medium (CMFM) (Sargent et al., 1986). At stage 8 these embryos are placed in fresh CMFM on agarose coated dishes and the vitelline membrane is removed using sharpened watchmakers forceps. The dissociated cells are dispersed further by gently blowing them with a Pasteur pipette. For incubations with growth factors, the vitelline membranes of the embryos are removed in CMFM supplemented with 0.1% BSA and the growth factor of interest. The dispersed blastomeres are allowed to develop until siblings reach the desired stage. Pasteur pipettes treated with polyHEMA are used to collect the dispersed embryos. This prevents the cells sticking to the glass. Briefly, polyHEMA treatment involves dipping the pipette into a 0.4% solution of polyHEMA in acetone and allowing the pipette to dry for 2 hours in a fume hood.

In order to assay for changes in the fates of dorsal or ventral regions of the embryo, it was necessary to isolate marginal zones. The appearance of the dorsal lip allowed definitive assignation of dorsal and ventral. At this stage (stage 10.5) the vitelline membrane of the embryo was removed and the embryo was placed in 75% NAM on an agarose coated dish so that the vegetal pole were facing upwards. Dissections were carried out using a tungsten needle that had been sharpened electrolytically. For dorsal marginal zones two radial cuts were made at the equator of the embryo, such that they subtended an angle of 30°, either side of the middle of the lip. A single cut was made to liberate the dissected piece of marginal zone from the rest of the embryo. This was done by making a cut slightly below the lip, through the vegetal region to the animal...
hemisphere. For ventral marginal zones, the radial cuts were made around a point 180° from the middle of the lip. The explanted marginal zones were placed in 75% NAM on an agarose coated dish and allowed to develop until siblings reached the desired stage.

2.1.4. Cycloheximide treatment
The co-incubation of animal caps or dissociated embryos in cycloheximide was used to ask whether a growth factor response required protein synthesis (Cascio and Gurdon, 1987). Cycloheximide blocks protein synthesis by destabilising the interaction of ribosomes and mRNA.

During the project two methods were used. The first is based on a regime used by Smith et al. (Smith et al., 1991). Animal caps or dissociated embryos were pre-treated with 7.5 μg ml⁻¹ of cycloheximide for 30 minutes before being transferred to medium containing both cycloheximide and the growth factor. After an incubation period of 30 minutes, the tissue is transferred to medium lacking cycloheximide for a maximum of 4 hours. All incubations were done on 35 mm petri dishes coated with agarose.

The second method is to simply co-incubate the tissue in both cycloheximide and growth factor for a maximum of three hours (Sokol, 1994). This method was used when dissociated cells were investigated to minimise the loss of cells during transfer.

The incorporation of a radiolabelled amino acid was used to determine the efficiency of protein synthesis inhibition. Animal caps or dissociated cells were incubated in ³⁵S labelled amino acids (Promix; Amersham) with or without 7.5 μg ml⁻¹ of cycloheximide. Incorporation was assayed using trichloroacetic acid (TCA) precipitation. Briefly, tissue was homogenised in 100 μl of homogenisation buffer (see Appendix A). To this was added 1 ml of ice cold 10% TCA and 400 μl of 1 mg ml⁻¹ BSA, which served as a carrier during the precipitation step. The acid insoluble fraction was separated using a Whatman glass fibre filter. After 5 rinses in 10% TCA, the filter was dried using
ethanol and the activity of the acid insoluble material was determined using a scintillation counter.

2.2. β-Galactosidase staining of *Xenopus* embryos

Individual blastomeres injected with a nuclear localised form of β-galactosidase can be lineage traced by staining the embryo for β-gal activity. The RNA used was supplied from Dr. Mike Jones.

Embryos were fixed for 1 hour at 4 °C in β gal fix (1 X PBS, 2 mM MgCl₂, 5 mM EGTA, 1% Paraformaldehyde, 0.2% Glutaraldehyde, 0.02% NP-40) and then extensively rinsed in PBS. β-gal activity was revealed by an incubation in X-gal buffer (5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆.3H₂O, 2 mM MgCl₂, 0.01% NP-40, 1 mg ml⁻¹ X-gal) for 2-4 hours at 37 °C. After staining the embryos were refixed in MEMFA overnight, dehydrated and cleared. Embryos were photographed in glass petri dishes (Schott) using Kodachrome 64 Tungsten film with light transmitted through a colour compensating filter.

2.3. Whole Mount Antibody Staining.

For antibody staining, embryos were removed from their vitelline membrane, fixed for 2 hours in MEMFA (see Appendix A) and then transferred into methanol for long term storage. All procedures were performed in either 2 ml or 5 ml screw top glass vials (Phase Separation). Embryos were then bleached by transferral into 80% methanol and 10% hydrogen peroxide before being rehydrated into phosphate buffered saline (PBS) containing 0.1% Triton-X-100. The free ends of any proteins were blocked by immersing the embryos into 1 M Glycine (pH 7) for 30 minutes, the embryo was then rinsed in PBT (PBS, 2 mg ml⁻¹ BSA, 0.1% Triton-X-100). Non-specific epitopes were blocked by incubating the embryos in 5% lamb serum mixed with PBT for 1 hour before the incubation with the primary antibody. For this study two antibodies were used, one
recognising notochord (MZ15; (Smith and Watt, 1985)) and the other recognising muscle (12/101; (Kintner and Brockes, 1984)). The MZ15 antibody was used at a 1:750 dilution; 12/101 was used at a 1:100 dilution. Incubation in these antibodies was performed overnight at 4°C.

The primary antibodies were then rinsed by 6 x 30 minute washes in PBT. The position of 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 the primary antibody. The horseradish peroxidase was stained using Diaminobenzedine (DAB; Sigma) and 0.01% hydrogen peroxide. The inclusion of 0.3 mg ml⁻¹ nickel chloride in the staining buffer intensified the stain. The embryos were then dehydrated in methanol and placed in Murray's clear (2 volumes of benzyl benzoate, 1 volume of benzoic acid) which rendered the embryos transparent.

2.4. Whole Mount in situ hybridisation

Whole mount in situ hybridisation was carried out essentially as described by Harland (Harland, 1991), with some minor modifications to the protocol.

A riboprobe labelled with digoxygenin was hybridised to a whole embryo. The riboprobe was prepared using a standard transcription reaction (Melton et al., 1984). The details of the reaction are given below:

\[
\begin{align*}
10 \mu l & \quad 5 x \text{ Transcription buffer} \\
5 \mu l & \quad 100 \text{ mM DTT} \\
10 \mu l & \quad \text{Digoxygenin nucleotide mix}^a \\
5 \mu l & \quad \text{Linearised template DNA} \\
2.5 \mu l & \quad \text{RNAsin} \\
90 U & \quad \text{RNA polymerase} \\
\text{DEPC-water to } 50 \mu l
\end{align*}
\]
Digoxygenin nucleotide mix is 10 µl of 10mM ATP, GTP and CTP each, 6.5 µl of 10 mM UTP and 3.5 µl of digoxygenin-11 UTP (Boehringer Mannheim).

The mixture was allowed to react at 37 °C for 2.5 hours after which time the template DNA was degraded by addition of 1 µl of RNAase-free DNAase I. The mixture was then phenol/chloroform extracted and the aqueous supernatant run through a R-50 spin column (IBI) to remove all unincorporated nucleotides. The resulting eluate was precipitated using 2 volumes of ethanol. The yield was estimated by determining the optical density, and the probe was diluted to a final concentration of 1 µg µl⁻¹. This was added to prepared embryos.

Embryos at the appropriate stage were fixed using MEMFA for 1-2 hours at room temperature, in 5 ml screw top glass vials (Phase Separation). Embryos were then placed into 100% methanol, in which they were occasionally left for long term storage. These were then rehydrated in PBS containing 0.1% Tween-20 and the embryos were permeabilised using 10 µg ml⁻¹ of proteinase K. Endogenous phosphatases were then blocked by immersion of the embryos in a solution of 0.1 M triethanolamine at pH 7.5 containing 5 µl of acetic anhydride ml⁻¹ of solution for 10 minutes and then the embryo was re-fixed using MEMFA for 30 minutes. The embryos were then placed in the hybridisation solution (see Appendix A) and allowed to prehybridise for 3 hours at 60 °C. This was done with gentle agitation in an environmental shaker. After this time the probe was added to a final concentration of 1 µg ml⁻¹ of solution and homologous sequences were allowed to hybridise overnight at 60 °C.

After the hybridisation was complete, the embryos were washed extensively to reduce the risk of non-specific staining. Washes were done at 60 °C and in 2 x SSC/0.3% CHAPS and then to 0.2 x SSC/0.3% CHAPS. An RNAase step was also included and the non-homologous sequences were removed by digesting single stranded RNA with 20 µg ml⁻¹ RNAase A and 10 units ml⁻¹ RNAase T1. The embryos were then washed twice at 60 °C in PBS containing 0.3% CHAPS.
In order to detect the hybridised sequences, an antibody recognising the digoxigenin moiety was used. This antibody was coupled to the enzyme alkaline phosphatase which allows its colorimetric detection. The embryos were transferred into PBT (PBS, 2 mg ml BSA, 0.1% Triton-X-100) and then into PBT containing 20% lamb serum. This step was used to block any potential sites that would bind the antibody in a non specific fashion. A 1:2000 dilution of sheep anti-dig FAB fragments coupled to alkaline phosphatase (Boerhinger Mannheim) were added to the embryos which were incubated overnight at 4 °C.

After any excess antibody had been washed, the alkaline phosphatase was detected using the Boehringer Mannheim purple AP substrate. Staining was performed in the dark in 24-well plastic dishes so that the extent of staining could be monitored. Once staining was completed the embryos were rinsed in methanol and then re-fixed.

In certain cases it was necessary to bleach the embryos. A solution of 1% hydrogen peroxide and 0.5% formamide was sufficient. If the embryo were to be cleared they were dehydrated using methanol and placed in Murray's clear (see above). Embryos (cleared and uncleared) were then photographed using Kodachrome 64 Tungsten film.

Some stained embryos were also sectioned. Embryos were placed in an ethanol series with three changes of 100% ethanol with each incubation lasting 30 minutes. The ethanol was slowly replaced with histoclear, which in turn was replaced by fibrawax (at 60 °C). These embryos were embedded in dispomoulds. 7 µm section were cut which were mounted on TESPA coated glass slides. The wax was removed by rinsing the slides in histoclear. DPX mountant was used to mount the slides.

2.5. Molecular Biology Techniques

All molecular biology procedures were carried out essentially as described in Sambrook et al. (Sambrook et al., 1989). For this reason, the operations are only briefly described here.
2.5.1. Introduction of DNA into bacteria

The DH5α strain of *Escherichia coli* was rendered competent for transformation using a buffered calcium chloride solution. Briefly an overnight culture of a single colony was diluted 200 times in L-broth and grown to the mid-log phase when the absorbence at 595 nm was 0.4. The cells were pelleted by centrifugation in a Beckman model J-6B at 3000 rpm for 5 minutes. The resulting pellet was gently resuspended in the frozen competent cell buffer. This process was repeated to wash the cells of any residual L-broth. The resuspended cells were then left on ice for 30 minutes after which time the cells were concentrated by centrifugation such that the final volume of the cells was 5% of the culture volume. The now competent cells were aliquoted in 200 µl amounts and frozen at -80 °C.

Plasmid DNA was introduced into these competent cells. For defined plasmids (for example, simple plasmid recovery) 10 µl of competent cells 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 on to L-agar plates containing 100 µg/ml of ampicillin. For more efficient transformations (e.g. for ligations), 200 µl of competent cells were thawed on ice and then added to a pre-chilled tube (Falcon 2059) containing the DNA to be transformed. After 30 minutes on ice, the bacteria were incubated in a 42 °C water bath for 2 minutes. After the heat shock, 900 µl of L-broth was added and the cells were allowed to recover at 37 °C for 30 minutes. The transformation was then plated on L-agar plates containing 100 µg ml⁻¹ of ampicillin and placed in a 37 °C incubator overnight.

2.5.2. Plasmid preparation

Depending on the purpose, plasmids were isolated either as large pure preparations or small scale preparations. Both used the same basic principle, which was to harvest cells and then to lyse them using an alkali. A single colony of transformed bacteria was picked
and used to inoculate L-broth containing 50 µg ml⁻¹ ampicillin. For small scale preparations, 3 ml of medium was used in snap cap 15 ml tubes, with overnight growth in a shaking 37 °C incubator. Large scale preps were done from 200 ml cultures. Cells were harvested by centrifugation and the resulting pellet was resuspended in solution 1 (Tris and EDTA at pH 7) containing 10 µg ml⁻¹ of RNAase A. For large preps the pellet was resuspended in 10 ml of solution, for small preps, 300 µl was used. An equal volume of solution 2 (0.2 M NaOH, 1% SDS) was added to lyse the bacteria. The mixture was allowed to lyse to completion on ice for 5 minutes. Proteins and genomic DNA was then precipitated by adding an equal volume of solution 3 (3 M potassium acetate pH 5.5) and then incubating on ice for a further 30 minutes. The precipitated material was then removed using another centrifugation step.

At this stage, the methods for large and small scale preps diverge. For small scale preps, the supernatant was removed to a fresh microcentrifuge tube, phenol/chloroform extracted and then precipitated with isopropanol. The resulting supernatant from the large scale prep was applied to a pre-equilibrated column from the QAIGEN maxi-prep kit (Hybaid). The column was then washed, and the DNA eluted into a corex tube, precipitated with 0.7 volumes of isopropanol and centrifuged at 10000 rpm in a Sorvall RJ 5 rotor. The final DNA concentration was calculated by reading the optical density at 260 and 280 nm. This data also gave information in the purity of the DNA.

2.5.3. Enzymatic digestion of DNA
Restriction enzyme digest 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 manufacturers was used. In most cases digests were for one hour but this was extended if the enzymes were of low efficiency or if a greater quantity of DNA was to be cut.
In certain cases, such as in the preparation of vectors for cloning, it was necessary to phosphatase the free ends after digestion, to prevent re-ligation of the vector. Calf intestinal alkaline phosphatase (CIAP; Boehringer Mannheim) was used. The restriction enzyme was heat inactivated at 70 °C for 10 minutes, the volume was doubled, this time using CIAP buffer, and 0.5 µl of enzyme. The reaction was incubated for 30 minutes at 37 °C, and the enzyme was heat inactivated. The treated DNA fragment was then either precipitated using ethanol or purified using agarose gel electrophoresis.

2.5.4. Agarose gel electrophoresis

DNA fraction and size estimation was performed by agarose gel electrophoresis. Gels were prepared by dissolving agarose in either 0.5 x TBE (Tris-borate EDTA) or in 1 x TAE (Tris-acetate-EDTA) to a final concentration of between 0.8%-1.6% depending on the expected size of the DNA fragment. To visualise the DNA, 0.5 mg ml⁻¹ ethidium bromide was added to the gel. DNA samples were mixed with gel loading buffer and electrophoresis was performed at 5-20 volts cm⁻¹ 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).

2.5.5. Gel purification of DNA

In order to purify DNA fragments of interested, 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 area 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, and then incubated at 50 °C for 10 minutes. After incubation, the glass beads were spun down and
washed in 3 times in solution B. The DNA was then eluted from the glass beads by incubation in water or TE for 15 minutes at room temperature.

2.5.6. Ligation

Fragments of DNA were ligated using T4 DNA polymerase (Promega). For ligations which involve cohesive ends, a 10 µl reaction was performed, using the buffer supplied by the manufacturer. The reaction was incubated at room temperature for 1-8 hours. For blunt-ended ligations, a buffer containing 5% polyethylene glycol (Gibco BRL) was used. The presence of PEG promotes the formation of intermolecular bonds, discouraging self ligation. These reactions were performed in 20 µl at 14 °C for 4-24 hours.

The ability of a ligation to transform competent cells was taken as an indication of its success. A comparison with a ligation performed with only vector, gave information about the background, though in most cases this was very low.

2.5.7. Polymerase chain reaction (PCR)

For the amplification of certain stretches of DNA, the method of the Polymerase chain reaction was used. Depending on the use of the DNA, either Taq polymerase was used or, if the amplified piece was to be used for injections, Vent (exo+) polymerase was used. The advantage of one over the other was that although Taq polymerase was easier to use and amplified longer stretches of DNA, it had a significant mutational frequency. Vent polymerase on the other hand amplified with a much lower mutational frequency.

The reactions were set up using the buffers supplied. Nucleotides were added to a final concentration of 200 µm and primers were added to a concentration of between 2-200 ng. PCR reactions were performed on a Perkin-Elmer Thermal Cycler using up to 30 cycles. After the reactions were completed, the success of the reaction was assessed by agarose gel electrophoresis. Details of the specific primers used are given in the appropriate chapters.
2.5.8. M13 DNA preparation

To obtain single stranded DNA, the two phases of the M13 phage life cycle was used. In its replication form, M13 is double stranded and can be used for cloning.

Cells containing the double stranded form of the M13 vector were grown at 37 °C for 5 hours in 2 x TY medium (see Appendix A). The cells were then centrifuged and the supernatant containing the packaged single stranded DNA was decanted into a fresh tube. 250 μl of PEG solution (30% polyethylene glycol-8000, 1.6 M NaCl) was added and the mixture incubated for 15 minutes before centrifugation. The supernatent was discarded and the pellet resuspended in 200 μl of TE. This was then subjected to phenol extraction. The single stranded DNA in the aqueous supernatent was then precipitated using 100% ethanol and the resulting pellet resuspended in 20 μl of TE.

2.5.9. Sequencing

The PCR products derived during the screen for homeobox genes were sequenced as single stranded DNA using the dideoxynucleotide chain termination method of Sanger (Sambrook et al., 1989). Single stranded DNA was annealed to primer at a concentration of 0.5 ng μl⁻¹ in annealing buffer (14 mM Tris pH 8; 7 mM magnesium chloride) together with nucleotides containing specific chain termination analogues. This mixture was incubated at 55 °C for 15 minutes and then cooled to room temperature. The reaction was labelled using ³⁵S-dATP and Klenow polymerase for 15 minutes at room temperature. A further incubation for 15 minutes was performed when a mixture of deoxynucleotides was added to the reaction. Following the addition of formamide loading dyes to the reaction and denaturation, the different chain termination products were size fractionated using polyacrylamide gel electrophoresis.

Xom cDNA was sequenced using a double stranded sequencing kit (Deaza T7 sequencing kit; Pharmacia) using T7 DNA polymerase to label the DNA fragment with
S-dATP. This was performed as instructed by the manufacturer. The use of an analogue of deoxyguanine, 7-deaza guanine, in this procedure eliminated band compressions in the sequencing gel. These are caused when fragments of different sizes but containing different secondary structures migrate with very similar mobilities.

All sequences were analysed using the MacVector programme. Overlapping sequences were assembled using the AssemblyLIGN programme. Homologies with known genes were assessed by a comparison of submitted sequences in the SWISS-PROT database using the BLAST programme.

2.6. RNA isolation and analysis

2.6.1. RNA isolation

RNA was isolated using the acid phenol/guanidinium isothiocyanate procedure (Chomczynski, 1993). Briefly, tissue was homogenised in a solution of guanidine isothiocyanate, sodium citrate and sarkosyl to which 0.7% β-mercaptoethanol has been added. The pH was then adjusted by adding one-tenth of the volume of 2M sodium acetate at pH 4. An equal volume of water-equilibrated phenol was added and the mixture was vortexed and allowed to remain on ice for 15 minutes. The phases were then separated by adding 0.2 volumes of chloroform. The aqueous upper phase was then precipitated using an equal volume of isopropanol. The resulting pellet was then resuspended in 50 μl of water and precipitated using a final concentration of 4M lithium chloride after an hour long incubation on ice.

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

Poly A+ RNA was isolated from total RNA using the poly ATtract system (Promega). A biotinylated oligo-dT primer was hybridised to the poly A+ fraction of total RNA extracted using the method described above. The biotinylated primer-poly A+ RNA hybrids were then separated from the total RNA using paramagnetic particles linked to
streptavidin. The poly A+ RNA was then released by incubating the washed beads in a low salt solution.

2.6.2. Northern Blotting Analysis

Northern analysis is used to determine the size, integrity and amount of a particular RNA. The RNA must first be size fractionated using agarose gel electrophoresis. However due to the extent of secondary structure formation of RNA, gel electrophoresis is carried out under conditions which eliminate this potential problem.

The agarose gel was run using the formaldehyde/MOPS buffer system. Agarose (between 1-1.6 g) was dissolved in 72 ml of DEPC-treated water. When the solution had cooled to about 60 °C, 18 ml of formalin and 10 ml of 10 x MOPS (see Appendix A) were added, and the gel was poured into a clean casting tray. The gel was then submerged in 1 x MOPS buffer and the RNA samples were loaded. The RNA was prepared for loading by mixing 4.5 μl with 2 μl of 10 x MOPS buffer, 3.5 μl of formalin and 10 μl of formamide. The sample was heated at 55 °C for 15 minutes and applied to the gel. A sample of RNA size markers was also run in duplicate, so that one lane could be stained using ethidium bromide. The applied RNA was then electrophoresised at 30 volts, overnight.

A nylon membrane (Hybond N; Amersham) was used to adsorb the RNA after size fractionation. The gel was washed successively in 50 mM NaOH, 0.1 M NaCl (for 20 minutes); 0.1 M Tris pH 7.6 (for 20 minutes) and then 2 x SSC for a further 20 minutes. The gel was then transferred using capillarity to the nylon membrane in 20 x SSC overnight. The RNA was cross-linked to the membrane by exposure to UV light in a Stratalinker (Stratagene) and then baked for 2 hours at 80 °C.

The filter was prehybridised using Church's buffer (7% SDS; 1% BSA; 0.5 M sodium phosphate pH 7; 1 mM EDTA) containing 100 μg ml⁻¹ salmon sperm DNA to block non specific sites for 2 hours at 60 °C. Radiolabelled probe, made as described
below, was added to the prehybridisation solution at 5 x 10 cpm ml⁻¹ and then hybridised overnight at 60 °C. Filters were then washed at 60 °C using 2 x SSC/0.1% SDS three times for 15 minutes before being washed in 0.2 x SSC/0.1% SDS twice for 15 minutes each. The washed filter was then wrapped in cling film and exposed to a Kodak X-OMAT film with intensifying screens to determine the position of the hybridised probe.

2.6.3. RNAase Protection

Although northern analysis yielded information about the quantity of RNA present, the inherent lack of sensitivity presented some problems. Instead, RNAase protection assays were used when source material was limiting. These were performed according to the method of Kreig and Melton (Krieg and Melton, 1987). A radiolabelled riboprobe was hybridised to total RNA. Non-homologous sequences were then removed using RNAases such as RNAase A or T1. These RNAases were insensitive to double stranded RNA hybrids but efficiently degraded any single stranded species. The result was resolved on a polyacrylamide gel.

The probes used during the project are listed in Table 1. Usually, the protection was performed using two probes, one corresponding to the gene of interest and the other, a loading control (either EF-1α or ornithine decarboxylase).

Full length cDNAs or fragments were subcloned in the Bluescript vector, which contains promoters for the T7 and T3 viral RNA polymerases. After linearisation with the appropriate restriction enzyme, the 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>Gene</th>
<th>Linearising site/ polymerase</th>
<th>Probe length</th>
<th>Protected length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ef-1α</td>
<td>Hinf I/ SP6</td>
<td>150</td>
<td>114</td>
</tr>
<tr>
<td>Ornithine decarboylase</td>
<td>Bgl II/ T7</td>
<td>120</td>
<td>90</td>
</tr>
<tr>
<td>Xom</td>
<td>Not I/ T3</td>
<td>247</td>
<td>198</td>
</tr>
<tr>
<td>Xbra</td>
<td>Ssp I/ SP6</td>
<td>293</td>
<td>214</td>
</tr>
<tr>
<td>BMP-4</td>
<td>Hpa I/ T7</td>
<td>360</td>
<td>300</td>
</tr>
<tr>
<td>Muscle and cytoskeletal actin</td>
<td>EcoR I/ SP6</td>
<td>380</td>
<td>270 (muscle actin)</td>
</tr>
<tr>
<td>αT4-Globin</td>
<td>EcoR I/ SP6</td>
<td>319</td>
<td>250</td>
</tr>
</tbody>
</table>

Table 1: The probes used during the course of this study are presented above, together with the linearisation sites and the RNA polymerase used to transcribe the probe.
2 µl  5 x Transcription buffer (see Appendix A)
0.5 µl  0.2 M DTT
1 µl  10 mM Nucleotides (-UTP)
0.5 µl  RNAsin (Promega)
5 µl  32P-UTP
1 µl  Linearised template DNA
1 µl  RNA polymerase

The reaction was incubated for 1.5 hours at 37 ºC, after which time 20 µl of formamide-containing loading dye was added. The reaction was denatured by heating to 80 ºC for 2 minutes and then size fractionated using a short polyacrylamide gel (6% polyacrylamide, 7 M Urea). This gel was run at 35 W for roughly 1 hour, or 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. This gel piece was macerated and the labelled RNA eluted for 2-3 hours at 50 ºC. The eluate was then precipitated and resuspended so that 1 µl contained at least 5 x 10^5 counts. The EF-1α riboprobe was labelled to one-fifth of the activity by the inclusion of some cold UTP (Sargent and Bennett, 1990). This prevented occlusion of the final result by a very strong EF-1α signal. The labelled probes were then added to RNA that had been dissolved in 70% formamide and 1 X hybridisation salts (see Appendix A), heated to 80 ºC and then hybrids were allowed to form for 8-14 hours at 50 ºC. After hybridisation, non-homologous sequences were degraded by digestion with either RNAase A or RNAase T1 or both at 37 ºC for 30-45 minutes. The RNAases were then themselves destroyed by incubation with proteinase K for 15 minutes at 37 ºC and the whole reaction subjected to phenol/chloroform extraction followed by precipitation using ethanol. The resulting pellet was then washed extensively in 70% ethanol and then resuspended in 2 µl of DEPC-treated water. Formamide loading
dyes were then added to the solution and the mixture was applied to a large 8%
polyacrylamide gel containing 7 M urea. The RNA was electrophoresised until the xylene
cyanol had migrated 75% of the total gel length at 55 W. The gel was then fixed using a
solution containing 10% methanol and 10% acetic acid in water, dried and then exposed
at -70 °C with intensifying screens to X-ray film.

2.6.4. In vitro Transcription

RNA that was to be injected into *Xenopus* embryos was transcribed from a construct that
contained the 5' and 3' untranslated regions of β-globin. This increased the efficiency of
translation from the RNA of choice (Kreig and Melton, 1984). The transcription method
used allowed for the synthesis of capped RNA. This was also necessary for the translation
of the RNA. After cloning the cDNA into the *Bgl* II site of the vector pSP64T, which
placed the cDNA in the middle of the UTRs of β-globin, the transcription reaction was
assembled as described below:

10 µl 5 x Transcription buffer
5 µl 0.1 M DTT
5 µl 10 mM ATP
5 µl 10 mM CTP
5 µl 10 mM UTP
5 µl 1 mM GTP
5 µl 5 mM Cap analogue (m7-G; New England Biolabs)
2.5 µl RNAsin (Promega)
2.5 µl SP6 RNA polymerase (Promega)
5 µl Linearised template DNA (1 mg ml⁻¹)

The reaction was incubated for 30 minutes at 37 °C after which time 2.5 µl of 10
mM GTP was added and the reaction was incubated at 37 °C for a further 90 minutes.
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 and the nucleotides were then removed by passing the reaction through a spin column (R-50; IBI). The resulting eluate was then precipitated by the addition of one-third volume 7.5 M ammonium acetate and 2.5 volume of ethanol. The exact constructs used during the course of this project are described in the appropriate chapters.

To determine the efficiency of translation, the synthetic RNA was translated using the rabbit reticulolysate system (Promega). Briefly, 200 ng of synthetic RNA was added to 20 μl of reticulolysate, together with 1 μl of 35S labelled methionine and 1 μl of amino acids minus methionine. The reaction was incubated at 30 °C for 1 hour and then the results analysed using SDS-PAGE, using the buffer system of Laemmli (Laemmli, 1970). Sizes were estimated by comparison to pre stained "Rainbow" molecular weight markers (Amersham).

2.6.5. cDNA Library Construction

Two cDNA libraries were made during the course of my studies. One was made from Xenopus ovaries, and the other from FGF treated animal caps. The ovary cDNA library was not completely characterised and so its attributes are presented here rather than in the results section. The cDNA library derived from FGF treated animal caps was characterised more thoroughly and data from this is presented in the next chapter.

Xenopus ovaries were obtained by sacrificing a Xenopus female. RNA was extracted from this using the method described above with some minor modifications. Due to the robustness of the ovaries it was necessary to use a tissue homogeniser to extract the RNA. Consequently the volume of the extraction was scaled up by a factor of twenty. The material was resuspended in 10 ml of solution D contain 0.7% β-mercaptoethanol and homogenised in a baked glass homogeniser. The homogenised tissue was decanted into a sterile 50 ml screw top polyurethane tube (Falcon), containing
10 ml of phenol extraction buffer (100 mM Tris pH 8; 10 mM EDTA; 0.1% SDS) layered on top of 10 ml of phenol. This mixture was vortex vigorously and then 10 ml of chloroform added to separate the phases. Centrifugation was used to clarify the mixture, and the aqueous phase removed to a fresh tube containing 20 ml of buffered phenol/chloroform. This was vortex once more and spun. The upper aqueous phase was once more decanted to another tube containing 20 ml of chloroform, mixed and then centrifuged. This aqueous phase as precipitated with an equal volume of isopropanol. The pellet was then resuspended in TE and then re-precipitated using a 3.3 M Lithium chloride at 0 °C for 1 hour. Poly A+ RNA was purified as described above.

For the FGF treated animal caps library, the source RNA was derived from 1500 caps cut at stage 8 and treated until stage 10.5 with 100 units of bFGF (see above). The animal caps were frozen at stage 10.5 and the RNA extracted as described above.

The isolated RNA was subjected to a reverse transcription reaction using reverse transcriptase from the Murine Moloney Leukaemia Virus (MMLV-RT) (Stratagene; La Jolla, Calif.). The reaction was primed from the poly A+ RNA using an adapter primer consisting of a chain of deoxytymidine (oligo-dT) attached to a site for the restriction enzyme Xho I. The priming RNA was then removed by digestion with RNAase H (Stratagene; La Jolla, Calif.) and the resulting strand of DNA was rendered double stranded using T4 DNA polymerase (Stratagene; La Jolla, Calif.). This double stranded cDNA was hemi-methylated during the polymerase reaction by replacing deoxycytidine (dCTP) with a methylated analogue which was incorporated into the DNA molecule. The purpose of the hemi-methylation is to ensure that no internal restriction sites are digested by the methylation sensitive restriction enzymes EcoRI and XhoI. EcoRI linkers were ligated and the double stranded cDNA was then digested with EcoRI and XhoI. The digest was size selected using a Sephacryl S-400 spin column and the largest two fractions were ligated into EcoR I and Xho I half arms from the phage vector λZAP II.
The phage was packaged using a Gigapack Gold XL kit (Stratagene; La Jolla, Calif.) according to the manufacturer's instructions.

The efficiency and complexity of the library construction was assessed by plating the phage particles on a bacterial lawn and counting the number of plaques (spots on the bacterial lawn were the phage particle has infected the host bacteria and caused it to lyse). Briefly, plating was carried out by mixing phage particles with a suspension of *E. coli* SURE cells. These had been grown for 8 hours and then resuspended in 10 mM magnesium sulphate such that the optical density of the resulting suspension at 595 nm was 0.5. The phage particles and bacteria mixture was incubated at 37 °C for 20 minutes before being added to 3 ml of top agarose (NZY broth+0.7% agarose; see Appendix A) at 48 °C before being plated onto 100 mm petri dishes containing NZY agar. These plates were then incubated at either 37 °C for 6-10 hours or overnight at 30 °C. The integration of an insert into the vector disrupted the reading frame of a subunit of the *lac Z* gene. By supplementing the plating mixture with IPTG (to induce synthesis of the *lac Z* gene) and X-gal (a coloured substrate for the *lac Z* gene), selection for those plaques containing inserts (white) against those without inserts (blue) can be applied.

Both the ovary cDNA library and the FGF treated animal caps library were calculated to contain about 10% non-recombinants. The ovary cDNA library contained 1.2 x 10^6 clones. The average insert size of 24 randomly picked clones was 1 kb, the largest being over 3kb.

Another very useful characteristic that the λZAP II vector possessed was the ease with which the plasmid form could be liberated from the phage vector. λZAP II contains all the sequences of the plasmid Bluescript, including the intergenic region of a filamentous f1 phage. The effect of this is that the region in between this intergenic region is replicated and packaged as a filamentous phage particle (a phagemid) when the lambda phage and a helper phage (ExAssist; Stratagene) co-infect a host bacteria. The filamentous phage DNA is single stranded. The double stranded plasmid can be rescued
from the packaged phagemid by infecting another bacterial strain, SOLR. This strain of bacteria does not allow replication of the ExAssist helper phage and Lambda phage is unable to infect. Only the single stranded phagemid can replicate. The double stranded form is rescued by plating on L-agar plates containing 100 μg ml⁻¹ of ampicillin. These plates were then incubated overnight at 37 °C.

2.7. Library Screening

To obtain a full-length cDNA of the clones, the FGF treated animal caps library in λZAP II was screened. Bacteriophage plaques contain large amounts of DNA and these can be transferred to a filter membrane. The location of the plaques that contain particular stretches of DNA can be revealed by hybridising the bound DNA to a radiolabelled probe derived from the sequence of interest.

2.7.1. Plating Libraries and Transfer to Filter Membranes

The cDNA library made from FGF treated animal caps was plated on to 245 mm x 245 mm square plates (Nunc) that had been lined with NZY agar. The phage were plated with the *E. coli* strain, XL-1B as described above, using top NZY agarose but without the addition of IPTG or X-gal. The phage were plated such that the expected density was 200 000 plaque forming units per plate on four plates. These were grown to near confluence at 37 °C for 8 hours, after which time they were placed at 4 °C for 2 hours.

Nylon filters (Hybond N; Amersham) were used to adsorb the DNA from each plaque. Each plate was transferred to two filters, so that patterns of hybridisation could be checked by comparison with duplicates. A filter was lain on top of the cold plates for either 2 minutes (primary filter) or 4 minutes (duplicate filter). Whilst on top of the plate the position of the filter was marked using an 8 gauge needle containing India ink and each filter was labelled with a ball point pen. The filter was removed from the plate using blunt flat faced forceps and transferred to 3MM filter paper (Whatman) that had been
soaked in 0.2 M NaOH/1.5 M NaCl in order to denature the DNA. The filter was placed on the paper such that the side with the DNA is facing upwards. The alkali is the neutralised by transferring the filter to another piece of 3MM paper soaked in 0.4 M Tris pH 7/2 x SSC and then to one soaked in 2 x SSC. Each step is for 1 minute. The filter was then dried in a vacuum oven at 80 °C for 2 hours.

2.7.2. Probe preparation

A stretch of DNA of interest was gel purified such that it did not contain any vector sequences. This piece of between 100-1000 bases was labelled using the Mega Prime kit (Amersham). This involves the hybridisation of random nonamer oligonucleotides to initiate DNA synthesis using the Klenow fragment of DNA polymerase I. By replacing one of the nucleotide components with 32P labelled nucleotides it is possible to produce a series of overlapping DNA fragments with a high specific activity (around 10^9 cpm µg^-1).

Approximately 25-100 ng of DNA was denatured in the presence of the primers by boiling for 2 minutes. The primers and DNA were allowed to anneal by slowly cooling the mixture to room temperature. To this was added 10 mM each of dATP, dTTP and dGTP, MBq (in 5 µl) 32P-dCTP and 2 µl of the Klenow fragment of DNA polymerase I. The reaction was incubated at 37 °C for 15 minutes and then passed through a G-50 spin column to remove all unincorporated nucleotides. A small amount of the probe (1 µl) was mixed with 5 ml of scintillation fluid and the specific activity of the probe determined.

2.7.3. Hybridisation

Hybridisation was performed essentially as described for Northern blotting analysis (see above). For library filters they were always performed in heat sealed polythene bags in an environmental shaker at 60 °C. Pre-hybridisation was performed by briefly immersing the filters in a solution of 10 x Denhardts (see Appendix A) and 0.4 x SSC and then baking in
a vacuum oven at 80 °C for 20 minutes. The hybridisation solution was also different to that used for Northern analysis. This was made up of 5 x SSPE; 5 x Denhardt's solution; 1% SDS; 0.1% sodium pyrophosphate; 10% Dextran sulphate and 100 μg ml⁻¹ Torula RNA to block non-specific sites. The probe was added to 10⁶ cpm ml⁻¹ of hyb solution. The probe was then allowed to hybridise at 60 °C for 8-14 hours.

After hybridisation, the filters were washed down to 0.2 x SSC/0.1% SDS at 65 °C, wrapped in cling film and exposed to an X-ray film (X-OMAT; Kodak).

Positive plaques were cored from the plates using the India ink marks as guides. In order to purify the plaques the cores were placed into λ suspension buffer (see Appendix A). After an incubation of 1 hour to liberate the phage particles, this suspension was plated on to NZY agar in 150 mm petri dishes using *E. coli* XL-1B as host using the method described above. Filters were lifted from these plates and re-screened as described. This process was repeated until a single positive plaque could be picked with certainty.
Chapter 3

3. Cloning by Subtractive Hybridisation

As described in the introduction, *Xenopus* development seems to depend on the action of various signalling molecules. However, the response to such signalling molecules is still unclear. Although some downstream genes had been found (see Introduction), the identification of more genes is important so that a complete picture of the inductive processes can be gained. In an attempt to understand the response to inducing factors, two methods were used to search for inducible genes. The first, presented in this chapter, involved subtractive hybridisation where a cDNA library made from untreated animal caps was subtracted from a cDNA library made from animal caps that had been treated with FGF. The other, using homology screening to look for members of a gene family, is presented in the next chapter.

Identification of inducible genes by subtractive hybridisation provided an attractive alternative to other methods of gene isolation employed successfully in Drosophila (Nüsslein-Volhard et al., 1984) and in mouse (for example see (Herrmann et al., 1990). Both these techniques involve the characterisation of genetic mutants, an approach unsuited to the *Xenopus* embryo.

Unfortunately, in my hands, the procedure did not yield any useful clones. This chapter describes the characteristics of the FGF treated animal caps library that I had constructed and the method used to enrich this library for sequences specific for FGF induction. The characterisation of the subtracted library is presented and I will describe the problems that were encountered.

This work was carried out in collaboration with Dr. Alison Snape also at the Division of Developmental Biology.
3.1. Materials and Methods

3.1.1. Subtractive Hybridisation

In an attempt to make a cDNA library enriched for genes induced by FGF, RNA derived from the FGF-induced library was hybridised with an excess of RNA synthesised from a directional cDNA library made from RNA derived from untreated caps (kindly provided by Dr Alison Snape). The procedure, described below is represented in figure 3.1.

The cDNA libraries were converted into their plasmid form (pBluescript SK-) according to the manufacturer’s directions. The plasmid forms of both libraries were linearised using NotI and RNA was transcribed from the T7 promoter using the Ribomax protocol (Promega technical bulletin 166, 1992). RNA from the untreated caps library was biotinylated by supplementing the nucleotide mix with 0.5 mM biotin-11 UTP.

15 ng of RNA from the FGF-induced caps library was converted to cDNA, priming from the SK sequence upstream of the 5' end of the insert. 32P-dCTP was added as a tracer. The template RNA was then hydrolysed, and the cDNA purified by running through a Sephadex G-50 spin column. Approximately 800 ng of this cDNA was then hybridised with a 75-fold excess of biotinylated RNA from the untreated caps library in 0.12 M sodium phosphate (pH 7), 0.82 M NaCl, 1 mM Na2EDTA and 0.1% SDS at 65°C for 20 hours.

Hybridised sequences were removed using streptavidin-linked paramagnetic beads (Promega). The beads were prepared by washing them three times in 0.5 X SSC. These were then added to a ten-fold dilution of the above hybridisation mix and incubated at room temperature for 10 minutes. The beads were collected at the side of the tube using a magnet (Promega) and the supernatant was removed into a fresh tube. This process was repeated until no counts were detected on the magnetic beads, indicating that all hybridised sequences had been removed. The cDNA, now enriched for genes activated by bFGF, was rendered double stranded by hybridising with RNA derived from the FGF treated caps library in order to prime second strand synthesis (Sambrook et al., 1989).
Figure 3.1

Subtractive hybridisation of an FGF-treated animal cap cDNA library

Below is shown a representation of the protocol that was followed to enrich for clones that were specifically induced by FGF. See text for further explanation.
The DNA was size selected using a Sephacryl S-400 spin column, cloned into the EcoRI and XhoI sites of λZAP and plated using blue/white colour selection. 700 white recombinants were then individually picked into 200 µl of Lambda suspension medium (100 mM NaCl; 10mM MgSO4; 50 mM Tris, pH 7; 0.01% gelatin) for further analysis.

The clones were further characterised by analysing their insert size and taking a random sample for whole mount in situ hybridisation analysis. These steps were performed in collaboration with Brenda Price and Maneesha Imandar also in the Division of Developmental Biology.

3.2. Results

3.2.1. Characteristics of the FGF-treated animal caps cDNA Library

A cDNA library was made from animal caps treated with basic FGF from stage 8 (mid-blastulae) to stage 10.5 (early gastrulae). To verify that FGF treatment had exerted its effect, the library was assayed for the presence of Xbra (Smith et al., 1991) and for keratin (Jamrich et al., 1987). Xbra expression occurs as a response to mesoderm induction. This contrasts with the expression of keratin, a gene expressed in the non-neural ectoderm and is down-regulated in response to FGF and further repressed in response to activin. The cDNA libraries made from untreated animal caps and from animal caps that had been treated with activin were also tested. These had been constructed by Dr. Alison Snape.

RNA made from the plasmid forms of the library was subjected to Northern blot analysis. The results of this are shown in Figure 3.2. Keratin transcripts can be detected in RNA that has been transcribed from the untreated animal caps library. However, expression is down-regulated in both the activin treated and FGF treated animal caps library. Northern blot analysis with Xbra shows the reciprocal result; expression is found only in the treated animal caps library. It should be noted that in the FGF treated animal caps library none of the Xbra transcripts appear to be full length, when compared to stage
Figure 3.2
Northern analysis of RNA derived from cDNA libraries from induced and uninduced animal caps

RNA was transcribed from linearised plasmid from each library. Each sample was run in duplicate on an agarose gel and blotted onto a nylon filter. These were then probed with Xbra and keratin. Xbra is present in the cDNA libraries derived from activin and FGF treated animal caps and is absent in those that were untreated.

Conversely the ectodermal marker keratin is present in the untreated caps library, considerably down-regulated in the FGF treated caps library and absent in the activin treated caps library.
10 embryos. Full length transcripts encoding Xbra appear to be present in the activin
treated animal caps library

All the transcripts that hybridised with the keratin probe are the same length,
though no judgement on the integrity of these transcripts could be made as transcripts
were not detected in RNA made from stage 10 embryos, presumably due to its low
abundance.

The unamplified FGF-treated caps library contained 1.5 x 10^6 plaque forming
unit. Clones from this library were picked and converted to the plasmid form and digested
with the appropriate restriction enzymes with which to judge the length of the inserts. 24
clones were picked and revealed an average insert size of about 1.2 kb, with the largest
being 2.4 kb and the shortest being 0.8 kb.

3.2.2. Subtractive hybridisation
The subtracted procedure outlined in the Materials and Methods section resulted in cDNA
cloned into the phage vector λZAP II. This was packaged and then plated. Plating
allowed the distinction between the those clones that contained inserts from those that did
not. Plaques resulting from phage that do not contain inserts have an intact β-
galactosidase and are therefore blue when plated on a medium containing IPTG and X-
gal (see chapter 2). Phage that possess inserts form white plaques.

The ratio of nine blue plaques to one white plaque was observed indicating that
only 10% of the phage plated were recombinants. By picking each white plaque, it was
hoped that each subsequent manipulation would be easier. Only 700 clones could be
identified and these were individually cored and placed in 250 μl of lambda suspension
buffer in a 98 well microtitre plate.

Three lines of analysis were employed, each confirming the suspicion that the
subtractive procedure had not been performed to its full potential. The first involved the
screening of each clone with Xbra, which should be amplified by the subtraction
procedure, and cytoskeletal actin which should be absent. Each clone was manually spotted on to a lawn of bacteria. Filters were then lifted and hybridised using the methods already described. Probes specific for Xbra and for the isoform of cytoskeletal actin used (Actin type V; supplied by Tim Mohun) failed to detect any hybridising signals.

Analysis of the inserts indicated that clones were present in approximately 50% of the picked clones and that these had an average insert size of 300 bases. The largest clone was around 800 bases long. A random selection of clones, all containing inserts, were then subjected to whole mount in situ hybridisation analysis. If the subtraction procedure had worked, some of the clones would be expected to show a localised expression. Of the 20 clones analysed in this fashion none showed any specific signal in the embryo when compared with sense controls.

A limited analysis of the sequence of these clones was also performed. Twelve recombinant clones were sequenced from both sides of the vector. Sequence analysis revealed that none of the clones showed any homologies to any known genes.

3.3. Discussion

In this chapter I first described the characterisation of a cDNA library constructed for this project. I then used this library in a subtractive hybridisation approach to search for genes that are induced by FGF in animal caps. By the analysing the number of clones, the insert size and the types of clones that were present, the FGF treated animal caps library was acceptable. I then used this library in a subtractive procedure to enrich genes that were specific for FGF mediated mesoderm induction. However, the method for subtraction was flawed.

The failure of the subtractive method was probably due to the inclusion of homologous vector sequences in the transcribed RNA from the libraries and then used for the subtraction. Although this stretch was small, with 70 bases in the vector and another 20 corresponding to the poly T tail of the cDNA, the sensitivity of the procedure to
common sequences meant that these pseudo-complements were also removed. Perversely, the procedure preferentially selected for non-recombinant clones as these short sequences are reverse transcribed faster during the manufacture of cDNA for subtraction. They are therefore more abundantly represented in the subtracted library. This was highlighted by the high number of blue non-recombinants that were observed when the subtracted library was plated in the presence of IPTG and X-gal.

The criteria used to screen the subtracted library do not completely eliminate the possibility that the subtraction procedure did identify some cDNAs expressed specifically in response to FGF. Firstly, whole mount \textit{in situ} analysis may not have resulted in the identification of any localised signal due to the size of the probes. Secondly, the fact that sequence analysis did not yield any information may result from the fact that only a relatively short stretch of DNA is being inspected, and that any homologies with other vertebrate genes, for example, may not be apparent in this region. This point is reinforced when one considers that the subtraction method may have results in the cloning of the most 3' sequences. This is due to the nature of reverse transcription, which prefers sequences close to the priming site. As the reverse transcriptase was primed using an oligo dT prime the 3' untranslated regions of the genes would be preferentially reverse transcribed. This region varies considerably from species to species.

The fact that only 700 clones were obtained indicates that vector-cross hybridisation was indeed a problem. One solution might be the inclusion of an oligonucleotide primer corresponding to the common sequences in the vector during the hybridisation step. This would block all these homologous sites in the vector, only allowing the subtraction of complementary sequences in the cDNA. Another method that was used successfully in the Laboratory of Mammalian Development (Harrison \textit{et al.}, 1995) was to re-clone one of the libraries into another vector. This method has the potential pitfall that certain inserts may be lost during the cloning process.
While the subtractive strategy was being considered, the second approach to cloning responsive genes was yielding interesting results. In the next chapter I describe this approach which led to the cloning of Xom, the subject of this thesis.
4. The Cloning of Xom

As an alternative to using subtractive hybridisation, another approach to identify new genes was employed. This was to use homologies present in a gene family to pick out new members. The cloning of genes that are part of a wider family has frequently identified new members (for example (Blumberg et al., 1991; Christian et al., 1991; Jones et al., 1995)) and as a second approach towards identifying potential targets of mesoderm inducing factors, this proved to be successful.

The homeobox-containing family provides an example of a family that has a well defined consensus sequence and whose members are important for positional and cellular identity during development. As mentioned previously (see Introduction), the homeodomain is a 60 amino acid long motif that mediates DNA binding. This motif is remarkably well-conserved, with some residues being almost invariant.

This chapter describes the utilisation of these conserved regions to screen the FGF-treated caps library for clones. One clone was picked for further study and here I discuss information that can be gleaned from the sequence data.

The initial stages of this work were carried out with the help of Surendra Koetcha and Norma Towers both in the Division of Developmental Biology.

4.1. Material and Methods

4.1.1. Homology screening strategy

A polymerase chain reaction based strategy was used to amplify sequences in the FGF-treated animal caps library that contained a homeodomain. A degenerate primer was used with an anchoring priming, which was found in the vector of the cDNA library, to selectively amplify clones that contained a homeobox. The degenerate primer was
directed against the sequence QIKIWF and this sequence represents one of the most conserved stretches in the homeodomain (see figure 1.7) (Bürglin, 1993; Scott et al., 1989). Another useful characteristic is the NRR motif next to the primer. This sequence is present in virtually all homeobox containing genes and allows rapid identification of homeoboxes.

The plasmid form of the FGF treated library was used as a substrate for the reaction. This was obtained using a modification of the protocol presented in chapter 2 using the manufacturer's mass excision protocol. The anchoring primer was directed against the T7 promoter sequence present 3' to the insert. The primer corresponding to the homeodomain incorporated a restriction site so that the subcloning of any products would be made easier. The nucleotide sequences of the primers are below; underlining in the 5' primer indicates an EcoRI site:

5' primer: 5′ GGAATTCAGATATGTTTCA 3'
           G T A C C
3' primer: 5′ GTAATCAGACTCAGCTATAGG 3'

The conditions for amplification were: 94°C for 30 seconds, 50°C for 1.5 minutes and 72°C for 2.5 minutes for 5 cycles, and then at 94°C for 30 seconds, 65°C for 1.5 minutes and 72°C for 21/2 minutes for 25 cycles. The reaction was then digested with EcoRI and Kpn I and ligated into M13.

Recombinants were selected using blue/white colour selection and 96 white recombinants were picked and grown as previously described. Single-stranded DNA was prepared from the 96 recombinant plaques.
**Figure 4.1**

**Procedure for cloning homeobox containing genes by PCR**

This figure shows the protocol followed for the cloning of genes that contained a homeodomain. Further details can be found in the text.
In order to estimate the number of different clones that the PCR strategy had amplified, the T-reaction from a dideoxy sequencing reaction was run on a 6% denaturing acrylamide gel using Klenow DNA polymerase (Promega) and $^{32}$P dATP as the label. This formed an initial screen; any clones that did not contain the primer were not further sequenced.

A representative of each class of clone that contained the homeobox primer was then sequenced using the deaza T7 sequencing kit (Pharmacia) as suggested by the manufacturer's instructions, and the sequences were analysed for existing homologies by database searching. Clones that warranted further study were amplified from the M13 vector by PCR using primers directed against flanking sequences. This PCR product was then subcloned into pBluescript KS+.

### 4.2. Results

#### 4.2.1. PCR Cloning

T-tracking the products obtained from the PCR screen allowed the classification of 7 different classes of clones. A representative from each class was then sequenced. This is shown in Table 4.1. Only three of these classes proved to be homeobox containing genes, *goosecoid* (Cho et al., 1991) was represented five times and *X-ANF 1* (Zaraisky et al., 1992) was found twice. The presence of *goosecoid* in the FGF-treated caps library was somewhat surprising. Although *goosecoid* transcription can be induced in response to activin-mediated mesoderm induction, it is insensitive to FGF (Cho et al., 1991). The RNA species of *goosecoid* found in the library may represent a maternal form of this gene.

Three clones appeared to be novel. Two were represented once and upon sequencing were found not to contain a homeodomain. No further work was carried out on these. The third was also the most abundant clone, representing 21 of the 96 clones that were T-tracked. Upon sequencing this clone was found to contain a homeodomain,
<table>
<thead>
<tr>
<th>Gene</th>
<th>Representation /96</th>
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<tr>
<td>goosecoid</td>
<td>5</td>
</tr>
<tr>
<td>XANF-1</td>
<td>2</td>
</tr>
<tr>
<td>Xom</td>
<td>21</td>
</tr>
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<td>tubulin</td>
<td>1</td>
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<tr>
<td>cytochrome C oxidase</td>
<td>1</td>
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<td>Unknown 1</td>
<td>1</td>
</tr>
<tr>
<td>Unknown 2</td>
<td>1</td>
</tr>
<tr>
<td>Not determined a</td>
<td>58</td>
</tr>
</tbody>
</table>

Table 4.1

**Classes of clones obtained using a degenerate homeobox primer**

From a total of 96 clones, seven distinct classes contained the homeobox primer. Each was sequenced and analysed for homologies. Xom represents the most highly represented gene

\(^a\) Not determined as T-tracking indicated the absence of the homeobox primer
Primer

\begin{verbatim}
GGAATT CCAQTTAAA ATCTGGTTTC TGAACCGCAG GATGAATAC AAACGGGAAA
\end{verbatim}

\begin{verbatim}
Q I K I W F Q N R R M K Y K R E
\end{verbatim}

\begin{verbatim}
TCCAAGATGG CAGACACGCAC TCATACCACC CAGCCCAAGTT CCTTGGTGTC TACGGCTATG
\end{verbatim}

\begin{verbatim}
I Q D G R P D S Y H P A Q F L G V Y G Y
\end{verbatim}

\begin{verbatim}
CACAGCAGCC CACTCCTGTA TTCCAGCATG CAGTCCAACA TCCCTACCCA GTTTATAACC
\end{verbatim}

\begin{verbatim}
A Q Q P T P V F Q H A V C H P Y P G Y N
\end{verbatim}

\begin{verbatim}
Kpn I site
\end{verbatim}

\begin{verbatim}
CACTAAATGA AACCTGCGT GGTACC
\end{verbatim}

\begin{verbatim}
P L M E T L P G
\end{verbatim}

Figure 4.2

Sequence of the partial Xom clone obtained by PCR

Shown is the sequence of the Xom PCR clone obtained using the strategy outlined in figure 4.1. The sequence corresponding to the primer is boxed. This clone was used throughout this project as a riboprobe for RNAase protection.
and a full length version of this clone was pursued. Figure 4.2 shows the sequence of the polymerase chain reaction generated clone.

4.2.2. Sequence of Xom
The FGF-treated animal caps library was screened for a full length version of this clone which was later called "Xom". The PCR product was used as a probe to screen 800,000 plaques. Of these 147 were positive, and twenty were cored and purified. Each clone was converted to the plasmid form and the size of each analysed. The longest clone, of about 1.3 kb, was sequenced using the deaza T7 sequencing kit (Pharmacia), using a combination of overlapping primers and subclones generated by restriction digests. To verify that the sequence was the longest obtainable, a gastrula staged library made by Dr. Bruce Blumberg in the University of California, Los Angeles, was screened. 247 clones strongly hybridised with a probe made from the 5' region of Xom. Of the 24 clones that were analysed the longest was also about 1.3 kb. This agrees with data from Northern analysis predicting a Xom mRNA encoding Xom is around 1.3 kb (see later).

The sequence of Xom cDNA is presented in figure 4.3. The sequence was analysed using the MacVector program (Eastman Kodak company). Conceptual translation of the sequence of Xom predicted a protein with a Mr of approximately 32 kilodaltons from the longest open-reading frame of 860 bases. To verify that the clone contained an the open reading frame, RNA made from the clone was translated using rabbit reticulolysate (Promega). The results were size fractionated by SDS-PAGE and are presented in figure 4.4. Rather than the 32 kilodalton protein expected, a major band migrating at around 55 kilodaltons was observed. This discrepancy between the expected size and the size obtained by in vitro translation may result from an unusual charge composition of the protein which the SDS in the gel cannot overcome. Indeed, as I will discuss later, the homeodomain region is very basic (with a pI of 11) and upstream to this
Figure 4.3

The Xom cDNA sequence

The Xom cDNA sequence is shown on the upper line. Underneath is the deduced amino acid sequence. The homeodomain is represented by amino acids highlighted in bold. The unique threonine at position 47 of the homeodomain is underlined.
Figure 4.4

In vitro translation of Xom cDNA

The Xom was cloned into pSP64T (see later) and linearised with three separate restriction enzymes. Asp 718 cuts at position 866 in the homeodomain and gives a transcript lacking a 3' untranslated region (UTR) or a poly A tail. Xho I cuts after the poly A tail of the gene, Pst I cuts after the 3' UTR of β-globin. RNA was then made from each transcript and translated using the rabbit reticulolysate system (Pomega).

Translation of the full length transcripts gave a band larger than expected (lanes 5 and 6). The difference between the full-length and truncated version of Xom was as expected (about 5KDa). An transcript made from Xom in the antisense orientation did not translate.
is a region of low pH (a pI of about 4). These regions may play a central role in the function of Xom.

4.2.3. Xom is a member of an unusual class of homeobox gene

The deduced amino acid sequence of the homeodomain of Xom was used in a database search. The sequence of a Drosophila annasae gene called Om ID (Tanda and Corces, 1991) and its D. melanogaster homologue, Bar H1 (Higashijima et al., 1992) showed the highest degree of homology to Xom. Both these genes are involved in eye development and in peripheral neuron specification in Drosophila and it was this limited homology in both sequence and expression pattern (see later) that suggested the name Xom for this gene. Figure 4.5 shows the homology of Xom with Om ID.

A further characteristic of the homeodomain is a threonine at position 47 of the homeodomain. This threonine places Xom in a family of homeobox proteins that include both Bar H1 and Om ID as well as Barx-1 (Tissier-Seta et al., 1995), a gene expressed in craniofacial mesenchyme of the mouse embryo, Hox 11 (Kennedy et al., 1991) which is involved in a T cell lymphoblastic leukaemia, and a gene involved in haematopoiesis, prh (Crompton et al., 1992). Figure 4.6 shows the sequences of these genes. Interestingly, Xom may define a sub group in this family with other Xenopus genes that appear to contain a threonine at position 47. Though more mention of these will be made later, these include Xvent-1, Xbrl-3 (Papalopulu and Kintner, 1996), pv-1 (M. Jamrich; personal communication) and Vox (Schmidt et al., 1996). It is intriguing that these appear to be expressed in very similar domains to Xom.

4.2.4. Other domains in Xom

The presence of a homeodomain in Xom implies that Xom should act as a DNA binding transcription factor. Examination of regions outside the homeodomain reveal domains that may modulate transcription. A representation of the domain structure of
Figure 4.5

Homology between Xom and Om 1D

Shown below is the homology of Xom with the Drosophila annasae gene Om 1D and its melanogaster homologue BarH1. The homeodomain is marked in **bold**. Residues that are conserved between the two are shown as a line in the sequence of Om 1D (•; bottom row). Conserved substitutions are represented in lower case. The homology between Xom and Om 1D though highest in the homeodomain also extends into the N-terminal region.
Figure 4.6
Comparison of the homeodomain sequences of the *Hox 11/TCL* subfamily of proteins with the homeodomain sequence of *Xom*.

Shown is the alignment of other members of the Hox 11 family. Conserved substitutions are shown in lower case. The percentage homologies are shown, taking into account the conserved amino acid changes. References can be found in the main body of the text.
**Xom** is shown in figure 4.7, together with a charge plot of the conceptual protein. The homeodomain is basic in nature, as are all DNA-binding motifs. It is flanked by a very acidic domain that also contains a serine/threonine rich domain, and a proline rich domain. The acidic domain (amino acids 41 to 172; red) is defined as a region in which the average charge over 20 amino acids is consistently negative, the charge being calculated using the DNAstar sequence analysis programme. The proline rich domain (amino acids 255-320) contains 16 proline residues in a stretch of 66 amino acids (yellow). Both these domains are thought to act as transcriptional activation domains, by allowing the interaction of other proteins (Ma and Ptashne, 1987; Mermod *et al.*, 1989). These domains do not show any homology with other acidic or proline rich domains found in other transcription factors.

### 4.3. Discussion

In this chapter I have described the cloning of **Xom** using a PCR based screen to identify homeobox containing genes. **Xom** does indeed contain a homeodomain and is hypothesised to act as a transcription factor. Consistent with this proposal is data from the charge profile of the conceptual **Xom** protein. This reveals that as well as a basic DNA binding domain, **Xom** contains two domains that have been shown, in other systems, to act as transcriptional activators. The fact that these domains are thought to mediate protein-protein interactions is intriguing and suggests that in the embryo, **Xom** may form part of a transcriptional complex.

The homeodomain of **Xom** is unusual due to a substitution of the normal valine or isoleucine at position 47 by a threonine residue. This substitution in another member of this sub family, **Hox 11**, has been shown to alter the DNA binding specificity (Dear *et al.*, 1993). Whereas a homeobox containing gene will usually bind a core consensus sequence of TAAT, **Hox 11** binds the sequence TAAC. The usual core consensus sequence can be bound if the threonine residue is mutated to a valine. It is possible that
The acidic domain (amino acids 41-172) is defined as the region in which the average charge over 20 amino acids is consistently negative. In the middle of this domain is a region rich in serine and threonine (stippled). The homeodomain is contained within amino acids 173-233 (purple). The proline rich domain (amino acids 255-320) contains 16 proline residues in a stretch of 65 amino acids (yellow).
the contribution threonone makes to the altered binding specificity of Hox 11 is a characteristic shared by other members of this class.

* Xom shows limited homology with the Drosophila genes Om ID/Bar H1. This homology extends to the flanking sequences. These flanking sequences in Om ID are important in the function of this gene (Akimura and Saigo, 1991). The homology of Xom and Om ID/Bar H1 is not just restricted to sequence. The next chapter describes the expression pattern of Xom, and it is apparent that both Xom and Om ID show expression in common structures.
Chapter 5

5. The Expression of Xom

The expression pattern of a gene can yield information about the role it may play in development and by comparing this pattern with the expression of other genes, information about its regulation can be inferred.

In this chapter I describe the expression of Xom during Xenopus development. The temporal expression was examined using Northern analysis and RNAase protection. Xom transcripts were spatially localised using whole mount in situ hybridisation. The methods used are all as described in chapter 2.

5.1. Xom is expressed at MBT and then throughout gastrulation

Northern analysis was used to determine the size of the Xom transcript and the time of expression. RNA derived from embryos between stage 1 and stage 42 was extracted, size separated on an agarose gel and transferred on to a nylon membrane. The resulting blot was hybridised to a probe derived from the PCR fragment encoding 200 bases of Xom sequence (see chapter 3). After washing at high stringency (0.2 x SSC at 65 °C) the blot was exposed for 3 days at -70°C with intensifying screens. The autoradiograph is shown in figure 5.1. The blot was rehybridised with an ornithine decarboxylase probe to confirm even loading of the RNA. The autoradiograph reveals that a single transcript of 1.3 kb is detected, suggesting that no isoforms of Xom resulting from differential RNA splicing exist. Xom cannot be detected as a maternally provided gene and Xom transcription commences at the mid-blastula transition (MBT). Transcripts increase to a steady state level from gastrulation until late tailbud stages (stage 36) at which time they are down regulated.
Figure 5.1

Northern analysis of Xom expression

The 195 base par Xom PCR fragment was used to probe a blot containing total embryonic RNA from different stages of Xenopus development. The blot was washed to 0.2 X SSC/0.1% SDS at 65°C and exposed for 2 days at -70°C with screens. A probe against ornithine decarboxylase (ODC) was used to check for equivalent loading in each lane. Transcript size was determined using RNA size markers (Promega).

A single transcript specific for Xom was found at approximately 1.3 kb. This transcript was detectable at stage 9, and transcripts persisted until stage 24, at which time expression appeared to be down-regulated.
To confirm this temporal profile, *Xom* expression was studied by the more sensitive technique of RNAase protection. Again, *Xom* can be detected prior to gastrulation at stage 9 (Fig. 5.2, lane 4). Levels of transcripts peak at mid-gastrulation stages (stage 10; Fig. 5.2, lane 5), decrease slightly at late gastrula (stage 12; Fig. 5.2, lane 6) and then peak once more at mid-neurula stages (stage 17; Fig. 5.2, lane 7). Transcription appears to be down-regulated after this stage, although RNA is still detectable in the oldest stages assayed (Fig. 5.2, lane 9). In other experiments transcripts were not detectable in swimming-tadpole staged embryos (stage 42, data not shown).

5.2. Spatial Expression of *Xom*

5.2.1. Localisation of transcripts by RNAase protection

RNAase protection analysis was used to provide a preliminary impression of the localisation of *Xom* transcripts. Embryos were dissected into animal, marginal and vegetal regions at stage 10.5 (early gastrula). The marginal region was then subdivided into dorsal, ventral and lateral marginal zones and then frozen. RNA derived from these pieces was analysed for the expression of *Xom* compared to the expression of EF-1a. Figure 5.3 shows one such experiment. The experiment revealed that levels of *Xom* RNA were higher in the ventral and lateral marginal zone when compared to the dorsal marginal zone. Transcripts are detected in both the animal and vegetal pole at levels comparable to those seen in the ventral and lateral marginal zone.

5.2.2. Localisation of transcripts by whole mount in situ hybridisation

For a more accurate picture of the expression of *Xom*, whole mount in situ hybridisation was performed. The result of this experiment, performed at a range of embryonic stages, is shown in Figure 5.4.
Figure 5.2

Temporal Profile of *Xom*

RNA extracted from embryos at the stages of development indicated above was assayed for the presence of *Xom* and a loading control, ornithine decarboxylase (ODC), in a RNAase protection. The above experiment represents the loading of 1.5 embryo equivalents in each lane. The products of the RNAase digestion were size separated on a 6% polyacrylamide gel and exposed overnight. *Xom* transcripts are detected from stage 9, peak at stage 10.5 and then after a slight decrease peak once more at stage 17. Transcripts decline after stage 26. Not shown in the above figure are the tRNA control for non-specific hybridisation (no signal was detected) and the undigested probes. The undigested probe could not be detected in the experimental tracks (track 1-9) indicating that the RNAase reaction was complete.
Figure 5.2
Temporal Profile of Xom
Figure 5.3
Spatial localisation of Xom transcripts

Six embryos at early gastrula stages (stage 10.5) were dissected into animal, vegetal, dorsal marginal, lateral marginal and ventral marginal explants and immediately frozen. The RNA was then extracted and then subjected to an RNAase protection assay to determine the presence of Xom transcripts in the pieces, and comparing the levels of RNA between samples with reference to a loading control (EF-1 α). The digestion reaction was resolved on a 6% polyacrylamide gel. The resulting autoradiograph is shown above. Transcripts can be detected in the lateral and ventral marginal zones at levels higher than seen in the dorsal marginal zone. This is clearer on a shorter exposure of the gel. Transcripts are also present in the vegetal pole. In other experiments transcripts were seen in the animal cap (see figure 6.1), however in the one shown above, RNA was poorly extracted from this tissue. Not shown are the tRNA control for non-specific hybridisation (this was blank), and the undigested probes to monitor the extent of the RNA digestion reaction.
Figure 5.3
Spatial localisation of Xom transcripts
The earliest expression of Xom appears to be localised to a discrete patch in the marginal zone of the embryo at approximately stage 8. However, expression at this stage is very weak and difficult to photograph. This expression expands slightly so that transcripts are localised to one side of the equator of the embryo (Figure 5.4A). Due to the lack of any landmarks at this stage it is unclear whether this corresponds to the dorsal or ventral regions of the embryo. From the expression later, and from data presented elsewhere in this thesis, a ventral localisation of Xom at these stages seems probable.

As mentioned already, RNAase protection analysis on dissected regions of the embryo suggests that at gastrulation stages Xom is found throughout the embryo, but is less abundant in the dorsal organiser region. Whole mount in situ hybridisation supports this idea (Figure 5.4B), although the levels on the dorsal side are much lower than those predicted by RNAase protection on dissected pieces of the embryo. The higher levels predicted by the dissection data may be due to the fact that the exact extent of the dorsal marginal zone is very hard to judge. Another discrepancy between the RNAase protection data and using whole mount in situ hybridisation is the apparent lack of Xom transcripts in the vegetal pole in the whole mount embryo. It is known that the whole mount in situ hybridisation technique detects vegetally localised transcripts poorly. This is thought to be due to poor probe penetration into the yolk-rich vegetal hemisphere (Hemmati-Brivanlou et al., 1990). However, in some cases these transcripts can be observed in sections of overstained embryos. The vegetally localised transcripts appear as a punctate pattern and these can be seen in figure 5.5 A.

As gastrulation proceeds, Xom expressing cells encroach into the dorsal domain, though never reaching as far as the most dorsal marginal zone. This may reflect the cell movements that occur during gastrulation; as the most dorsal cells move away from the equatorial region, more lateral cells replace them. The result of this can be seen at stage 12 (Figure 5.4C), where Xom expression is excluded from the descendants of the dorsal region, the anterior neural plate and the dorsal midline in the ectoderm. The underlying
Figure 5.4

Whole mount in situ analysis of Xom expression

(A) Vegetal view of a Xenopus embryo at stage 9 shows asymmetric expression of Xom. It is likely that Xom is most highly expressed in ventral cells, because by stage 10.5 (B) transcripts are clearly excluded from the organiser. Apparent lack of expression in vegetal tissue is likely to be due to poor probe penetration. (C) Dorsal view of an embryo at stage 12.5; expression is excluded from the anterior neural plate and the dorsal midline. (D) Dorsal view of an embryo at stage 14. Expression is becoming restricted to two domains, one anterior and one posterior. The posterior domain surrounds the lateral and ventral regions of the embryo. (E) Lateral view of an embryo at stage 20. The anterior domain has resolved to mark the dorsal region of the eye. The posterior domain includes the ventral region of the embryo and (out of the plane of the photograph) the dorsal midline in the trunk. (F) Lateral view of an embryo at stage 32 shows expression of Xom in the dorsal part of the eye, in the proctodeum and in the tailbud. Scattered expression is also visible in the dorsal midline, which may be due to migrating neural crest cells.
mesoderm (the prechordal mesoderm and the notochord) also fail to express Xom. This exclusion probably reflects the inheritance of these regions of the embryo.

At stage 14 (Figure 5.4D), Xom expression begins to resolve to form three domains of expression. The first domain, marking what will eventually become the optic cup of the embryo, can be seen in the figure as paired patches of expression in the anterior part of the embryo. The second domain marks the region that will form the roof plate of the trunk neural tube. This can also be seen in figure 5.4D as a pair of posteriorly localised patches flanking the midline. The final domain, out of view in figure 5.4D shows expression in the ventral regions of the embryo.

By stage 15/16 (not shown) this resolution is complete: the broad domains of expression tighten so that the posterior pair of patches are two lines of expression flanking the neural plate and the anterior expression is now two small arcs of expression. This is more clearly seen at stage 20 when the anterior expression marks the dorsal part of the eye. Also visible is the expression in the ventral regions of the embryo (figure 5.4D).

The roof plate expression of Xom cannot be seen in this view. However, this can be discerned in section (Figure 5.5B). It should be noted that sections are performed on embryos that have been over-stained. This gives a somewhat false impression of the levels of transcripts. In the section shown, as well as the roof plate expression of Xom, transcripts are also detected in all three germ layers in the ventral and lateral regions of the embryo. Compared with the whole mount figure, it is clear that rather then being expressed uniformly in this region, less transcripts are present in the lateral regions.

This is also the case with the dorsal expression in the eye (figure 5.5C). Sections seem to suggest that this expression is uniform in the retina of the eye, contrary to what is seen in whole mount. It is therefore probable that in the eye, Xom transcripts, though present in the rest of the eye, are highly enriched on the dorsal part. Over-staining merely causes the levels of transcripts to appear more or less equal due to the saturation by the stain.
Figure 5.5

Sections of embryos processed for whole mount in situ analysis using a probe specific for Xom.

(A) Section of stage 10.5 embryo. Transcripts are absent from the organiser, but are present in ventral and lateral regions of the embryo in all three germ layers. The punctate staining in vegetal tissue may be due to poor probe penetration into this yolky tissue. (B) Section of a stage 19 embryo in the trunk region. Staining is visible in all three germ layers of the ventral and lateral regions of the embryo, and in the dorsal cells of the roof plate of the neural tube. (C) Section of the embryo shown in (B) at the level of the head. Transcripts are present in the presumptive eye.
The roof plate of the posterior neural tube gives rise to the neural crest of the trunk region. *Xom* expression also seems to follow this lineage and at stage 32 (Figure 5.4F) expression can be detected in typical derivatives of the trunk neural crest. These include cells of the peripheral nervous system, the fin and the lateral line organ. By now, *Xom* has become localised to the dorsal part of the retina of the future eye, and is also seen in the proctodeum. This gives rise to the anal region of the gut and has a complex origin arising in part from trunk neural crest. The speckled appearance of the trunk midline is probably due to the migration of neural crest cells. Also seen is expression in the tailbud. This may reflect the composite of two domains of expression. The dorsal contribution is from the base of the spinal cord, with cells about to converge to become trunk roof plate cells. The ventral expression marks a general territory populated by new cells from the tailbud that are destined to become ventral cell types.

5.3. Discussion
In this chapter I have described the expression pattern of *Xom* as studied by Northern analysis, RNAase protection and whole mount *in situ* hybridisation. The first two techniques were used to determine the temporal pattern of *Xom* expression. Whole mount *in situ* hybridisation was used to determine the spatial pattern of transcripts.

Northern analysis indicates that only one *Xom* transcript is present in the embryo. *Xom* transcripts rapidly accumulate on or around the onset of zygotic transcription. A finer time course around this period is presented in the next chapter. It is clear that the initial expression already shows some localisation and by stage 9 *Xom* transcripts can be detected in a gradient in the margin of the embryo. From the later expression pattern, where *Xom* is excluded from the dorsal most tissue, and from data on the regulation of *Xom* by BMP-4 (see later), it is probable that this expression marks the ventral side of the embryo. The implications of this are interesting. Is a ventrally localised signal initiating
Xom expression in the ventral equator of the Xenopus embryo? I discuss this further in the final chapter of this thesis.

Xom expression is excluded from dorsal tissues at the start of gastrulation, and remains excluded from the dorsal tissues at stage 12, the onset of neuralation. This expression pattern is intriguing because it represents an early distinction between non-neural and neural regions of the embryo. The sharp boundary between the two domains is also suggestive of a repressive mechanism and it is interesting that the expression pattern of Xom at both stage 10.5 and stage 12 is the reciprocal of noggin which at stage 12 is expressed in the future notochord and anterior neural plate, and of chordin which at stage 12 is expressed in the prechordal mesoderm underlying the anterior neural plate. The relationship between Xom and noggin is investigated in the next chapter.

Xom also marks the dorsal part of the optic cup. In fact from an early stage (stage 14; see figure 5.4D), two patches of anterior expression can be seen. This region of the neural plate gives rise to the dorsal part of the eye, the ventral part arising from a more anterior part of the neural plate (Eagleson and Harris, 1989). The purpose of this dorsal expression in the retina of the eye is unclear. One possible role is in establishing the dorsal-ventral differences necessary for axonal guidance. An alternative possible role comes from studies on a highly related Xenopus gene that appears to be expressed in an identical fashion to Xom. Xbr-1 (Papalopulu and Kintner, 1996) is ultimately expressed in the periphery of the dorsal ciliary margin of the eye. The authors suggest that these cells represent a stem cell population of cells that constantly add differentiated neurons to the retina of the growing animal. The role of Xom to maintain a stem cell population may also be conserved in the trunk neural crest.

In the next chapter, I describe the regulatory events that determine the early expression pattern of Xom.
Chapter 6

6. The Regulation of Xom

In the previous chapter I described the expression pattern of Xom. In this chapter, the regulation of Xom localisation is investigated. In particular, two aspects of the expression pattern are studied. The first aspect is the exclusion of Xom from dorsal tissue types. The second aspect concerns signals that may initiate Xom in the embryo. Using a combination of RNAase protection and whole mount in situ hybridisation on embryos treated in a variety of ways, I show that Xom is dependent on signalling by the TGF-β family member BMP-4.

6.1. Materials and Methods

All the methods were as described in Chapter 2. In this chapter various constructs were used for in vitro transcription, and these are described below;

- **noggin**: The construct nogginΔ5′ was used to make RNA for injections (Smith and Harland, 1992)

- **BMP-4**: Messenger RNA was transcribed from a pSP64T construct containing the coding region of BMP-4. Transcription was performed with an *Eco* RI linearised construct using SP6 RNA polymerase (Dale *et al.*, 1992)

- **Xwnt-8**: A plasmid derived from pSP64T containing the Xwnt-8 coding region was used for the transcription of mRNA (Christian *et al.*, 1991)

- **ΔBMPR**: mRNA encoding a truncated form of a receptor specific for BMP-2, -4 and -7 was transcribed from a plasmid that was the very kind gift of Atasashi Suzuki and Naoto Ueno (Schmidt *et al.*, 1995; Suzuki *et al.*, 1994). RNA was made by linearising the plasmid with *Eco* R1 and transcribing with SP6 RNA polymerase.
6.2. Results

6.2.1. Induction of Xom in animal caps

The use of animal caps to assay the induction of genes as a response to growth factor treatments is a common technique in *Xenopus* embryology. However, the assay does rely on the fact that the gene of interest is itself not expressed in the animal cap. As described in the previous chapter, Xom is expressed in the animal cap. Although this makes any definitive statements about the induction of the genes more difficult, the assay still provided a rough guide to genes that may induce Xom transcription. Furthermore, the endogenous level of Xom did prove useful. It provided an easy assay for genes that repressed Xom transcription.

Figure 6.1 shows the result of an animal cap assay in which caps were dissected at stage 7.5 and cultured until sibling controls had reached stage 10.5. Not shown is data from a similar experiment with caps taken at stage 17. The results are similar at both stages.

In this experiment, animal caps were explanted and treated with 16 units ml⁻¹ of activin or 100 units ml⁻¹ of bFGF. Animal caps were also taken from embryos that had been previously injected with 1 ng of BMP-4 RNA, 1 ng of noggin RNA, 1 ng of Xwnt-8 RNA or 100 pg of Xwnt-8 DNA under the control of the cytoskeletal actin promoter. After the culture period, the animal caps were frozen and the level of Xom was determined by RNAase protection.

As already mentioned, Xom is expressed in the animal cap. Upon treatment of the animal caps with activin, FGF, BMP-4 or Xwnt-8 DNA, Xom levels were found to be higher than in untreated caps at both stage 10 (Figure 6.1) and stage 17 (data not shown). Xwnt-8 RNA had little effect on the levels of Xom transcripts, however noggin markedly down-regulated Xom transcription. Even after the autoradiograph was exposed for two weeks, no Xom transcripts could be detected.
Figure 6.1

Expression of Xom in treated animal caps

Expression of Xom in isolated animal pole regions is enhanced by mesoderm-inducing factors and the ventralizing agent BMP-4 and is suppressed by noggin. Animal pole regions were dissected from uninjected embryos or from embryos which had received injections of RNA encoding the indicated proteins. Animal caps from uninjected embryos were cultured in the absence of factors or in the presence of various inducing factors. Expression of Xom was analysed by RNAase protection after culture to the equivalent of stage 10.5. EF-1α was used as a loading control.
6.2.2. Repression of *Xom* by noggin

The repression of *Xom* by noggin was potentially of interest; *Xom* and noggin are reciprocally expressed and so this repression may reflect an *in vivo* mechanism for regulating *Xom* expression.

In order to determine whether noggin was able to repress *Xom* in the whole embryo, whole mount *in situ* hybridisations were performed on noggin injected embryos. In this experiment, one cell of a two cell stage *Xenopus* embryo was injected with 500 pg of noggin RNA. At this stage a blastomere will give rise to either the right or the left half of the embryo. The embryo was allowed to develop until early gastrula stages (stage 10-10.5) and then fixed. The localisation of *Xom* was determined by using the entire *Xom* cDNA as a probe in a whole mount *in situ* hybridisation assay.

Figure 6.2 shows the result of this experiment. In this experiment repression of *Xom* transcription has occurred in one half of the embryo.

6.2.3. Inhibition of BMP signalling down regulates *Xom* expression

As described in the introduction, the function of noggin can be ascribed to the inhibition of BMP signalling. It was therefore conceivable that the mechanism of *Xom* repression was by an inhibition of BMP signalling.

To investigate this further, a truncated BMP-4 receptor was obtained from Atashasi Suzuki and Naoto Ueno (Sapporo, Japan). Like the dominant negative FGF receptor (Amaya *et al.*, 1991) and activin receptor (Hemmati-Brivanlou and Melton, 1992), this receptor as been truncated in its intracellular domain and so can presumably bind ligand but is unable to transduce a signal (see (Schmidt *et al.*, 1995; Suzuki *et al.*, 1994; Wilson and Melton, 1994))

In the first experiment, embryos were injected at the one cell stage with RNA made from the truncated *Xenopus* BMP receptor that has been shown to bind BMP-2, -4
Figure 6.2

The repression of *Xom* by noggin RNA injection

Noggin RNA (1 ng) was injected into one blastomere of *Xenopus* embryos at the two-cell stage and the embryos were allowed to develop to the early gastrula stage (stage 10), when the expression of *Xom* was analysed by whole mount in situ hybridisation. (A) Noggin-injected embryo viewed from the animal hemisphere. Note down-regulation of *Xom* in half the embryo. (B) Control embryo viewed from the animal hemisphere.
and -7 but not activin or TGF-β1 (Suzuki et al., 1994). These embryos were allowed to develop and then frozen at various stages after the mid-blastula transition. Xom transcript levels were determined by RNAase protection.

Figure 6.3A shows an RNAase protection analysis studying Xom expression in embryos injected with a truncated BMP receptor. Xom expression is delayed by the injection of the truncated receptor, so that at stage 8 expression is absent in injected embryos, but this recovers by stage 9, and by stage 11 expression is at control levels.

The RNAase protection data, however, does not show the change in localisation seen at stage 10-11. This is shown in figure 6.3B. Embryos injected with the truncated receptor at the one cell stage of development were allowed to develop to stage 10.5. They were then fixed and subjected to whole mount in situ hybridisation to determine the localisation of Xom transcripts. Expression is absent from the ventral side but is seen in a pair of lateral stripes. In separate experiments, RNA for the truncated BMP receptor was injected equatorially into all cell of the four cell stage embryo. In this experiment, although the levels of Xom are reduced, the localisation of the Xom transcripts is the same as that seen for the unicellular injection. This implies that the lateral striped pattern seen in embryo after injection with the truncated BMP receptor does not result from the differential inheritance of the injected RNA. It is unclear if this expression pattern is caused by the degradation of the injected RNA with the lateral expression of Xom being less sensitive to the levels of truncated receptor. An alternative possibility is that Xom is subject to two modes of regulation; the first, dependent on the BMP receptor, causes the early ventral expression, the other, responsible for the lateral expression, is controlled by a pathway that can be independent of the BMP receptor.
Figure 6.3

Perturbations in BMP signalling alter the expression pattern of Xom

(A) RNA encoding a truncated BMP-2/4 receptor was injected into Xenopus embryos at the one-cell stage and the embryos were allowed to develop to the indicated stages before being analysed by RNAase protection using a probe specific for Xom. At stages 8 and 9 there is a dramatic down-regulation of Xom, but this has recovered by stage 11. (B) In situ hybridisation analysis of embryos injected with a truncated BMP-2/4 at stage 10 also reveals inhibition of Xom expression. (C) Over-expression of BMP-4 causes expression of Xom to invade the dorsal marginal zone as well as the ventral tissues.
6.2.4. Induction of *Xom* by BMP-4

As described in the above experiments, the repression of *Xom* occurs by the inhibition of the BMP signalling pathway either by using an artificial antagonist (a truncated BMP-4 receptor) or an "in vivo" one, namely noggin. This suggests that the reverse may be true: that *Xom* expression is positively regulated by BMP signalling. This section tests this idea.

Embryos, injected unilaterally with 500 pg BMP-4, were fixed for whole mount *in situ* hybridisation at stage 10. *Xom* transcripts now invade the whole of the dorsal marginal zone (6.3C). Even though only one half of the embryo should over-express *Xom*, it was clear from both the expression pattern of *Xom* and the later ventralised phenotype of the injected embryo, that the injected blastomere also influenced its uninjected neighbour.

The requirement for intercellular signalling for *Xom* expression of was investigated by dispersing whole *Xenopus* embryos in a calcium-magnesium free medium. If *Xom* expression was controlled by cell to cell communication, in cases where cell interactions have been disrupted, *Xom* expression should be down regulated.

Embryos were placed in calcium-magnesium free medium from the four-cell stage of development until stage 7.5 (see chapter 2). Their vitelline membranes were then removed and the dispersed embryos were placed in fresh calcium-magnesium free medium. The dispersed blastomeres were then treated with either 50 ng ml\(^{-1}\) of FGF-2, 8 units of activin or 100 ng ml\(^{-1}\) of BMP-4 protein. At stage 10.5 the cells were collected and *Xom* transcripts were assayed using RNAase protection. The resulting autoradiograph is shown in figure 6.4. Dissociation of whole embryos causes down-regulation of *Xom* expression, indicating that *Xom* requires cell-cell contact for expression. This expression cannot be rescued by the addition of FGF-2, but is rescued by BMP-4 or activin. In other
Figure 6.4

Xom expression requires cell contact

The dispersal of whole embryos prevents the expression of Xom. This expression can be restored by addition of BMP-4 and activin to the medium. *Xenopus* embryos were transferred to calcium- and magnesium-free medium at stage 7.5, their vitelline membranes were removed, and the blastomeres were kept dispersed by passing a gentle stream of medium over the cells from a Pasteur pipette. Dispersed blastomeres were cultured to stage 10.5 in the presence of the indicated factors and analysed for expression of Xom by RNAase protection. EF-1α was used as a loading control. Lane 1: intact control embryos; lane 2: dispersed blastomeres with no additional factors; lane 3: dispersed blastomeres plus 50 ng/ml FGF-2; lane 4: dispersed blastomeres plus 100 ng/ml BMP-4; lane 5: dispersed blastomeres plus 8 units/ml activin.
experiments a range of activin concentrations (4-24 units ml⁻¹) were also able to rescue *Xom* expression.

Using a modification of this experiment, it was possible to determine whether the rescue of *Xom* expression in dissociated cells by BMP-4 or activin was dependent on protein synthesis; that is, if the induction of *Xom* is an immediate early response. This was simply achieved by modifying the experiment above and adding cycloheximide to the medium in order to inhibit protein synthesis (see chapter 2). The levels of *Xom* transcripts were determined by RNAase protection analysis and is shown in figure 6.5. In this particular experiment 94% of protein synthesis had been inhibited.

Cycloheximide causes a slight super-induction of *Xom* when it is added to untreated, dissociated embryos, though this is a normal effect of cycloheximide on immediate-early genes (Sokol, 1994). However, the stimulation seen for BMP-4 and activin is not diminished by the addition of cycloheximide, and is greater than that seen in the untreated dissociated embryos, indicating that *Xom* is an immediate early response to BMP-4 and activin stimulation.

The immediate early response of *Xom* to BMP-4 was interesting because they are expressed in similar expression domains during early development: both are excluded from the organiser and mark ventral and lateral territories in the embryo. The implication, therefore, is that *Xom* is induced, in vivo, by BMP-4. To investigate this relationship further, the timing of the expression of BMP-4 and *Xom* during normal development were compared. Figure 6.6 shows an RNAase protection of BMP-4 and *Xom* levels at defined time points after fertilisation. Although low levels of BMP-4 are detected as a maternal transcript (see also (Dale et al., 1992)), the mid-blastula transition seems to be marked by a down regulation of BMP-4 transcripts, and this allows easy assignation of the onset of zygotic BMP-4 transcription. From this it appears that *Xom* expression precedes the zygotic expression of BMP-4. It is therefore possible that *Xom* expression is controlled by maternal BMP-4 transcripts or by BMP-2, which is abundantly expressed as a maternal
Xom activation by BMP-4 and activin is not inhibited by cycloheximide

*Figures 6.5*

*Xenopus* embryos were transferred to fresh calcium- and magnesium-free medium at stage 7.5 and their vitelline membranes were removed. Dispersed blastomeres were cultured to stage 10.5 in the absence of additional factors (lanes 1 and 2) or in the presence of FGF-2 (lanes 3 and 4), BMP-4 (lanes 5 and 6) or activin (lanes 7 and 8). Samples in even-numbered lanes were cultured in the continual presence of 7.5 μg ml⁻¹ cycloheximide. This was sufficient to reduce incorporation of [³⁵S]methionine into trichloroacetic acid-insoluble material by over 94%. Expression of *Xom* was analysed by RNAase protection.
Figure 6.6

Comparison of the time of onset of BMP-4 and of Xom

Xenopus embryos at the indicated times after fertilisation (hr) were analysed by RNAase protection simultaneously for the expression of BMP-4, Xom, and EF-1α. Low maternal levels of BMP-4 RNA are visible and zygotic expression of Xom slightly precedes that of BMP-4.
6.3. Discussion

In this chapter I have presented evidence that suggests that Xom expression is subject to both negative and positive regulation. Both of these facets can be accounted for by considering the signalling of the bone morphogenetic protein group of signalling molecules, particularly of BMP-4. The similarity of the BMP-4 expression pattern to that of Xom is seen not only during gastrula stages where both are excluded from the organiser region of the embryo, but also at later stages. Figure 6.7 shows the expression of BMP-4 at stage 32. At this point in development, BMP-4 is expressed in the dorsal part of the eye, in the roof plate of the trunk region, in derivatives of the trunk neural crest and in the proctodeum, all sites of Xom expression. Unlike Xom, BMP-4 is also expressed in the heart region. It is not expressed in the tailbud.

I have shown that BMP-4 is able to induce Xom both in animal caps and in dissociated whole embryos. Indeed this induction can occur in an immediate early fashion. By blocking the function of one of the receptors for BMP-4, the requirement for BMP-4 was determined. A truncated BMP-2/4 receptor blocked the early expression and although expression levels recovered by the start of gastrulation, whole mount in situ hybridisation analysis of these embryo revealed that the ventral expression of Xom was absent. This may represent two mechanisms that bring about Xom expression or alternatively it may result from decreasing levels of the truncated BMP receptor.

Data from the dissociated cell experiment (figure 6.4) suggests that a large range of activin concentrations are able to induce Xom. It is possible that two separate signalling events serve to bring about Xom expression: one by an activin-like protein (in the lateral regions) and the other by BMP-4 (in the ventral regions). This is consistent with the view that the ventral and lateral regions of the embryo have different lineages:

transcript (Clements et al., 1995).
The expression pattern of BMP-4 in a tailbud staged Xenopus embryo

The expression pattern of BMP-4 in a stage 32 *Xenopus* tadpole is shown. For comparison, the *Xom* expression pattern is presented elsewhere in this thesis (figure 5.4F). Expression is visible in the dorsal part of the eye, the ventral region of the otic vesicles, the fin, the proctodeum and around the heart.
the most ventral expression marks ventral tissue during development., the lateral expression may mark those cells that will become the eye and the trunk neural crest.

Another possibility is that only BMP signalling is necessary for Xom expression, and that activin in the dissociated cells experiment is merely stimulating the BMP-4 signalling pathway. Data from the noggin experiment supports this. Noggin directly inhibits BMP-4 action; over-expression of noggin in half of the embryo inhibits the expression of Xom in the whole of that half and not just in the ventral regions of the embryo. This data argues against Xom being subject to two modes of regulation. The lateral stripes expression seen after the injection of the truncated BMP receptor may reflect degradation of the injected RNA. This is consistent with the expression of Xom. The expression can be considered as a progress from ventral to more lateral areas during stage 8 to stage 10.5 and that the lateral expression of Xom is the most recent. This expression may also be the least sensitive to levels of the truncated BMP receptor.

Xom expression appears first on the ventral side of the embryo and it appears that this expression is controlled by maternally provided BMP-4 or by maternal BMP-2 protein which also has a similar activity (Clements et al., 1995). Is this maternal protein somehow localised so that it is active only on the ventral side of the embryo? Equally possible is the fact that the activity of BMP-2/4 may be repressed by another maternal gene. Noggin transcripts are known to be maternally provided and may repress Xom at these early stages as well. This may be addressed by using antisense oligonucleotides directed against maternal noggin to inhibit its expression. Whole mount in situ hybridisation can be performed to detect any changes in the early expression of Xom.

This chapter presents evidence that Xom is an immediate early response to BMP-2/4. In the next chapter, data will be described that suggests that the function of BMP-4 in ventralising the Xenopus embryo may occur through the activation of Xom.
Chapter 7

7. The Function of Xom

In the previous chapter, I explored the induction of Xom by the signalling molecule BMP-4. The role of BMP-4 in normal development is to provide a ventralising influence in the embryo and it is possible that Xom mediates this. The effect of Xom over-expression using injected RNA was compared to the effects reported for BMP-4 both in the whole embryo and in explanted pieces (Dale et al., 1992; Jones et al., 1992). The effects of Xom are broadly similar to BMP-4, however the ventralising phenomena is less pronounced.

7.1. Materials and Methods

The general methods used in this chapter are described in the main Material and Methods section (Chapter 2). In the present chapter, the effects of the over-expression of Xom were investigated by injecting RNA encoding either Xom or a control construct ΔXom. The following constructs were used to allow efficient translation from the injected RNA.

**Xom**  The full length cDNA was cloned by blunt ended ligation into pSP64T. RNA was made from this by linearising the plasmid with Eco RI and transcribing with SP6 polymerase.

**ΔXom**  A truncated version of Xom was used to control for non-specific effects due to the injection RNA. It was constructed by cloning a Bgl II fragment from the Xom cDNA into pSP64T. RNA was transcribed using SP6 RNA polymerase after linearisation with Eco RI.

**β-gal**  This was transcribed from a pSP64T construct contain a nuclear localised form of the β-galactosidase gene (Smith and Harland, 1991).
7.2. Results

7.2.1. Xom over expression blocks the formation of dorsal mesoderm.

The role of BMP-4 as an agent mediating ventralisation has been well documented. It is known that the over expression of BMP-4 in the dorsal part of the embryo leads to an inhibition of dorsal and anterior structures (Jones et al., 1992) with embryos developing a range of phenotypes from an absence of anterior structures to a complete inhibition of all except the most ventral tissue. The next series of experiments investigates whether over-expression of Xom in the whole embryo also has the same effect.

2 ng of Xom RNA were injected into each of the two dorsal blastomeres of a four cell staged embryo. The development of these embryos was compared to the development of embryos that had been dorsally injected with ΔXom, a construct which controlled for the effects of injecting an RNA (see above). In most cases (124/125), the development of embryos injected dorsally with ΔXom RNA was identical to the development of control uninjected embryos (see Figure 7.1A and Table 7.1).

The effects of injecting Xom into dorsal blastomeres is shown in Figure 7.1B. In the experiment shown, 65% of cases these embryos suffered anterior truncations (Table 7.1). However these anterior truncations were variable in their extent ranging from minor abnormalities where only a small part of the head was absent to an almost complete inhibition of axial structures. The cause of this variability is as yet unclear.

In order to investigate whether other dorsal mesodermal cell types were absent, embryos injected dorsally with Xom were subjected to whole mount antibody staining using the antibody MZ15 as a marker for notochordal differentiation (Smith and Watt, 1985). In 48% of Xom-injected embryos, notochord differentiation was inhibited (Table 7.1 and Figure 7.1D). ΔXom did not block the ability of embryos to make notochord (Figure 7.1C).
Table 7.1

Effects of over-expression of Xom in dorsal blastomeres of Xenopus embryos

The two dorsal blastomeres of Xenopus embryos at the four-cell stage were injected with the indicated RNAs (4 ng total), and embryos were allowed to develop to stage 36. The presence of notochord was established by whole-mount antibody staining using the monoclonal antibody MZ15.
The injection of Xom RNA into dorsal blastomeres causes the loss of anterior structures and of notochord

The prospective dorsal blastomeres of embryos at the four cell stage of development were injected with a total of 4 ng of Xom or of ΔXom RNA. Embryos were allowed to develop until stage 32. (A) Embryos expressing ΔXom develop normally. (B) Embryos injected with Xom lack anterior structures. The embryos were also fixed for whole mount antibody staining with the monoclonal antibody MZ15. (C) Embryos injected with ΔXom RNA form a notochord. (D) Embryos injected with RNA encoding Xom lack a notochord.
7.2.2. Xom expressing cells have a more ventral character

The fates of individual cells in which Xom was mis-expressed were traced using a lineage label. In this experiment RNA derived from a nuclear localised form of the β-galactosidase gene was co-injected with RNA encoding Xom. This experiment was also used to assess whether the phenotype seen after dorsal injections of RNA encoding Xom were due to a change in the fate of cells or were the result of cell death. The injections were performed at the 32 cell stage of Xenopus development as an accurate fate map exists for this point (Dale and Slack, 1987a).

The C1 blastomere was targeted for injection. Not only do the derivatives of this cell give rise to the notochord, from whole mount in situ hybridisation experiments these cells also appear to express the lowest levels of Xom. This blastomere was injected with 2 ng of either Xom or ΔXom RNA and 100 pg of β-galactosidase RNA and the embryos were then allowed to develop until stage 42 of development. They were then processed for β-galactosidase expression. Table 7.2 shows the results obtained from one such experiment, with Figure 7.2 showing the localisation of β-galactosidase activity. Xom expressing cells only very rarely contribute to the notochord; in most cases they populate the somites. In 50% of the cases, ΔXom expressing cells do populate the notochord, in the remainder labelled cells are seen in the head region. The staining that is observed in the region of the gut is considered to be non-specific as it is not localised to the nuclei of these cells.

7.2.3. Xom ventralises the effects of activin

In the above experiments, I have shown that Xom is able to change the fate of dorsal cells. Xom may do this by ventralising the response of a cell of the Xenopus embryo to dorsalising agents. In a similar fashion, BMP-4 has been shown to over-ride dorsal
Table 7.2

Over-expression of Xom in blastomere C1 of the 32-cell Xenopus embryo

One of the C1 blastomeres of the 32 cell staged Xenopus embryo was injected with 2 ng RNA encoding either Xom or ΔXom together with 100 pg β-galactosidase RNA. The embryos were allowed to develop until stage 42 and then stained for β-galactosidase expression. In embryos where ΔXom was injected into the C1 blastomere, β-galactosidase staining was seen in the notochord (in about 50% of the cases) or the head. In embryos injected Xom, staining was rarely seen in the notochord.
Figure 7.2

*Xom* changes the fate of dorsal blastomeres

2 ng RNA encoding Δ*Xom* (A, B) or *Xom* (C, D) was injected into blastomere C1 of the 32 cell stage *Xenopus* embryo along with β-galactosidase RNA as a lineage marker. Whereas Δ*Xom* expressing blastomeres are able to contribute to the notochord (A, B), those expressing *Xom* do not (C, D).
mesoderm formation elicited by high concentrations of activin. Thus, incubating an animal cap in activin alone causes the differentiation of dorsal mesoderm, whereas animal caps incubated in BMP-4 and activin protein differentiate as ventral mesoderm. BMP-4 protein alone does not induce mesoderm (Jones et al., 1992).

To investigate whether Xom had a similar effect, embryos at the one cell stage were injected with 4 ng of Xom or 4 ng of ΔXom. The embryos were cultured until uninjected siblings had reached stage 8 (mid-blastula), at which point animal caps were isolated and incubated with or without activin. The explants were then cultured until sibling controls had reached the desired stage.

The type of mesoderm induced was measured by two criteria, the extent of elongation at stage 17 and the alteration in the expression of molecular markers. Figure 7.3 shows the difference in the morphology of the animal caps taken from uninjected (A and B), ΔXom injected (C and D) and Xom injected (E and F) embryos that were either untreated (A, C and E) or treated with 4 units ml\(^{-1}\) of actin (B, D and F). The normal elongation exhibited by activin treated animal caps is severely inhibited if the cap is expressing high levels of Xom. The lack of extensive movements in the animal cap is indicative of ventral mesoderm (Symes and Smith, 1987).

Although high levels of Xom appear to block the ability of activin to induce dorsal mesoderm, this inhibition can be relieved when the cap is incubated in higher concentrations of activin. This is shown in figure 7.4. Here the elongation of animal caps taken from ΔXom injected (A and C) and Xom injected embryos (B and D) are compared when treated with 8 units ml\(^{-1}\) of activin (A and B) or 16 units ml\(^{-1}\) of activin (C and D). The extension of Xom expressing animal caps at 16 units ml\(^{-1}\) of activin is equivalent to that seen when ΔXom expressing caps are incubated at the same concentration.

Figure 7.5 shows the expression of Xbra as determined by RNAase protection on treated animal caps frozen at stage 10.5. This experiment shows that Xom over-expression does
Figure 7.3

\textit{Xom blocks the elongation of animal caps in response to activin}

Animal caps were dissected from control embryos or from embryos injected with RNA encoding \textit{Xom} or \textit{\textDelta Xom}. They were left untreated or were exposed to 4 units/ml activin. Caps were photographed at the equivalent of stage 17. (A) Control animal caps remain spherical. (B) Activin treatment of animal caps derived from uninjected embryos causes dramatic elongation. (C) Animal caps derived from embryos injected with RNA encoding \textit{\textDelta Xom} remain spherical. (D) Activin treatment of animal caps derived from embryos injected with RNA encoding \textit{\textDelta Xom} causes elongation. (E) Animal caps derived from embryos injected with RNA encoding \textit{Xom} remain spherical. (F) Activin treatment of animal caps derived from embryos injected with RNA encoding \textit{Xom} does not cause elongation.
Figure 7.4

Higher concentration of activin relieve the inhibition of Xom on the elongation of animal caps

Animal caps were explanted from embryos injected with either \(\Delta Xom\) (A, C) or \(Xom\) (B, D) at stage 8 and treated with 8 units/ml (A, B) or 16 (C, D) units/ml of activin. They were photographed at the equivalent of stage 17. (A, C) Animal caps taken from embryo expressing \(\Delta Xom\) show a similar elongation at both concentrations of activin used. (B, D) Animal caps taken from \(Xom\) injected embryos elongate less than controls after treatment with 8 units/ml of activin. At 16 units/ml the extent of elongation is indistinguishable between \(Xom\) and \(\Delta Xom\) expressing animal caps.
Figure 7.5

*Xom* does not induce mesoderm or affect the ability of activin to induce mesoderm

Animal caps were dissected from control embryos or from embryos injected with RNA encoding *Xom* or Δ*Xom*. They were left untreated or were exposed to 4 units/ml activin. Caps were cultured until the equivalent of stage 10.5 and analysed for the expression of *Xbra*. All the samples treated with activin expressed *Xbra* normally. *Xom* did not perturb the induction of *Xbra* by activin and it did not induce *Xbra* itself.
Figure 7.6

**Xom inhibits induction of dorsal mesoderm by activin**

Animal caps derived from embryos injected with RNA encoding *Xom* or *ΔXom* were exposed to activin (4 units/ml) and cultured to the equivalent of stage 25 for analysis of cardiac actin expression (A) and for analysis of αT4-globin expression (B). Induction of cardiac (muscle-specific) actin is inhibited by *Xom* RNA but not by *ΔXom*. Expression of αT4-globin is not induced even though activin mediated elongation was blocked.
not induce ectopic formation of mesoderm in the animal cap. It also does not effect the ability of activin to induce mesoderm.

The above data suggests that Xom inhibits the formation of dorsal mesoderm. Further evidence in support of this idea comes from studying expression of a muscle-specific actin. The differentiation of muscle in this assay is used as a marker for dorsal to dorso-lateral types of mesoderm. The levels of muscle specific actin were determined by RNAase protection and the resulting autoradiograph is shown in Figure 7.6A. Activin induces muscle differentiation in animal caps derived from both uninjected control embryos and embryos injected with ΔXom. However, in activin treated animal caps taken from embryos injected with Xom muscle differentiation is inhibited as indicate by the lower levels of muscle actin. It is noted that these animal caps also fail to express αT4-Globin (Figure 7.6B). This gene is localised to the blood islands of the early embryo and is therefore a marker of differentiated ventral mesoderm. Perhaps Xom-injected animal caps that have been treated with activin are not sufficiently ventralised to enable the expression of globin to occur.

7.2.4. Xom partially inhibits the effects of noggin

The secreted factor noggin directs the formation of neural tissue and dorsal mesoderm by directly inhibiting BMP-4. The above data suggest that Xom is able to inhibit the formation of dorsal mesoderm, perhaps by mimicking the BMP-4 pathway. A suggestion from this is, therefore, that a downstream component of the BMP-4 pathway should be able to relieve the antagonism of BMP-4 by noggin. By investigating the ability of Xom to inhibit noggin, this idea was tested.

20 pg of a DNA construct placing noggin under the control of the cytoskeletal actin promoter was co-injected with 2 ng of Xom into each ventral blastomere of the four cell stage embryo. The embryos were cultured until stage 10.5 at which time the ventral marginal zone was dissected and then cultured until sibling embryos had reached stage
Figure 7.7  
*Xom partially inhibits noggin-mediated dorsalisation*

Embryos were injected with either *Xom* or Δ*Xom* RNA into the ventral blastomeres of the 4 cell stage *Xenopus* embryo together with 20 pg noggin-DNA driven by the cytoskeletal actin promoter. Ventral marginal zones were dissected from these embryos when they had reached stage 10.5 and these explants were then cultured to the equivalent of stage 32. They were then fixed and analysed, using whole mount staining, for the presence of muscle by staining with the monoclonal antibody 12/101. Shown above is such an experiment, the staining having derived from the oxidation product of diaminobenzidine (DAB). (A) shows the presence of 12/101 positive cells in the whole embryo. Staining marks the muscle blocks in the embryo. In explanted ventral marginal zones taken from untreated embryos (B), 12/101 staining cannot be detected. In ventral marginal zones taken from embryos that have been injected with noggin (C) or noggin and Δ*Xom* (D) do stain with 12/101, indicating that they have taken a more dorsal character. 12/101 staining is, however, reduced in ventral marginal zone taken from embryos injected with noggin and *Xom* (E).
Figure 7.7

*Xom* partially inhibits noggin mediated dorsalisation
32. The explants were then fixed in MEMFA for 2 hours at room temperature. The presence of muscle in these explants was investigated by using the antibody 12/101 (Kintner and Brockes, 1984).

Figure 7.7 shows explants that have been subjected to a whole mount antibody staining procedure against muscle. The 12/101 antibody stains the muscle blocks of the stage 32 embryo (fig. 7.8A) but does not stain explanted ventral marginal zones taken from untreated embryos.

Explants of the ventral marginal zone from embryos injected with noggin (Fig 7.7C) or with noggin and ΔXom (Fig 7.7D) possess high levels of muscle in almost all explants (85% were 12/101 positive), whereas in explants taken from embryos injected with noggin and Xom (Fig 7.7E), the number of explants showing patches of 12/101 staining was reduced (45% of explants were 12/101 positive). These patches were also smaller than the 12/101 positive patches in noggin injected or noggin and ΔXom injected marginal zones. This suggests that Xom can at least partially inhibit noggin.

It must be noted that although this experiment provided the best example of the inhibition of dorsalisation mediated by noggin, other experiments were not so clear. This variability is consistent with the variability of the phenotype seen in the dorsal injections of Xom.

7.3. Discussion

The effects of Xom in blocking dorsal differentiation are similar to those seen for BMP-4. Although it is possible that the effects are mediated by Xom, it must be noted that the effects of Xom are nowhere near as potent as those seen for BMP-4. This is illustrated by the fact that 4 ng of Xom needs to be injected into the embryo in order to elicit a ventralising response, even though it appears that Xom is efficiently translated (see figure 4.5). The failure to induce markers for the most ventral mesoderm in combination with activin (Figure 7.7B) and the incomplete inhibition of noggin also highlight this problem.
The variability and the low efficiency seen when using Xom may be due to my inability to express Xom to high levels. Another possibility relates to the fact that Xom is expressed at high levels in vivo. Over-expression may not make much difference to the levels of Xom bound to target promoter sites. It could also be due to the requirement for other genes. In support of this idea are several Xom-related genes that appear to ventralise the embryo (Gawantka et al., 1995; Jamrich, 1996; Schmidt et al., 1996). It is therefore possible that Xom co-operates with these genes to elicit the full BMP-4 response.

Xom blocks the ability of activin to induce dorsal mesoderm in animal caps, but in the previous chapter, it is clear that activin can induce Xom to high levels in both animal caps and in dissociated cells (figures 6.2 and 6.7). This apparent paradox, in which activin induces the expression of a protein that inhibits its dorsal mesoderm inducing effects, might be resolved by looking at the function of other genes that are induced by activin. These genes may serve to repress the activity of Xom and in so doing so, induce dorsal mesoderm. This idea is also consistent with the morphology of animal caps from Xom injected embryos treated with higher concentrations of activin (Figure 7.5). Here it is clear that the response of animal caps treated with higher concentrations of activin is sufficient to overcome the block on elongation provided by Xom. An interesting experiment to test this idea would be to somehow inhibit the function of Xom and then see how the response to activin is modified.

In fact this paradox is not limited to Xom. Another gene shown to ventralise the embryo, Mix-1 (P. E. Mead and L. Zon; personal communication), has been shown to be an immediate early response to activin. Indeed higher concentrations of activin in the animal cap assay lead to a progressive increase in the expression levels of this gene (Green et al., 1994; Rosa, 1989).
Chapter 8

8. General Discussion

The organisation of the *Xenopus* embryo is established by a series of inductive interactions, which together give rise to the final body plan of the embryo. In this thesis I have used two approaches to clone genes involved in early *Xenopus* development. The first involved subtractive hybridisation to enrich for genes that were expressed as a response to FGF mediated signalling. This method was not successful due to the reasons elaborated in chapter 3. The second, utilising the homology between the homeobox containing gene family, proved more successful. The remainder of this thesis described the cloning and characterisation of a homeodomain-containing transcription factor that is an immediate early response to BMP-4, that has the same expression at gastrulation as BMP-4 and that may mediate some of the functions of BMP-4.

8.1. *Xom* is a homeobox-containing gene

In this thesis I have described the cloning of *Xom*, based on its homology with the homeobox-containing gene family. *Xom* contains a homeodomain and is therefore postulated to act as a DNA binding protein. The homeodomain of *Xom* is of an unusual class, marked by the presence of a threonine in position 47 of the homeodomain, in place of the more usual valine or isoleucine. In another member of this class this residue has been shown to be important in determining the DNA binding specificity of the homeodomain. *Hox 11*, a gene required for the development of the mouse spleen (Dear et al., 1995), also contains a threonine at position 47 of the homeodomain. In *Hox 11* this threonine causes the binding specificity of the homeodomain to change from 5'-TAAT-3' to the more unusual 5'-TAAC-3' (Dear et al., 1993). Similarly, the DNA binding activity of another member of this class, *Bar H1*, is also different from most other homeodomains (Kalionis and O'Farrell, 1993). It should be noted that the homeodomain of *Bar H1* is
most related to the homeodomain of Xom. It is therefore possible that the DNA binding specificity of Xom is also changed. It is also interesting that the homeodomains of other ventralising Xenopus homeobox genes have the threonine substitution at position 47 (Gawantka et al., 1995; Schmidt et al., 1996). It is unclear why the embryo has so many homeobox genes with a similar expression pattern, similar activity and with a threonine at position 47 of the homeodomain. Perhaps these genes act as a complex to bind a DNA. This may be why Xom has a rather weak ventralising activity. A prediction from these observations is that downstream genes of the ventralising homeobox genes will all contain a similar promoter sequence, possibly containing the TAAC motif.

8.2. The expression of Xom

Xom is expressed very early, probably with the onset of zygotic transcription. It is important therefore to compare the expression of Xom with other genes that are expressed around this time, such as goosecoid and Xwnt-8, in order to place these genes into a potential regulatory hierarchy. It is known, for example, that the gene siamois is expressed before goosecoid and Xwnt-8 (Lemaire et al., 1995). RNAase protection data similar to that performed for Xom and BMP-4 (figure 6.8) would indicate position of Xom in this chain.

In a similar fashion, the onset of transcription of the ventralising homeobox genes could be compared to reveal some clues to their function and to determine if a similar hierarchy exists amongst them. As yet, data on the onset of the transcription of Vox (Schmidt et al., 1996) and pv-1 (M. Jamrich; personal communication) is unavailable, however reports that Xvent expression only commences at gastrula stages (Gawantka et al., 1995), places its transcription after that of Xom.

8.3. The induction of Xom

From the data presented in this thesis, the action of BMP-4 is central to the expression of Xom; Xom expression can be induced by BMP-4 in an immediate early-fashion and
perturbations in BMP-4 signalling also perturb the expression pattern of Xom. This is of interest as the suggestion that BMP-4 performs an inductive function in the embryo is supported by a strong body of evidence. BMP-4 is expressed in the correct place at the correct time for its proposed ventralising role (Schmidt et al., 1995). Its effects on isolated tissue also have the postulated effects (Dale et al., 1992; Jones et al., 1996; Jones et al., 1992). Inhibition of the BMP pathway using either a truncated BMP receptor (for example see (Suzuki et al., 1994)) or by using "natural" inhibitors such as noggin (Smith and Harland, 1992; Smith et al., 1993) or chordin (Holley et al., 1995; Sasai et al., 1994) inhibit the formation of ventral tissue, with a compensatory increase in dorsal structures.

Xom expression is localised as soon as it is detectable by whole mount in situ hybridisation, and this localisation probably marks the ventral side of the embryo. Data using the truncated BMP receptor suggests that this expression is controlled by maternal BMP signalling. However it is unclear whether maternal levels of BMP-2 or BMP-4 are responsible for this induction. Experiments using antisense oligonucleotides to prevent translation from a maternal RNA such as BMP-2 or 4 may go some way to addressing the exact identity of the inducing molecule (see, for example, (Sasai et al., 1995)), but would still leave the conundrum of how Xom is localised in the first place. It is possible that a maternal BMP is able to act throughout the margin of the embryo but is prevented from doing so on the dorsal side by the action of localised maternal noggin. Again experiments studying the change in Xom localisation when antisense oligonucleotides prevent maternal noggin translation should address this. Another possibility is that cortical rotation not only establishes the dorsal side of the embryo; it also molecularly defines the ventral side. If this is true, Xom should be expressed throughout the embryo, at pre-gastrulation stages of development, when cortical rotation is inhibited by ultra-violet irradiation of the vegetal pole.

The expression pattern of Xom seems to be almost identical with that of BMP-4. This, together with the observation that BMP-4 can induce Xom in an immediate early
fashion, strengthens the argument that \textit{Xom} is a transcriptional response to BMP-4 activity. However there are regions where the later expression of the two genes do not overlap. BMP-4 is in the heart region of the embryo and Xom is not; Xom is in the tailbud and BMP-4 is not. The tailbud expression is intriguing, suggesting that another mechanism, not dependent on BMP-4 signalling, is responsible for expression here. Alternatively, the levels of BMP-4 in the tailbud may be too low to be detected by the whole mount \textit{in situ} hybridisation procedure.

8.4. The Function of \textit{Xom}

The presence of a homeobox in the sequence of Xom suggests that it can act as a transcription factor. An acidic domain N-terminal to the homeodomain and a proline-rich domain on the C-terminal side suggest that Xom is able to act as a transcriptional activator (Ma and Ptashne, 1987; Mermod \textit{et al.}, 1989). The identity of target genes of Xom is unknown, and it would be of interest to screen for possible downstream responses of Xom.

Further clues on the function of Xom may be gained from the using a "dominant negative" approach to interfere the activity of Xom. One possible method that can be used is a procedure developed for the homeobox gene, \textit{Mix-1} (P. Mead and L. Zon; unpublished observations). Here an interfering allele of \textit{Mix-1} was constructed by mutating a residue in the homeodomain. This allele blocks the activity of Mix-1. The effects of a similar construct made for Xom could be compared to the effects of the dominant negative BMP receptor to provide more information on the activity of \textit{Xom}.
References


**Nieuwkoop, P. D., and Faber, J.** (1967). "Normal Table of *Xenopus laevis* (Daudin)." North Holland, Amsterdam.


Appendix A

Solutions for Embryo work

**NAM**

<table>
<thead>
<tr>
<th>1 x NAM</th>
<th>g/l for 10 x NAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>110 mM NaCl</td>
<td>65</td>
</tr>
<tr>
<td>2 mM KCl</td>
<td>1.5</td>
</tr>
<tr>
<td>1 mM Ca(NO$_3$)$_2$</td>
<td>2.4</td>
</tr>
<tr>
<td>1 mM MgSO$_4$</td>
<td>2.5</td>
</tr>
<tr>
<td>0.1 mM Na$_2$EDTA</td>
<td>0.37</td>
</tr>
</tbody>
</table>

For 500 ml:

<table>
<thead>
<tr>
<th>75%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>37.5 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td>10 ml</td>
<td>10 ml</td>
</tr>
<tr>
<td>5 ml</td>
<td>-</td>
</tr>
<tr>
<td>2.5 ml</td>
<td>2.5 ml</td>
</tr>
</tbody>
</table>

10 x NAM for 10 x NAM solutions:
- 10 ml 0.1 M Na phosphate (pH 7.4)
- 10 ml 0.1 M NaHCO$_3$
- 2.5 ml 10 mg ml$^{-1}$ gentamycin

**Calcium-Magnesium Free medium (CMFM)**

- 88 mM NaCl
- 2 mM KCl
- 2 mM Na phosphate (pH 7.4)

**MEMFA**

10x stock of MEM

- 1M MOPS
- 20mM EGTA
- 10mM MgSO$_4$

MEMFA (for 50 ml)

- 5 ml 10 x MEM salts
- 5 ml 37% formaldehyde
### PBS

<table>
<thead>
<tr>
<th>Component</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.0</td>
</tr>
<tr>
<td>KCl</td>
<td>0.25</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>1.437</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.25</td>
</tr>
</tbody>
</table>

### Whole Mount *in situ* hybridisation buffer

- 50% Formamide
- 5 X SSC
- 1 mg/ml Torula RNA
- 100μg/ml Heparin
- 1X Denhardts
- 0.1% Tween-20
- 0.1% CHAPS
- 5mM EDTA

### 2. Solutions for Molecular Biology

#### L-Broth (Agar)

<table>
<thead>
<tr>
<th>Component</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-tryptone</td>
<td>10</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>5</td>
</tr>
<tr>
<td>NaCl</td>
<td>10</td>
</tr>
<tr>
<td>(Agar)</td>
<td>(15)</td>
</tr>
</tbody>
</table>

#### NZY Broth (Agar)

<table>
<thead>
<tr>
<th>Component</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein hydrolysate</td>
<td>10</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>5</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>2</td>
</tr>
<tr>
<td>(Agar)</td>
<td>(15)</td>
</tr>
</tbody>
</table>
### 2 x TY

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Difco tryptone</td>
<td>16 g/l</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>10 g/l</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g/l</td>
</tr>
</tbody>
</table>

### TAE-50 x stock

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>242 g/l</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>57.1 g/l</td>
</tr>
<tr>
<td>0.5 M EDTA (pH 8)</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

### TBE-10 x stock

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>121.1 g/l</td>
</tr>
<tr>
<td>Boric Acid</td>
<td>61.83 g/l</td>
</tr>
<tr>
<td>Na$_2$EDTA</td>
<td>3.72 g/l</td>
</tr>
</tbody>
</table>

### MOPS buffer for RNA agarose gels

- 20 mM MOPS (morpholine propanosulphonic acid)
- 5 mM Na acetate
- 1 mM EDTA

- Equilibrate to pH 7 and filter.

### Agarose gel loading buffer-in water

- 0.25% bromophenol blue
- 0.25% xylene cyanol FF
- 30% glycerol

### TE

- 10 mM Tris-Cl (pH 8.0)
- 1 mM EDTA

### Church’s buffer

- 7% sodium dodecylsulphate (SDS)
- 1% bovine serum albumin (BSA)
- 0.5 M Na phosphate (pH 7.0)
- 1 mM EDTA
Elution Buffer for riboprobes
- 0.5 M NH₄ Acetate
- 1 mM EDTA
- 0.1% SDS

5x Hybe Salts for RNAase protection
- 2M NaCl
- 0.2M PIPES pH 6.4
- 5 mM EDTA

RNA Digestion buffer for RNAase protection
- 10 mM Tris pH 7.5
- 5 mM EDTA
- 300 mM NaCl

Poly acrylamide gel DNA loading Buffer
- 100 ml de ionised formamide
- 2 ml 0.5 M EDTA
- 0.1% xylene cyanol
- 0.1% bromophenol blue