The regulation of derrière: a direct target of VegT

By

Richard J. White

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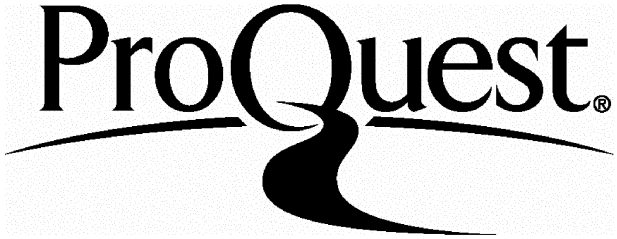

Abstract

The T-box transcription factor *VegT*, whose transcripts are restricted to the vegetal hemisphere of the *Xenopus* embryo, is required for both the specification of endoderm and also to regulate the production of mesoderm-inducing signals. Since *VegT* is a transcriptional activator, it is likely to exert its effects by regulating the expression of target genes.

This thesis describes an analysis of the regulation of one such target gene, *derrière*. *Derrière* is a TGF-β superfamily member expressed in the vegetal pole and marginal zone of *Xenopus laevis* embryos. First, several candidate target genes, which exhibit mesoderm-inducing activity, were tested using a hormone-inducible version of VegT to identifying direct downstream effectors of *VegT*. These include the TGF-β superfamily members *Xnr1-4* and *derrière*. In agreement with other groups it was found that *Xnr1*, *Xnr2, Xnr4* and *derrière* are direct downstream targets of *VegT*, whereas *Xnr3* (a neural inducer) is not.

Second, a fragment of the *derrière* promoter was isolated and studied using reporter gene assays, electrophoretic mobility shift assays and transgenesis. The promoter contains two putative T-box binding sites, one of which binds VegT protein *in vitro*, that are required for expression of a reporter gene in oocytes, but are not required for expression in embryos. This is due to at least one positive feedback loop mediated by TGF-β signalling, through two Fast binding sites in the *derrière* promoter. This causes the rapid accumulation of transcripts in the marginal zone in the hour preceding gastrulation even in the absence of the T-box binding sites.

Finally, the question of the function of *derrière* in the *Xenopus* embryo was addressed using animal cap recombination assays and the injection of an antisense morpholino oligonucleotide.

These results demonstrate that *derrière* is subject to complex regulation and is a good candidate for one of the endogenous mesoderm-inducing signals activated by *VegT*.

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"Always do good things, never do bad things."

– Eddie Izzard.

Contents

Chapter 1. Introduction

INTRODUCTION

All the adult cells of triploblastic animals arise from one of three primordial germ layers: ectoderm, which forms epidermis and neural tissue; mesoderm, which forms muscle, bone, connective tissue and blood; and endoderm, which principally forms the gut and contributes to its derivatives (e.g. liver and pancreas) (Nieuwkoop, 1969). How these three distinct populations of cells are specified, and how they are distinguished at the molecular level, are central questions in developmental biology. By the time gastrulation begins, the embryo has been regionalised by the expression of specific genes, such that particular cell types form from specific regions of the embryo. The earliest broad regionalisation that subdivides the embryo is the specification of germ layers. This process involves a combination of mechanisms, including segregation of maternal determinants during oogenesis and cell–cell communication via the secretion of various signalling molecules. These secreted signalling molecules include members of the transforming growth factor-β (TGF-β) and fibroblast growth factor (FGF) families of growth factors.

This thesis studies the regulation of one of these genes, *derrière*, which is involved in the specification of germ layers. *Derrière* is a TGF-β superfamily member, expressed in the marginal zone and the vegetal pole of *Xenopus* blastula and gastrula embryos (Sun et al., 1999), and its expression requires the presence of the maternally expressed transcription factor VegT (also known as Antipodean, Brat and Xombi; Horb and Thomsen, 1997; Kofron et al., 1999; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996). Therefore, this introduction will focus on the T-box transcription factor family and TGF-β signalling molecules involved in early mesoderm induction.

Xenopus development

The amphibian *Xenopus laevis* has been used extensively as a experimental system for the study of developmental biology. Due to their relatively large size and external development, *Xenopus* embryos are amenable to embryological manipulations not possible in other higher chordates. Genetic analysis is not possible as *Xenopus laevis* are tetraploid (Bisbee et al., 1977; Tymowska and Kobel, 1972) and have a long generation time (18–24 months), although experiments using gain-of-function and dominant-negative approaches have been used extensively. A related species, *Xenopus tropicalis*, which is diploid and has a relatively short generation time, is beginning to be used to complement studies in *Xenopus laevis* using a genetic approach (Amaya et al., 1998; Offield et al., 2000). The start of *Xenopus* development can be considered to be the production of the egg by oogenesis.

Oogenesis – the animal–vegetal axis

The mature *Xenopus* oocyte is obviously polarised, with a darkly pigmented animal hemisphere and a more yolky vegetal hemisphere. This animal–vegetal axis is set up during oogenesis, although it is currently unclear how this polarity is established. It does not depend on the orientation of the oocytes with respect to gravity or any aspect of the ovary's structure (reviewed in Gerhart et al., 1983). However, this polarity can be related to the later organisation of the embryo. The three embryonic germ layers form in defined regions along the animal–vegetal axis, with ectoderm being derived from the most animal cells, endoderm forming from the most vegetal cells and mesoderm forming in the region between the two.

At the start of oogenesis, a primary oogonium undergoes four mitotic divisions to produce a nest of 16 oocytes connected by cytoplasmic bridges formed by incomplete cytokenesis. From then on, oogenesis can be divided into six stages by size and appearance of the oocytes (Dumont, 1972) and these stages correlate with defined processes occurring at those times. As the oocytes develop they take up vitellogenin from the blood, pigment appears and is segregated to the animal pole (along with the germinal vesicle, the oocyte's nucleus) and the oocyte grows until it reaches 1200–1300 μm in diameter.

In terms of patterning the embryo, the most important process to occur during oogenesis is the production of particular mRNAs and their localisation to specific sites at the oocyte cortex. This is probably how the underlying polarity of the egg (perhaps maintained during oogenesis by the cytoskeleton) is translated into the three regions of developmental potential along the animal–vegetal axis.

Maternal determinants

Many maternal RNAs are localised to either the animal or vegetal cortex, although this localisation has been intensively studied only for RNAs localised to the vegetal cortex (e.g. *Vg1*, *VegT*, *Xcat2* and *XWnt11*). It occurs by two distinct pathways, one occurring early and associated with the movement of a cluster of organelles known as the mitochodrial cloud (Xcat2 and XWnt11; Kloc and Etkin, 1995; Kloc et al., 1996) and one occurring at later stages independently of the movement of the mitochondrial cloud (Vg1 and VegT; Kloc and Etkin, 1995; Zhang and King, 1996). In the latter case, localisation is controlled by sequences in the 3' untranslated region (UTR) of the message, resembling mechanisms studied in *Drosophila melanogaster* (Davis and Ish-Horowicz, 1991; Micklem, 1995).

This process is cytoskeleton-dependent, as is anchorage to the cortex. In the case of both *Vg1* and *VegT*, the anchorage of the RNA to the cortex is released during oocyte maturation, when intermediate filaments break down, and the RNA begins to diffuse away from the vegetal pole (Yisraeli et al., 1989; Yisraeli et al., 1990; Zhang and King, 1996). However, the distribution of *VegT* RNA is still limited, because the RNA is still only found in the vegetal hemisphere of the fertilised egg. Whether this asymmetric distribution of *VegT* RNA is achieved by selective transport, degradation of the message in the animal hemisphere, or some other mechanism is unknown.

Fertilisation – the 'dorsal–ventral' axis

To produce a fully patterned embryo it is necessary to define a second axis to complement the animal–vegetal axis laid down during oogenesis. This occurs at fertilisation, when a single sperm penetrates the egg in the animal hemisphere (apparently at random). The point where the sperm enters is known as the sperm entry point (SEP) and it defines a new axis for the embryo.

This new axis is conventionally referred to as the 'dorsal–ventral' axis. However, it does not correspond exactly to either the dorsal–ventral or the anterior–posterior axis of the later embryo, but to a combination of the two. More recent fate-mapping studies suggest that the terminology be changed to reflect this phenomenon (Lane and Sheets, 2000; Lane and Smith, 1999). However, in this thesis, I shall use the accepted terminology.

Establishment of the dorsal–ventral axis: Cortical rotation

The SEP is converted to the dorsal–ventral axis by a process known as cortical rotation, which occurs approximately 40 min after fertilisation. During cortical rotation the cortical cytoplasm rotates relative to the core of the embryo by approximately 30° (Fig. 1.1). Thus, the vegetal cortical cytoplasm (shown in green in Fig. 1.1) moves to the dorsal side of the embryo. This vegetal cortical cytoplasm contains a transplantable dorsalising activity which defines the dorsal side of the embryo. The process of cortical rotation is dependent on microtubules; an array of microtubules running parallel to the direction of rotation is set up during the first cell cycle and microtubule inhibitors block rotation and lead to embryos with no axis (Elinson and Rowning, 1988). It has also been shown that a subset of the embryo's endogenous organelles are transported approximately 90° towards the future dorsal equator by this microtubule array (Fig. 1.1; Rowning et al., 1997).

The rotation of the vegetal cortical cytoplasm is thought to specify the dorsal side by redistributing dorsal determinants, originally localised to the vegetal pole, to the future dorsal side of the embryo. This can be shown by transplanting cytoplasm from either the vegetal pole of an embryo before rotation or the dorsal equatorial region of an embryo after rotation to the ventral vegetal cells of a host embryo. Either of these procedures will give rise to a secondary axis, which forms on the opposite side of the host (Fujisue et al., 1993; Holowacz and Elinson, 1993). Subsequently, nuclear accumulation of β-catenin on the dorsal side of the embryo occurs (Schneider et al., 1996), and this accumulation of β-catenin is essential for correct dorsal development (Heasman et al., 1994).

The identity of the dorsal determinants is unresolved, although candidate molecules exist. For example, a maternal frizzled receptor, *frizzled7* (*Xfz7*), is required for the development of dorsal structures (Sumanas and Ekker, 2001; Sumanas et al., 2000) and *XWnt8b* is maternally expressed in the animal pole region (Cui et al., 1995). However, intracellular components of the Wnt signalling pathway are also affected by cortical rotation. The dishevelled protein (dsh), a key component of the *Wnt/β-catenin* signalling pathway has been shown to be present at the vegetal cortex in unfertilised eggs and to move to the dorsal side during cortical rotation (Fig. 1.1; Miller et al., 1999). Also, a negative regulator of the pathway, glycogen synthase kinase 3β (GSK-3β in Fig. 1.1) has been shown to be depleted in the dorsal cortical region after cortical rotation (Dominguez and Green, 2000).

The overall scheme for dorsal development is shown in Fig. 1.1. Cortical rotation leads to the transport of determinants to the future dorsal side of the embryo. This leads, via interactions with components of the Wnt signalling pathway to the enrichment of β-catenin on the dorsal side, where it is able to associate with XTcf-3 and activate transcription of target genes such as *siamois*, *twin* and *Xnr3*. On the ventral side, where β-catenin is low, XTcf-3 functions on its own as a transcriptional repressor.

Cleavage and gastrulation

At room temperature, the first cell cycle occurs approximately 1.5 hrs after fertilisation and subsequent divisions occur regularly every 30 minutes. These rapid cell cycles, a constant cycling through S and M phases only, are known as cleavage. No growth occurs and the embryo simply subdivides its cytoplasm. After cleavage stages the embryo becomes known as a blastula, because there is a fluid filled cavity (the blastocoel) in the animal pole, just below a three-cell-thick layer of animal pole cells known as the animal cap (Fig. 1.2A).

Cleavage proceeds for 11–12 divisions (depending on the size of the egg; Newport and Kirschner, 1982a). At this point the divisions become asynchronous and the cell cycle elongates due to the incorporation of G-phases (G1 and G2). This event is known as the mid-blastula transition (MBT) and is accompanied by two other changes in cellular behaviour.

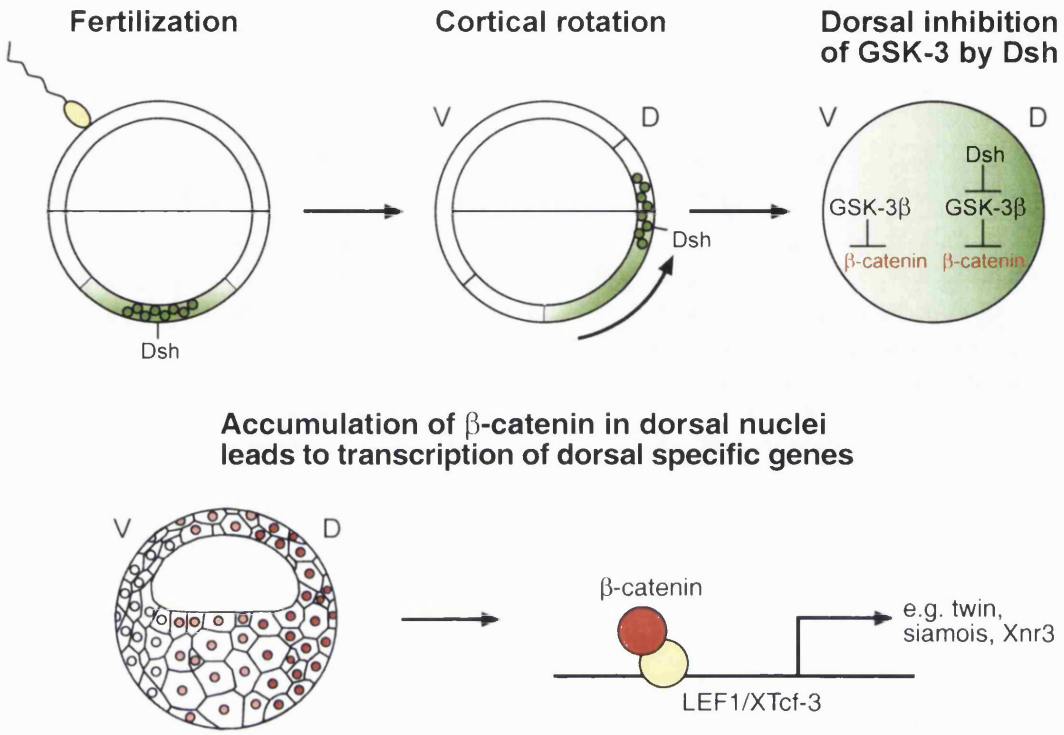

Fig. 1.1. Cortical rotation. Rotation of the cortical cytoplasm 40 min after fertilisation leads to the transport of determinants to the future dorsal side of the embryo. This leads, via interactions with components of the Wnt signalling pathway to the enrichment of β-catenin on the dorsal side, where it is able to associate with XTcf-3 and activate transcription of target genes such as *siamois*, *twin* and *Xnr3*. On the ventral side, where β-catenin is low, XTcf-3 functions on its own as a transcriptional repressor.

First, blastomeres exhibit motile behaviour after the onset of MBT. This mirrors the cell movements of epiboly, and later gastrulation, that occur from now on (see below). Second, the embryo begins to transcribe its zygotic genome (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b). Before the 11th/12th cleavage there are only very low levels of transcription and the egg relies solely on maternally deposited proteins and RNA.

Between MBT and the onset of gastrulation a process occurs called epiboly, which refers to the movement of cells of the animal cap that migrate vegetally and partially cover cells of the vegetal hemisphere. Consequently, the animal cap becomes thinner and is eventually only two cells thick. The overall effect of this process is to produce a ring of animal-cap-derived cells in the subequatorial region (known as the marginal zone) of the embryo. These cells principally give rise to mesodermal cell types. Following this stage, the embryo undergoes gastrulation, a process of cell movements that results in the formation of the visible axes of the embryo (head–tail and back–belly) and the morphological distinction of the three germ layers.

Gastrulation begins approximately 10 hours after fertilisation at room temperature and from this point the embryo is referred to as a gastrula. A group of cells known as bottle cells, which are located on the dorsal side of the embryo, undergo a characteristic shape change, invaginate into the embryo and migrate towards the animal pole (Fig. 1.2B). This involution leads to the formation of the 'dorsal blastopore lip'. The site of cell involution spreads laterally from the dorsal side to the ventral side until there is a ring of involuting cells throughout the equatorial zone (Fig. 1.2D). These cells movements, accompanying epiboly, continue until eventually the ectoderm (derived from the animal region) covers the entire outside of the embryo, the mesoderm forms an intermediate cell layer and the endoderm lies at the centre of the embryo.

The involuting cells form the mesoderm, one of the three germ layers, whose derivatives form the muscle, axial skeleton (cartilage and bone) and blood and contribute to most organs. The other two germ layers – the ectoderm and endoderm – come from the animal and vegetal poles respectively and form skin and neural tissue in the case of ectoderm and gut and associated organs (e.g. lungs) in the case of the endoderm. Involuted marginal cells contribute to muscle and notochord and undergo a cell movement known as convergent extension. Cells converge from ventro-lateral positions to the dorsal side and produce a longer, thinner array of cells by intercalating between their neighbours, which serves to extend the embryo along its anterior-posterior axis (Fig. 1.2C; Keller et al., 1985). Gastrulation and convergent extension movements rearrange the cells of the blastula, to produce the correct distribution and size of the tissues in the embryo such as the notochord and somites. However, this can only occur if the three primordial germ layers have been specified correctly so that the mesoderm forms from the marginal zone cells, which then involute.

Mesoderm induction

How do maternally encoded embryonic asymmetries give rise to several distinct precursor layers specified to produce the three germ layers at gastrulation?
Early experiments by Nieuwkoop (1969) demonstrated that the three germ layers will form from specific regions of the blastula stage embryo. Explants of tissue showed that the layer of cells overlying the blastocoel (the animal cap) would form ectoderm when cultured alone, the vegetal pole would form endoderm and that the equatorial cells would form mesoderm (Fig. 1.3A).

Nieuwkoop also showed that vegetal explants, which themselves form endoderm, were able to induce mesodermal derivatives from animal cap cells, which would not ordinarily form mesoderm. This suggested that there is a signal released by the vegetal cells, which induces the overlying equatorial cells to become mesodermal in nature (Fig. 1.3B). The molecular nature of this inducing signal and the timing of its release have been the subject of much debate with many candidate molecules being proposed as the endogenous 'mesoderm-inducing' signal (e.g. Vg1; Dale et al., 1993; Weeks and Melton, 1987).

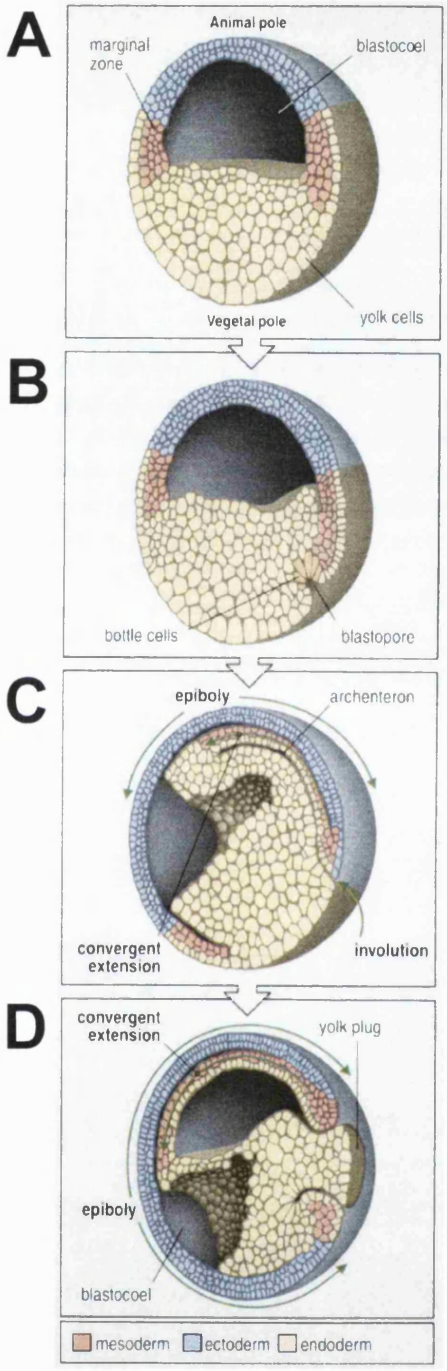

Fig. 1.2. Gastrulation in *Xenopus*. (A) At the late blastula stage, the blastocoel lies under the animal cap and the tissue that is fated to form the mesoderm lies in the marginal zone. (B) Gastrulation begins with the formation of bottle cells in the blastopore region. Cells of the marginal zone involute into the embryo and move towards the animal pole. (C) The axis of the embryo extends in the anterior-posterior direction in a process known as convergent extension. (D) The area of involution spreads ventrally and forms a circle around the yolk plug. Taken from Principles of Development, p. 247 (1998).

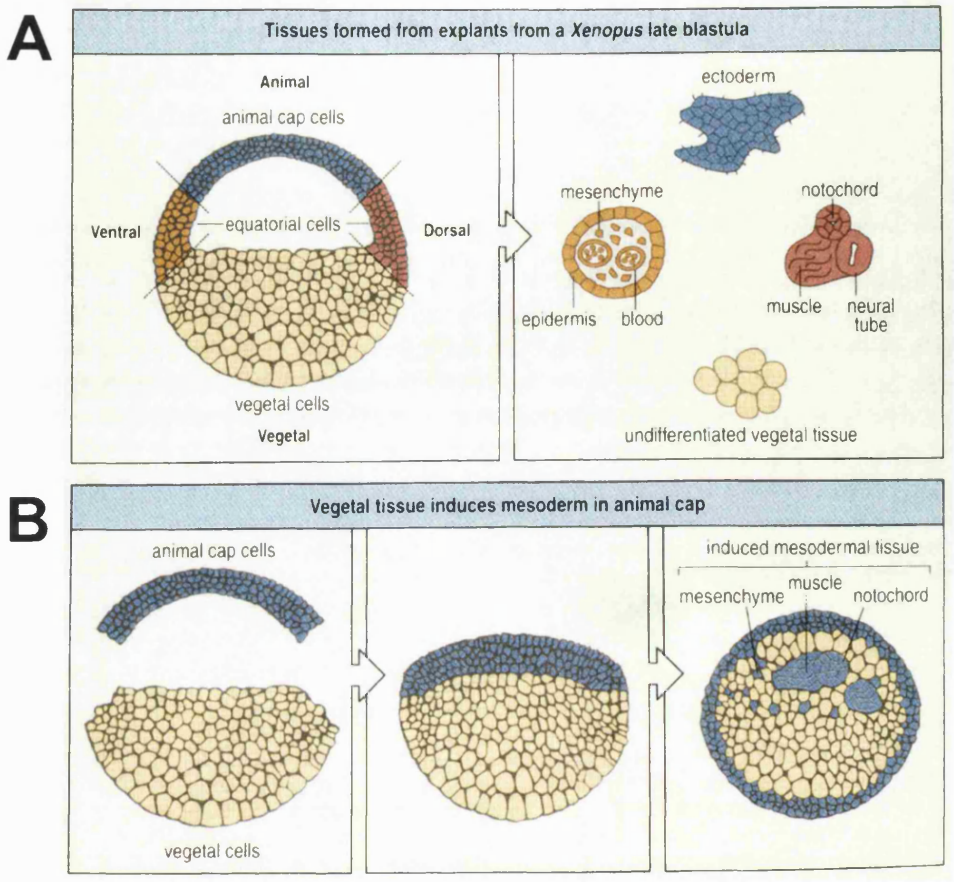

Fig. 1.3. Mesoderm induction in the *Xenopus* blastula. (A) The *Xenopus* blastula is divided into three germ layers. Explants of animal cap cells or vegetal cells form only ectoderm and endoderm respectively when cultured alone, whereas equatorial cells form mesodermal derivatives such as muscle, notochord and blood. (B) Vegetal pole cells release signals which can convert animal cells to a mesodermal fate. When animal caps are combined with vegetal pole region, mesodermal derivatives are formed from the animal cap cells in response to an inductive signal from the vegetal cells. Taken from Principles of Development, p. 82 (Wolpert et al., 1998).

Experiments by Jones and Woodland (1987) involving recombining embryonic blastomeres of differing ages attempted to define the time-scale of mesoderm induction. Recombining progressively older animal blastomeres with vegetal poles showed that animal cap cells are competent to respond to the mesoderm-inducing signal until early gastrula stages. The reciprocal experiments, where progressively younger vegetal blastomeres were recombined with animal cells nearing the end of competence, suggested that the mesoderm-inducing signal was present and acting at approximately the 64-cell stage (Jones and Woodland, 1987). This led to the idea that the endogenous mesoderm-inducing factor was a maternally encoded signalling molecule. However, recent evidence suggests that mesoderm induction actually takes place after the onset of zygotic transcription (see below), although events that take place before MBT may still be involved.

The fibroblast growth factor family

The search for the endogenous mesoderm-inducing factor produced many candidate molecules. The first defined growth-factor candidate was a member of the FGF family, basic FGF (bFGF), which was shown to possess mesoderm-inducing activity (Kimelman et al., 1988; Slack et al., 1987). The character of tissue induced by bFGF is generally of a ventro-lateral nature, such as mesenchyme, mesothelium, blood and less frequently muscle (Green et al., 1990; Slack et al., 1987). A better candidate from the FGF family came with the identification of *embryonic FGF* (eFGF; Isaacs et al., 1992). It is expressed in the right place to have a role in mesoderm induction and is able to induce muscle in animal pole explants: over-expression of *eFGF* induces expression of a mesodermal marker gene, *Xbra* (Isaacs et al., 1994).

To assess the requirement for FGF signalling during mesoderm induction, a truncated form of the receptor was constructed, which is predicted to interfere with endogenous signalling in a dominant-negative manner. Over-expression of the truncated receptor (XFD) leads to a loss of mesoderm induction (Amaya et al., 1991; Amaya et al., 1993). Somites and notochord do not form in cells injected with XFD. These results are supported by experiments in which expression of XFD was driven from the cytomegalovirus promoter in transgenic embryos (Kroll and Amaya, 1996).

In these embryos, where expression of XFD protein does not begin until early gastrula stages (stage 10), *Xbra* expression is lost after mid-gastrula, although the initial expression is normal. Also, *Xbra* is expressed transiently in activin-induced animal caps co-injected with XFD (Schulte-Merker and Smith, 1995). These results confirm that although FGF signalling is involved in the production of mesoderm, it is probably more likely to be required for a secondary maintenance phase rather than the initial induction itself.

The transforming growth factor β family

The most promising candidates for the endogenous mesoderm inducer are members of the TGF-β superfamily. Many factors with mesoderm-inducing activity, purified from various tissues and cell lines, were found to be TGF-β members. These include Tiedemann's vegetalizing factor, PIF, WIF and XTC-MIF (Albano et al., 1990; Born et al., 1972; Godsave et al., 1988; Smith et al., 1989; Smith et al., 1990; Smith et al., 1988; Sokol et al., 1990; Thomsen et al., 1990). TGF-β2 was shown to have mesoderm-inducing activity and a set of comparison experiments suggested that the active component of XTC-MIF was a TGF-β family member (Rosa et al., 1988).

Structure

The TGF-β superfamily comprises a large group of signalling molecules. These molecules are produced as large pro-proteins that homo-dimerise (and hetero-dimerise as well) and are then cleaved by a furin pro-protein convertase to release the active mature dimer (Constam and Robertson, 1999; Dubois et al., 1995; Molloy et al., 1992). The defining characteristic of the family is a set of conserved cysteine residues in the mature region, six of which form a structural feature known as the TGF-β cysteine knot (Fig. 1.4). This is composed of three intramolecular disulphide bridges between the six conserved cysteines, two of which are between the same two β-sheets. This generates a hole through which the third disulphide bridge is inserted, thus completing a knot structure (Fig. 1.4; Daopin et al., 1992; Schlunegger and Grutter, 1992). This structure confers rigidity and helps to maintain the overall structure of the protein. A seventh cysteine makes an intermolecular disulphide bridge between two TGF-β molecules to produce the dimer (Daopin et al., 1993).

It is thought that the processing step to produce the mature region is highly regulated and contributes to the activity or range of activity of the molecule. For example, the latent TGF-β binding protein (LTBP) is a protein that associates with the pro-peptide during secretion and interacts with the extracellular matrix (ECM). Its interaction with ECM may affect the range of action of the signalling molecule.

Also, the sequence of both the pro and mature regions appear to affect the range of action of TGF-β molecules. It has been reported that there is a second, more N-terminal, consensus furin-cleavage site in the pro region of bone morphogenic protein 4 (BMP4), which can affect the range of its action when mutated (Cui et al., 2001). Similarly, it has previously been shown that substituting the pro region from one TGF-β molecule with a pro region from a different one can switch molecules with short-range actions to ones with long-range actions and *vice versa* (Dale et al., 1993; Jones et al., 1996; Thomsen and Melton, 1993).

Receptors

The receptors for the TGF-β ligands are transmembrane serine/threonine kinases. A diagram of the TGF-β signal transduction pathway is shown in Fig. 1.4. There are two TGF-β receptors, which function in combination; the type II receptor binds the ligand via its extracellular domain, which brings together the type I and type II receptors. The type II receptor then phosphorylates the type I receptor, which initiates subsequent downstream signalling (Fig. 1.4). Dominant-negative versions of a type II and type I receptor have been constructed by removing the cytoplasmic kinase domain. This should allow these receptors to bind ligand and/or dimerise but prevent signalling, thus dominantly affecting the endogenous receptors. When these dominant-negative receptors (tActRIIB and tALK4) are over-expressed in embryos they cause a loss of mesoderm and also block the formation of mesoderm in animal cap explants treated with TGF-β family mesoderm inducers (Chang et al., 1997; Hemmati-Brivanlou and Melton, 1992).

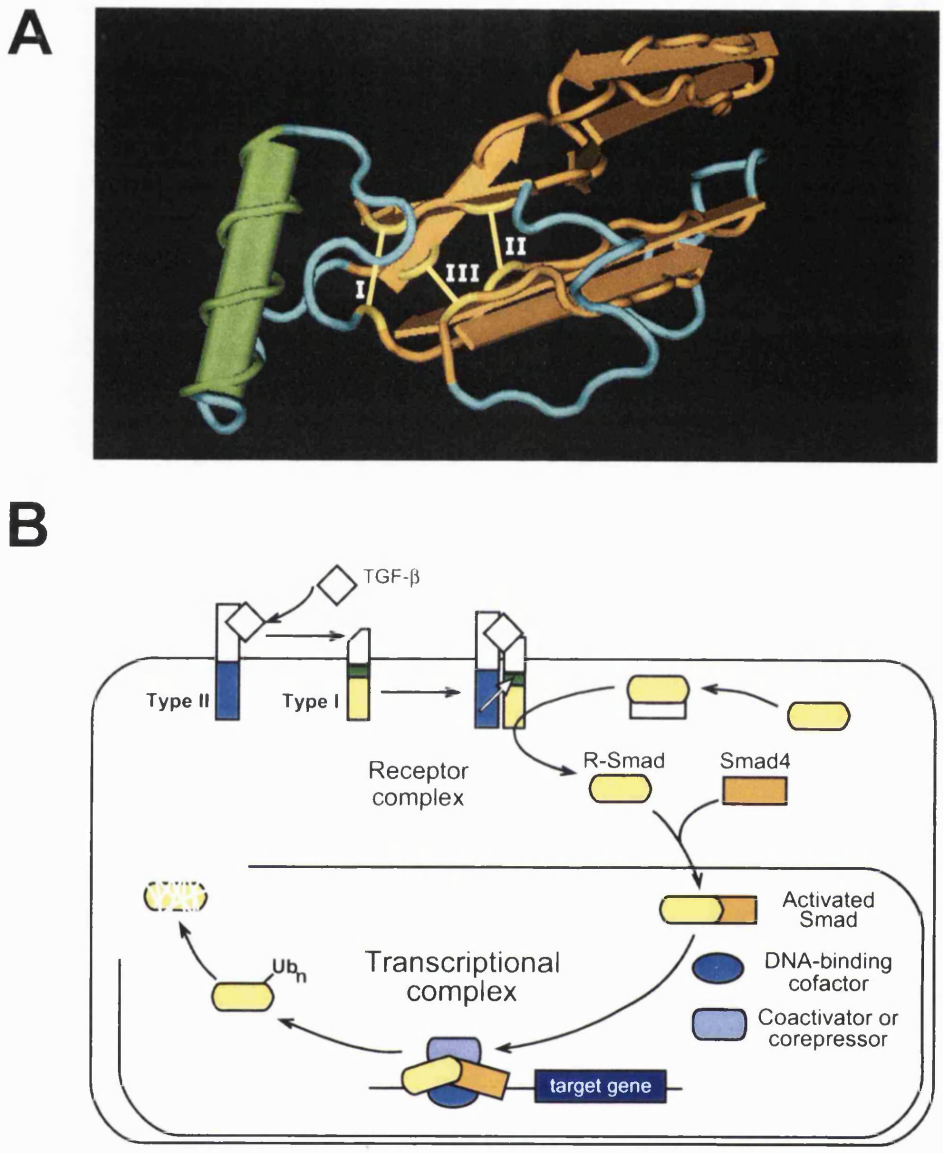

Fig. 1.4. Structure of TGF-β and diagram of signalling pathway. (A) The TGF-β cysteine knot. The characteristic structure of the TGF-β family is formed from three intramolecular disulphide bridges. Disulphide bridges I and II and the chains of the protein form a ring through which disulphide bridge III is inserted. (B) TGF-β signalling pathway. The TGF-β ligand binds the type II receptor and causes formation of a ligand–type II–type I receptor complex. The type II receptor phosphorylates and activates the type I receptor. The activated receptor then phosphorylates the receptor-specific Smad (R-Smad) which associates with the common co-Smad (Smad4) and translocates to the nucleus. In the nucleus, the R-Smad–Smad4 complex associates with other proteins to form a transcriptional complex on the promoter of target genes where it can either activate or repress gene activity. Taken from Massague and Wotton (2000).

Transcriptional responses

Smads

The major pathway downstream of these receptors utilises Smad proteins. Originally identified genetically in *Drosophila* and *Caenorhabditis elegans* as components of the TGF-β signalling pathway (Raftery et al., 1995; Sekelsky et al., 1995), these proteins transduce the signal from the type I receptor to the nucleus (Fig. 1.4). Upon receptor activation, a receptor-specific Smad (known as R-Smads) is recruited to the type I receptor, which phosphorylates the Smad on its C terminus (Macias-Silva et al., 1996). This phosphorylation allows the Smad to translocate to the nucleus with a common Smad (Smad4 or Smad4β in the context of early *Xenopus* development; Raftery et al., 1995; Howell et al., 1999), where it can affect gene transcription.

Fast proteins

Once in the nucleus, the R-Smad–Smad4 complex then associates with DNA-binding partners to produce complexes with particular target gene specificity. Smad complexes can activate transcription either by associating with co-activators or by blocking the activity of repressors (de Caestecker et al., 2000; Remacle et al., 1999). They can also repress transcription by recruiting co-repressors into the complex (Wotton et al., 1999).

One type of DNA-binding partner in *Xenopus* is represented by the winged-helix transcription factor family members Fast1 and Fast3 (Chen et al., 1996; Howell et al., 2002). For example, Fast1, Smad2 and Smad4β make up a complex (the activin-responsive factor), which interacts in a TGF-β-dependent manner with the *Mix2* promoter. Fast proteins are transcriptional activators that bind an eight base-pair sequence with the consensus AATNNACA (Labbe et al., 1998; Zhou et al., 1998). There are Fast-responsive sites in the *mix2*, *gsc*, *nodal* and *lefty2* promoters (Labbe et al., 1998; Saijoh et al., 2000; Zhou et al., 1998).

Loss-of-function studies of individual Fast proteins have been carried out in *Xenopus*, mouse and zebrafish. All show similar defects, but with differing degrees of severity. Knockdown of *XFast1* and *XFast3* in *Xenopus* gave rise to embryos that did not gastrulate properly, the most severely affected having a loss of axial structures and anterior truncations (Howell et al., 2002).

This phenotype is similar to that of zebrafish embryos with mutations in the *schmalspur* (*sur*) gene, the zebrafish *Fast1* orthologue (Pogoda et al., 2000; Sirotkin et al., 2000). Embryos with no maternal or zygotic *sur* (MZ*sur*) do not develop a shield, the zebrafish equivalent of the organiser, and consequently have a reduction of prechordal plate, floorplate and notochord with ventral brain defects and cyclopia. These phenotypes are also similar to the less severe of the phenotypes produced by the targeted disruption of the mouse *FoxH1* (*Fast1*) gene (Hoodless et al., 2001; Yamamoto et al., 2001). Due to background effects, a proportion of the homozygous mutant embryos actually do not form the embryo proper, although the extra-embryonic tissues appear to develop normally.

These experiments show that Fast proteins are key effectors of TGF-β signals and are required for the correct formation and patterning of the mesoderm. However, these embryos also demonstrate that they are not the only effectors of TGF-β signalling. The phenotype of MZ*sur* mutant embryos is less severe than those of the TGF-β ligands *squint* (*sqt*) and *cyclops* (*cyc*). *sqt;cyc* double mutants lack trunk somites and blood, which are present in the MZ*sur* embryos. Therefore, there are Fast-independent actions of TGF-β signalling and it has already been shown that Smad2 and Smad4 are able to form a complex containing the paired-class homeodomain protein Mixer (Germain et al., 2000).

Candidate TGF-β Inducers

Many TGF-β molecules expressed during early *Xenopus* development have been identified as candidates for the endogenous mesoderm-inducing signal.

Activin (XTC-MIF)

A mesoderm-inducing factor isolated from a tissue-culture cell line (XTC-MIF), was shown to be the *Xenopus* homologue of mouse *activin* A (a TGF-β family member; Smith et al., 1990; Smith et al., 1988). Activin is able to induce a range of mesodermal tissue types in isolated ectodermal explants in a concentration-dependent manner (Green et al., 1992; Green and Smith, 1990). Activin RNA is not expressed maternally, although activin protein is present in and has been purified from *Xenopus* eggs (Asashima et al., 1991; Fukui et al., 1994).

In support of a role for TGF-β signalling in mesoderm induction, over-expression of truncated versions of the activin type I and II receptors – predicted to act as dominant-negative molecules in the same manner as the truncated FGF receptor – leads to a loss of mesoderm in embryos. However, these dominant-negative receptors block the effects of many different TGF-β molecules. Although this experiment does not necessarily show a role for activin in mesoderm induction it does show that an activin-like signal is required for mesoderm formation (Chang et al., 1997; Hemmati-Brivanlou and Melton, 1992).

A more specific dominant-negative receptor was constructed by Dyson and Gurdon (1997) by removing the transmembrane domain as well as the intracellular domain from the activin type II receptor. This construct does not block the effects of other TGF-β molecules, and embryos over-expressing it lack head structures. These embryos still have mesoderm, but the mesodermal marker *Xbra* is slightly reduced and delayed in its expression (Dyson and Gurdon, 1997). These experiments were confirmed using an activin-binding protein, follistatin, which gave similar results (Marchant et al., 1998), although follistatin has recently been reported to also bind BMP4 (Fainsod et al., 1997; Iemura et al., 1998).

Vg1

Vg1 was isolated as a maternally expressed TGF-β member localised to the vegetal cortex (Melton, 1987; Rebagliati et al., 1985; Weeks and Melton, 1987) and as such was an excellent candidate for an endogenous mesoderm-inducing factor. The RNA is localised to the vegetal cortex during oogenesis and is released from the cortex but remains in the vegetal hemisphere on oocyte maturation. The large 42 kd precusor pro-protein was found to be present in oocytes and eggs, but the active processed mature dimer has never been detected *in vivo* (Dale et al., 1989; Tannahill and Melton, 1989). Over-expression of Vg1 RNA has no effect on development and is unable to induce mesoderm in animal caps (Dale et al., 1993; Thomsen and Melton, 1993).

In an attempt to demonstrate a function for the mature form of Vg1, chimeric molecules fusing the pro-region of either BMP-2 or BMP-4 to the mature region of Vg1 were constructed (Dale et al., 1993; Thomsen and Melton, 1993). Over-expression of RNA for these constructs produced a large pro-protein as well as a smaller, apparently correctly processed, mature Vg1 protein.

Both of these chimeric molecules are able to induce mesoderm in animal caps. Also, a fusion of the activin pro-region and the Vg1 mature region leads to effective secretion of a processed mature form of Vg1 from oocytes and this protein is also able to induce mesoderm (Kessler and Melton, 1995). What is clear from these results is that the particular pro-region that is attached to a TGF-β mature region significantly affects its processing and apparent activity. How this effect occurs is still not known.

To address the *in vivo* role of Vg1, a dominant-negative Vg1 was constructed (Joseph and Melton, 1998) and over-expression of this construct leads to a loss of dorsal mesodermal derivatives. It is possible, however, that this construct also interferes with the function of the closely related gene *derrière*, which was unknown at the time, and therefore it is difficult to assign relative importance to these molecules.

Mesoderm induction is a zygotic event

Recently, it has been suggested that mesoderm induction may not occur until after the onset of zygotic transcription. Wylie et al. (1996) carried out recombination experiments to investigate this question. Animal caps at late blastula stage (stage 9) were combined with vegetal pole regions either before or after the onset of zygotic transcription (stage 7 and stage 9). The recombinants were left together for only 1 hour, at which point the animal caps were removed and cultured alone. When the animal caps were exposed to post-MBT vegetal regions, they expressed the mesodermal marker genes *XWnt8* and *MyoD*. However, when the animal caps were exposed to pre-MBT vegetal regions, they expressed no *MyoD* and only very low levels of *XWnt8*. This suggests that, contrary to what had previously been thought, mesoderm induction is actually mediated zygotically and that the mesoderm-inducing factor is, in fact, a zygotically expressed activin-like TGF-β signal. Thus, zygotically expressed TGF-β molecules are now candidates for mesoderm-inducing signals.

Genetic evidence from other systems, chiefly mouse and zebrafish, has shown that molecules of the *nodal* subfamily are necessary for mesoderm formation. In homozygous *nodal* mutant mouse embryos, the primitive streak fails to form and there is a lack of embryonic mesoderm (Conlon et al., 1994; Zhou et al., 1993). In zebrafish, embryos lacking both of the nodal homologues, *squint* (*sqt*) and *cyclops* (*cyc*), fail to form almost all endoderm and mesoderm (although tail somites do form; Feldman et al., 1998).

In *Xenopus*, there are many candidate genes, although due to the limited value of dominant-negative approaches, it has been difficult to distinguish which of these are most important. The main contenders currently are the *Xenopus nodal-related* genes (*Xnr1,2 & 4–6*) and *derrière* (Jones et al., 1995; Joseph and Melton, 1997; Sun et al., 1999; Takahashi et al., 2000).

Xnr1-6

Xnr1–6 are members of the TGF-β superfamily and are most related to mouse *nodal* (Conlon et al., 1994; Zhou et al., 1993). They fall broadly into two types: *Xnr1*, *Xnr2* and *Xnr4–6* have mesoderm-inducing properties (Jones et al., 1995; Joseph and Melton, 1997; Takahashi et al., 2000), whereas *Xnr3* is a neural inducer (Ecochard et al., 1995; Smith et al., 1995). They are expressed in slightly different patterns, with *Xnr1* and *Xnr2* most closely resembling the expression pattern of *VegT*. These genes are first expressed in the vegetal hemisphere just after the onset of zygotic transcription, and later on in early gastrula stages they show expression in the marginal zone, with a higher level of expression on the dorsal side. Both will induce dorsal mesoderm in animal pole tissue, as assayed by both morphological criteria and the expression of the molecular markers *Xbra*, *muscle-actin* and *goosecoid* (*gsc*) (Jones et al., 1995).

In contrast, *Xnr3* will not induce mesoderm in an animal cap assay and its expression pattern differs in that it is expressed in a more restricted domain in the region of the embryo corresponding to the Spemann organiser, but it is only expressed in the epithelial layer of cells above and below the blastopore lip (Ecochard et al., 1995; Smith et al., 1995). *Xnr4* is also expressed in the organiser, but in a slightly different area. The expression is specifically restricted to the deep cells of the organiser rather than the superficial layer (Joseph and Melton, 1997). It has also recently been shown to be expressed in the vegetal hemisphere as well (Clements et al., 1999). *Xnr4* is able to induce mesoderm in animal cap assays (Joseph and Melton, 1997).

Recently, two more members of the family have been cloned from *Xenopus* — *Xnr5* and *Xnr6* (Takahashi et al., 2000). They are expressed as soon as zygotic transcription starts, initially in the dorsal–vegetal cells and then throughout the endoderm. Both these molecules are mesoderm inducers and have been shown to be downstream of *VegT* (Takahashi et al., 2000).

Interestingly, whereas *Xnr1*, *Xnr2* and *Xnr4* are inducible by activin-like signalling, *Xnr5* and *Xnr6* are not, although they are induced by *VegT* (Clements et al., 1999; Jones et al., 1995; Takahashi et al., 2000). This, coupled with the fact that they are expressed slightly earlier than the other nodal-related genes, puts them in an excellent position to make up the early activin-like signal downstream of *VegT*.

Various approaches have been used to test the requirement for the *Xnr* genes in mesoderm induction. Dominant-negative forms of *Xnr2*, *Xnr5* and *Xnr6* have been created to test the role of these genes. Over-expression of a dominant-negative *Xnr2* (cm–Xnr2), leads to a loss of anterior structures, which appears to be due to a defect in the anterior deep mesendoderm, the 'head-organiser' region, as shown by the decreased expression of markers such as *cerberus*, *Xhex-1*, *Frzb*, *dickkopf-1* (*dkk-1*) and *mixer*. cm–Xnr2 also slightly delays the expression of more general mesoderm markers such as *Xbra*, *Xwnt8* and *MyoD*. In contrast, over-expression of a dominant-negative *Xnr5* (cm–Xnr5) reduces expression of many mesodermal and endodermal genes (e.g. *Xbra*, *gsc*, *mixer*, *Xsox17*β).

The drawback to this approach is that these reagents are not entirely specific; cm–Xnr2 also affects the activity of *Xnr1*, *Xnr2*, *Xnr4*, *Xnr5* and *Xnr6*, whereas cm–Xnr5 can inhibit the effect of *Xnr2*, *Xnr4*, *Xnr5*, *Xnr6*, *derrière* and *BVg1*.

A more specific reagent is a truncated form of the cerberus protein (Cer-S), which is a specific inhibitor of nodal signalling. Over-expression of Cer-S in *Xenopus* embryos causes down-regulation of organiser genes such as *gsc*, *noggin* and *chordin* as well as more general markers such as *Xbra* and *Xwnt8* and the embryos display a lack of axial structures. All these data suggest that the nodal-related genes are excellent candidates for endogenous mesoderm-inducing signals.

Derrière

Derrière, another member of the TGF-β superfamily, is most closely related to *Vg1* (79% amino acid identity in the mature region) and has been shown to be an inducer of posterior mesoderm (Sun et al., 1999). There are no maternal transcripts, but it is expressed zygotically.

Expression is first detected at mid-blastula (stage 9, Fig.1.5A). Expression is seen in the vegetal pole as well as the marginal zone. The staining becomes more intense on the future dorsal side where the blastopore lip forms. As gastrulation proceeds, expression is lost from the vegetal cells but remains in the margin around the closing blastopore (stage 10.5, Fig. 1.5B). By late gastrula (stage 12.5), expression remains around the blastopore but is excluded from the dorsal midline, which will form the most axial mesoderm (Fig. 1.5C).

Over-expression of *derrière* in animal caps causes the dose-dependent induction of both mesodermal and endodermal marker genes and is able to dorsalise ventral marginal zone explants (Sun et al., 1999). Also, when over-expressed in embryos it can produce a partial secondary axis.

In order to disrupt *derrière* function, a cleavage mutant predicted to act in a dominant-negative fashion was constructed. This construct interferes with *derrière*, as assayed by marker gene induction and elongation of animal caps (Sun et al., 1999). It also has slight effects on some other TGF-β molecules. When over-expressed in whole embryos, this construct causes a lack of trunk and tail structures. Involution is impaired at gastrulation and whereas the head appears largely normal, somites do not develop, the neural tube remains open and the tail does not form (Sun et al., 1999). These results strongly suggest that *derrière* is involved in the formation of trunk and tail mesoderm.

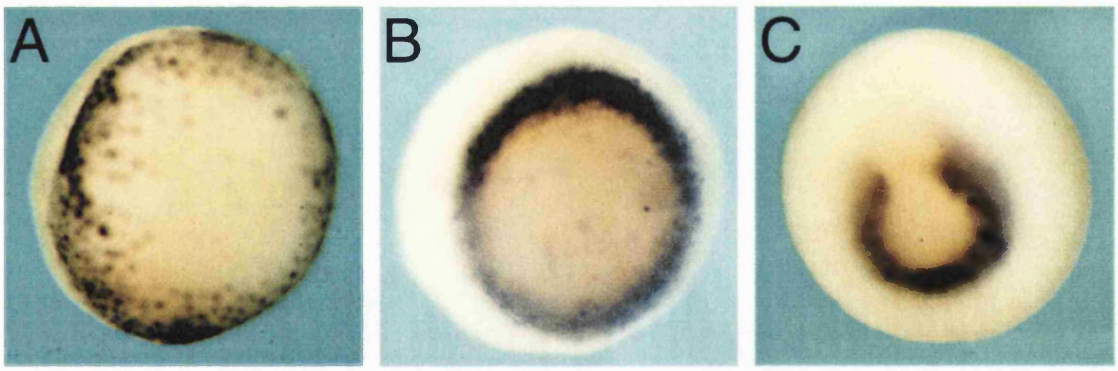

Fig. 1.5. *Derrière* expression pattern. (A) St 9 embryo, vegetal pole view. *Derrière* is expressed in vegetal pole cells as well as the marginal zone. Expression is patchy due to poor penetration of the probe into the yolky vegetal cells. (B) St 10.5 embryo, vegetal pole view. *Derrière* expression remains in the margin where it is expressed more strongly on the dorsal side. (C) St 12.5 embryo, posterior view. *Derrière* expression remains around the closing blastopore, but is down-regulated in the dorsal midline.

VegT

VegT (also known as *Antipodean*, *Brat* and *Xombi*) was cloned independently by four different groups (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996). It is a maternal determinant required for germ layer specification and is a member of the T-box family of transcription factors.

The T-box family

The T-box family of transcription factors is a large family of proteins characterised by a common DNA-binding domain, the T-domain. The family is named after the founding member, *Brachyury* or *T*, whose mutants were originally described in 1927 (Dobrovoslkaïa-Zavadskaïa, 1927) and then cloned in 1990 (Herrmann et al., 1990; Wilkinson et al., 1990). The mutation is semi-dominant, such that heterozygotes have short tails whereas homozygotes lack a differentiated notochord and posterior mesoderm, leading to a truncation of the embryo. The embryos die due to a failure to form the allantois. When the gene was cloned, the protein that *Brachyury* encoded was shown to be a transcriptional activator (Kispert et al., 1995).

Since then, many orthologues have been cloned in a wide variety of species – Hydra, *Caenorhabditis elegans*, *Drosophila melanogaster*, Ascidians, Zebrafish, *Xenopus*, mouse, chick and humans (reviewed in Papaioannou, 2001; Smith, 1999) – and have been shown to be important in a wide variety of developmental processes. There are three members of the T-box family that are known to be expressed at early stages of *Xenopus* development, Brachyury, Eomesodermin and VegT.

Brachyury

Xenopus Brachyury (*Xbra*), the *Xenopus* orthologue of *Brachyury*, is the original member of the T-box family (Smith et al., 1991). It is expressed at gastrula stages in the marginal zone of the embryo a few cell diameters away from the blastopore lip and in the most axial mesoderm (the notochord). Expression remains in the marginal zone around the closing blastopore during gastrulation and continues to be expressed in the notochord only during neurula stages (Smith et al., 1991). The encoded protein is a transcriptional activator, like its mouse orthologue. Over-expression of *Xbra* in animal cap explants is sufficient to induce mesoderm in the explant tissue (Cunliffe and Smith, 1992).

The mesodermal cell types induced are of differing character depending on the amount of injected *Xbra* RNA, in a similar manner to the induction of mesoderm by TGF-β signalling (Cunliffe and Smith, 1992; O'Reilly et al., 1995). In fact, *Xbra* itself is induced in a dose-dependent fashion by TGF-β signalling (Green et al., 1992).

The *Brachyury* othologue in the zebrafish, *no tail* (*ntl*), confirms the importance of this gene in early development. Like mouse embryos deficient in Brachyury, *ntl* mutant embryos have no notochord, abnormal anterior somites and defects in posterior development. The posterior somites are fused at the midline, due to the lack of a notochord, and the embryos also lack a tail (Halpern et al. 1993; Schulte-Merker et al. 1994).

In *Xenopus*, where loss-of-function experiments are much more difficult, the importance of *Xbra* was shown using a construct in which the DNA-binding domain of *Xbra* was fused in-frame to the repression domain of the *Drosophila* protein engrailed. This Xbra-En^R construct should bind to and repress all the normal transcriptional targets of Xbra. Injection of this construct into *Xenopus* embryos causes a loss of posterior stuctures and also causes a failure in gastrulation movements (Conlon et al., 1996; Conlon and Smith, 1999).

Brachyury is a transcription factor showing sequence-specific DNA-binding and transactivation activity

Significant insight into the activities of the T-box family first came with the definition of a consensus Brachyury binding site followed by the solution of the crystal structure of Brachyury protein bound to DNA. A consensus binding site was defined *in vitro* using a binding site selection approach (Kispert and Herrmann, 1993), in which random oligonucleotides were selected for their ability to bind Brachyury protein. After several rounds of selection the products were cloned and sequenced and aligned to give a consensus selected sequence. In this case, the sequence selected was a 24 base-pair palindrome:

AATTTCACACCTAGGTGTGAAATT

This sequence actually binds two molecules of Xbra and it became clear that only 8–10 base-pair (underlined) were needed to bind a single Xbra protein (Casey et al., 1998). In 1997, the crystal structure of Xbra bound to DNA was solved (Muller and Herrmann, 1997).

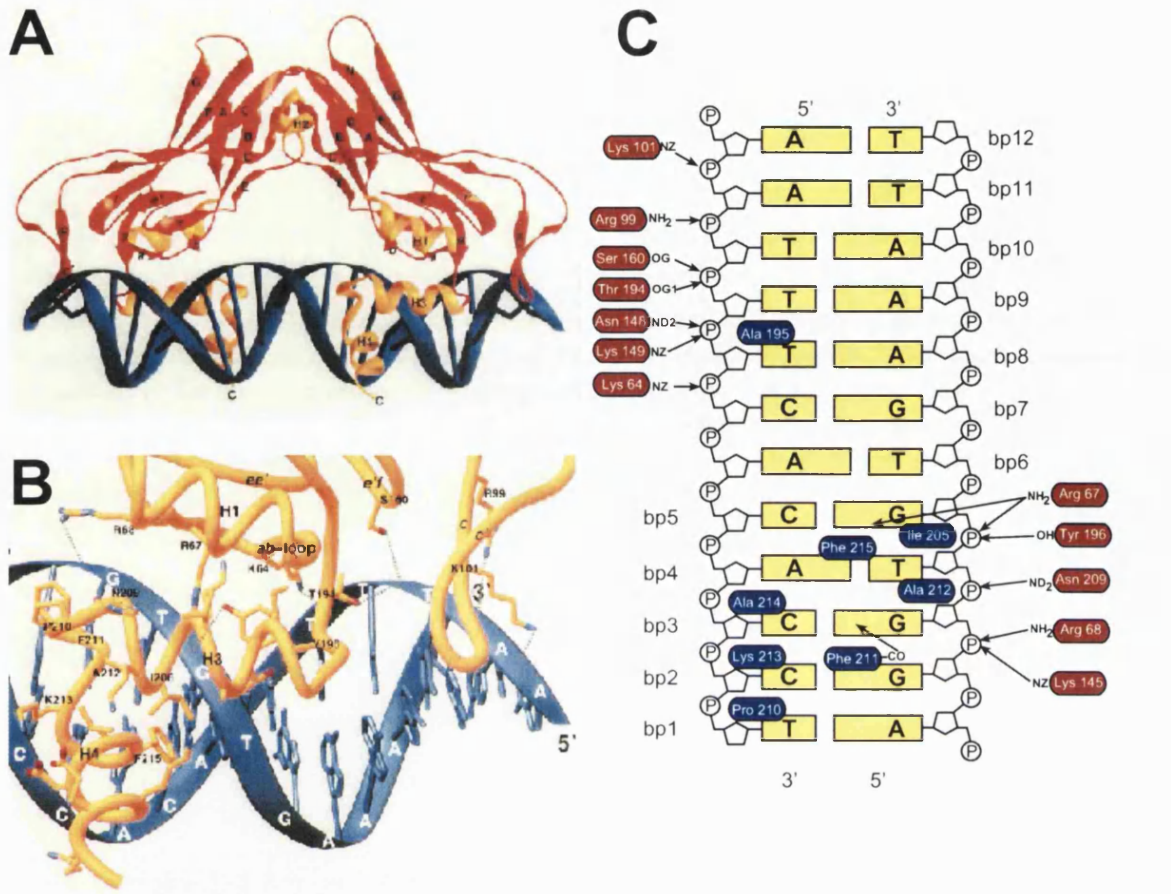

Fig. 1.6. Crystal structure of Xbra bound to DNA. (A) Ribbon diagram of the T-domain dimer bound to DNA. Depicted are residues 39–221 of both monomers (strands and loops: red, helices: yellow) and the 24-mer DNA duplex (blue). Labels mark strands of the central immunoglobulin fold (A–G) and the two smaller lid-forming sheets (bee'/c'cfga). (B) Interactions of one monomer with DNA. The view is the same as in (A) but with the contacts at the right half-site blown up. Polar interactions are indicated by dashed lines. For clarity, residues 145, 148 and 149, which also contact the DNA backbone, have been omitted. (C) DNA recognition by the T domain. Protein–DNA interactions in one half-site. Polar interactions are indicated by arrows. Residues against a red background are involved in polar interactions; those against a blue background make hydrophobic contacts and are placed next to the region of DNA that they contact. Taken from Müller and Herrmann (1997).

This showed the critical contact points in the DNA sequence and the mode of binding by Xbra protein. As shown in Fig. 1.6, sequence-specific contacts are made by two helices at the C-terminal end of the T-domain, while interactions between loops in the rest of the T-domain and the phosphate backbone of the DNA provide stability to the complex. The critical determinants of sequence specificity are shown in bold below:

TT**T**CA**CACCT**

These base-pairs are directly contacted by residues in the protein, whereas the more 5' bases have a tendency to be A•T rich as these are less sensitive to the distortion of the DNA helix, which occurs on insertion of the two protein-binding helices into the minor groove.

Eomesodermin

Eomesodermin (*Eomes*), another T-box protein, is also expressed in the prospective mesoderm of *Xenopus*. The gene is expressed soon after the onset of zygotic transcription throughout the marginal zone (in contrast to *Xbra*). Over-expression of *Eomes* in animal cap explants leads to the induction of mesodermal markers (e.g. *Xbra*, *XWnt8* and *Chd*) and injection of Eomes-En^{R} (a dominant-interfering construct) leads to a block in gastrulation and a decrease in muscle-actin expression (Ryan et al., 1996).

A mutation produced in the mouse gene by homologous recombination yields embryos with a block in differentiation of trophectoderm to trophoblast, an extra-embryonic tissue which does not exist in *Xenopus* embryos. However, if this block is overcome by combining mutant ES cells with tetraploid embryos, which will not form embryonic tissues but will form extra-embryonic structures, a requirement in mesoderm formation is uncovered. In these aggregate embryos, mesoderm is not produced properly: mesodermal markers are expressed but the tissue fails to migrate to the primitive streak where gastrulation occurs. This failure is not in making mesoderm *per se*, as mutant ES cells will form teratomas including mesodermal derivatives (Russ et al., 2000).

VegT and germ layer specification

VegT is expressed both maternally and zygotically and has been shown to be essential for both endoderm and mesoderm specification. Unlike the other two T-box genes discussed above, *VegT* is present in the developing oocyte as a maternally deposited mRNA localised to the vegetal cortex (Fig. 1.7A–C). It is later expressed zygotically, although the zygotic transcript is an alternatively spliced isoform of the same gene and is also known as *Antipodean* (Apod; Stennard et al., 1999). The protein produced has the N-terminal 25 amino acids replaced by five different amino acids. It is tempting to suggest that these two isoforms mediate different processes by virtue of this changed N-terminus, although it is currently unknown whether this confers any functional difference on the protein. The two isoforms are certainly regulated differently, with the maternal form expressed only during oogenesis (the RNA declines between stage 10–10.5) and the zygotic form expressed from stage 10 onwards. Also, *Apod* is inducible by TGF-β signalling whereas *VegT* is not.

At the onset of zygotic transcription, expression of *Apod* begins on the dorsal side of the marginal zone where the dorsal blastopore lip is forming (Fig. 1.7D–E). As gastrulation proceeds, the expression extends around the margin, laterally and ventrally, until the entire margin is expressing by stage 10.5 (Fig. 1.7F–G). This expression persists around the closing blastopore throughout gastrulation, except that at later stages *Apod* is down-regulated in the dorsal midline (Fig. 1.7H), which is fated to become the most axial mesoderm (the notochord). *Apod* is expressed in the posterior paraxial and lateral mesoderm and persists here until tailbud stages (Fig. 1.7I).

Over-expression of synthetic *VegT* mRNA in animal caps leads to the formation of both endoderm and mesoderm (Horb and Thomsen, 1997; Stennard et al., 1996) and expression of a dominant-negative form of *VegT* (*VegT-En*^{R}) inhibits mesoderm induction and severely affects development (Horb and Thomsen, 1997). Both these results suggest that *VegT* plays an important role in germ layer specification.

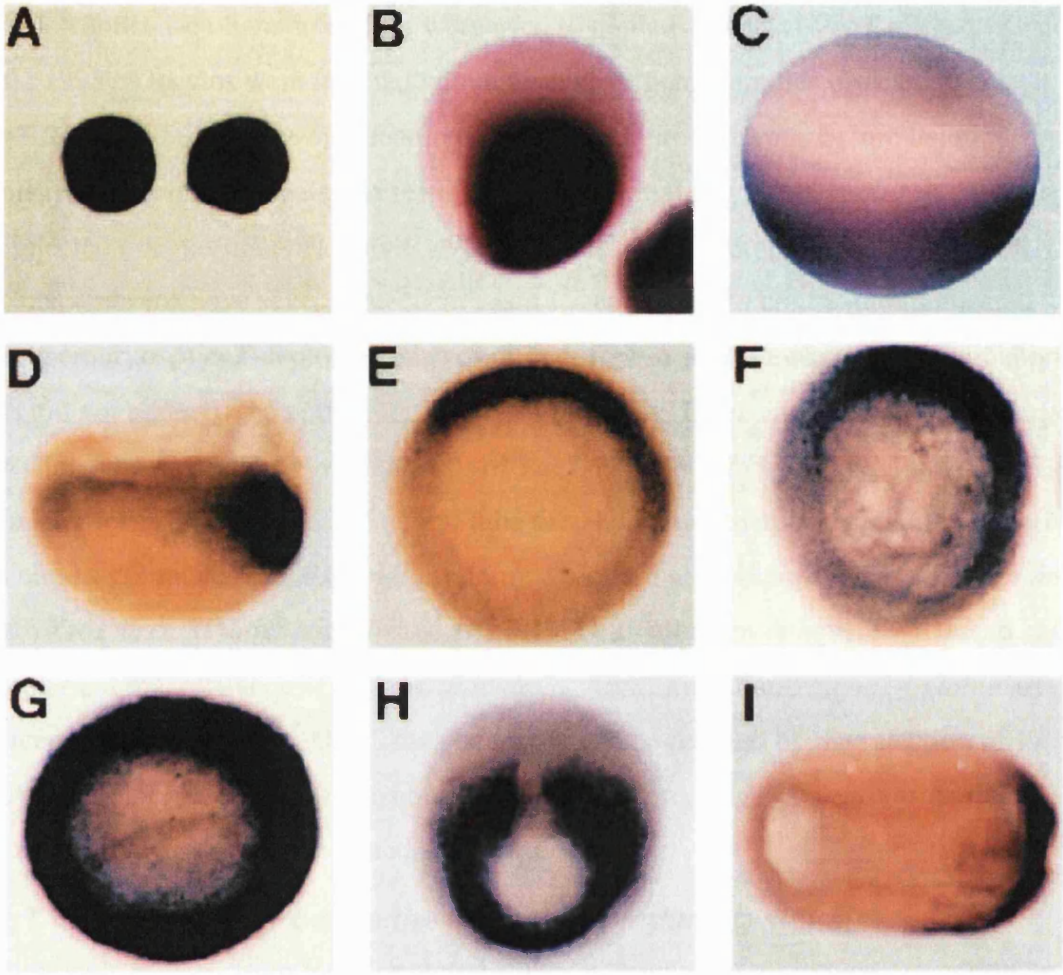

Fig. 1.7. Expression pattern of *VegT*. Taken from Zhang et al. (1996). (A) Stage I oocytes. *VegT* is uniformly distributed. (B) Stage IV oocytes. *VegT* is localised to the vegetal hemisphere. (C) Ovulated egg. *VegT* remains in the vegetal hemisphere. (D) Stage 9.5 embryo, lateral view. Zygotic *VegT* is expressed on the dorsal side, within the marginal zone. (E) Stage 9.5 embryo, vegetal view of embryo in D. (F) Stage 10.25 embryo, vegetal view. Staining stronger on dorsal side, but increasing along lateral and ventral sides. (G) Stage 10.5 embryo, vegetal view. Staining throughout the entire marginal zone. (H) Stage 12.5 embryo posterior view. Ventral and lateral staining around the blastopore. Expression has been excluded from the dorsal midline. (I) Stage 16 embryo, dorsal view. *VegT* expression in the posterior. Taken from Zhang et al. (1996)

VegT is required for normal germ layer specification

The evidence that *VegT* is required for germ layer specification came from experiments in which antisense oligonucleotides were used to deplete maternal *VegT* mRNA (Zhang et al., 1998). Oocytes were injected with antisense oligonucleotides directed against *VegT* RNA, and they were then matured *in vitro* with progesterone before being implanted back into a female and fertilised in the normal way. This approach depleted the injected oocytes of approximately 90% of their normal complement of *VegT* mRNA.

Such embryos have severe defects at gastrulation stages. In contrast to uninjected sibling embryos, *VegT*-depleted embryos did not form a blastopore ring and animal pole cells did not undergo the normal movements of epiboly. Later on, when sibling embryos were finishing gastrulation, vegetal cells of *VegT*-depleted embryos involuted and these cells later formed notochord and neural tube tissue. Overall there is a lack of endoderm and almost complete reduction in mesoderm; endodermal markers such as *endodermin* (*edd*), *Xsox 17α*, *Xlhbox8*, *insulin* and *IFABP* are either absent or severely reduced and mesodermal markers such *MyoD*, *cardiac-actin*, *Xbra*, *Xwnt8* and *alphaT4 globin* are reduced by approximately 90%. This phenotype can be rescued by the injection of *VegT* RNA (Kofron et al., 1999; Zhang et al., 1998). Therefore, *VegT* is clearly important for proper germ layer specification and morphogenesis.

VegT is required to produce the mesoderm-inducing signal

Vegetal pole regions from *VegT*-depleted embryos can no longer induce mesoderm in naive animal cap cells, suggesting that *VegT* is necessary for the production of the mesoderm-inducing signal (Zhang et al., 1998). This recent work agrees with the results of studies on heterochronic recombinants by Wylie et al. (1996), that mesoderm induction occurs later than had originally been thought (see ***Mesoderm induction is a zygotic event***). It implies that mesoderm induction is an event that is mediated by zygotic factors, not carried out by maternally expressed signalling molecules. The implication is that the mesoderm-inducing factor, rather than being a maternal factor is, in fact, a direct zygotic target of *VegT*.

This study: Screen for direct targets of *VegT*

In order to find out how *VegT* has its effects on cell fate specification and cell behaviour we need to know what genes it regulates. The aim of this project was to find direct downstream targets of *VegT* and to study their regulation and function. To do this, I adopted a candidate approach in which known genes that are likely to be targets of *VegT* were investigated. In order to screen these potential targets, a hormone-inducible version of *VegT* was constructed. This construct was then used in conjunction with a variant of the standard animal cap assay to screen potential target genes by RNase protection. In order to test whether inductions were direct, the protein synthesis inhibitor cycloheximide (CHX) was used. Inductions that occur even in the presence of CHX are considered to be direct.

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Chapter 2. Materials & Methods

Abbreviations

Ala	Alanine
BSA	Bovine serum albumin
Cys	Cysteine
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
EDTA	disodium ethylenediaminetetraacetate
EMSA	Electrophoretic mobility shift assays
NBCS	New born calf serum
Met	Methionine
PAGE	Polyacrylamide gel electrophoresis
pBSK	pBluescript II (SK)
PEG	Polyethylene glycol
SDS	Sodium dodecyl sulphate
Ser	Serine
SSC	Sodium chloride/sodium citrate buffer
TAE	Tris-acetate/EDTA buffer
TBE	Tris-borate/EDTA buffer
TCA	Trichloroacetic acid
ATP	Adenosine 5'-triphosphate
CTP	Cytidine 5'-triphosphate
GTP	Guanosine 5'-triphosphate
TTP	Thymidine 5'-triphosphate
UTP	Uridine 5'-triphosphate
d (as in dATP)	deoxy

Note: Items in bold and italics are references to other sections.

Molecular biology techniques

Preparation and storage of competent bacteria

Chemically competent DH5α bacteria were used to propagate plasmid DNA. Non-competent DH5α were streaked onto an LB-plate and grown overnight at 37°C. A single colony was picked into 4 ml LB and cultured at 37°C overnight. 2 ml of this culture was used to inoculate 400 ml 2 x TY, which was cultured for approximately 2–3 hours until the $OD_{\lambda=550} = 0.48$. All successive procedures were carried out at 4°C.

The culture was then transferred to pre-chilled 50 ml FALCON tubes, chilled on ice for 10 min and then centrifuged at 2500 rpm for 5 min. The cell pellet was gently resuspended in 1–2 ml ***Tfb I*** and then made up to 30 ml/100 ml starting culture and incubated on ice for 2 hours. The culture was centrifuged again for 5 min at 2500 rpm. The pellet was then resuspended in 4 ml/100 ml starting culture ***Tfb II***, separated into 200 µl aliquots, snap-frozen in liquid N_2 and stored at –80°C.

Tfb I

30 mM KOAc

50 mM $MnCl_2$

100 mM RbCl

10 mM $CaCl_2$

15% glycerol

Store at 4°C

Tfb II

10 mM NaMops (pH 7.0)

75 mM $CaCl_2$

10 mM RbCl

15% glycerol

Store at 4°C

Transformation of competent bacteria

200 µl aliquots of competent DH5α bacteria were defrosted on ice. Up to 8 µl of a 20 µl ligation mix was added to 100 µl cells and incubated on ice for 25 min. Heat shock was carried out at 42°C for 45 s followed by incubation on ice for 2 min. 900 µl of LB was added and the mix was cultured at 37°C for 30–60 min. 100–200 µl of culture was then plated on LB-ampicillin plates and incubated at 37°C overnight.

Mini, midi & maxi preps

Transformed colonies were picked into 2 ml LB (containing 100 μg/ml ampicillin) and cultured overnight at 37°C. 1.5 ml of culture was centrifuged at 13,000 rpm for 30 s. The resulting bacterial pellet was resuspended in 120 μl ***P1***. 120 μl ***P2*** was then added, the suspension was mixed gently by inversion and incubated for 5 min. The mixture was neutralised by adding 120 μl ***P3***, mixing gently and incubating on ice for 10 min.

Samples were centrifuged at 13,000 rpm for 5 min to spin down the precipitate of cellular debris and genomic DNA. The supernatant was treated by ***phenol/chloroform extraction*** and ***ethanol precipitation*** and the resulting pellet was resuspended in 50 μl H_2O. Larger-scale preparations were made using the Qiagen Midi and Maxi prep kits, according to the manufacturer's instructions.

P1	*P2*	*P3*
50 mM Tris-HCl (pH 8.0)	200 mM NaOH	3 M NaOAc (pH 5.5)
10 mM EDTA	1% SDS	
100 μg/ml RNase A		

DNA quantification

Solutions of nucleic acids were quantified by spectrophotometry. Samples of nucleic acids were diluted 1:100 in TE and readings were taken at a wavelength of 260 nm where an OD = 1 corresponds to 50 μg/ml for double-stranded DNA and 40 μg/ml for RNA. To obtain an estimate of the purity of the solution, the ratio of λ=260 to λ=280 was used. A pure preparation of DNA will have an $OD_{\lambda=260}$ / $OD_{\lambda=280}$ ratio of 1.80.

Phenol/chloroform extraction

Proteins were removed from DNA solutions after enzymatic reactions by phenol/chloroform extraction. An equal volume of a 25:24:1 mixture of Phenol:chloroform:isoamyl alcohol was added and vortexed. The mixture was centrifuged for 5 min at 13,000 rpm. The aqueous phase was removed and retained.

Precipitation of nucleic acids

DNA solutions were concentrated by ethanol precipitation. A 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol were added, mixed and incubated on dry ice until the mixture was viscous. This was centrifuged at 4°C at 13,000 rpm for 10 min.

The supernatant was removed and the pellet washed by adding 70% ethanol and centrifuging again at 13,000 rpm for 2 min. The 70% ethanol was then removed and the pellet was allowed to air-dry for 5 min before it was resuspended in water.

Isopropanol precipitation was used for both DNA and RNA. The procedure is essentially the same as ethanol precipitation except that a volume of isopropanol equal to the solution being precipitated is used.

To concentrate small-scale preparations of DNA for sequencing reactions, precipitation with PEG was used. 0.6 volumes of 20% PEG/2.5 M NaCl was added to the sample and incubated on ice for 30 min. This mixture was then centrifuged at 4°C at 13,000 rpm for 10 min to precipitate the DNA. The pellet was washed with 70% ethanol as above and resuspended in water.

Restriction digestion

Restriction digests were performed using commercially available enzymes and buffers (Roche and New England Biolabs) at the temperature specified by the manufacturer. In all reactions, the enzyme comprised no more than 10% of the reaction volume.

Agarose gels

DNA fragments were separated by agarose gel electrophoresis. Gels were made by dissolving agarose in 1 x TAE buffer at concentrations between 0.8% and 2.0%, samples were mixed with 6 x loading buffer and electrophoresis was carried out at 90 V for 30 to 60 min until the desired resolution was achieved. DNA fragments were visualised by the addition of ethidium bromide at 0.1 μg/ml to the gel, which intercalates into double-stranded DNA and fluoresces under ultraviolet light at a wavelength of 302 nm. The size of fragments was estimated by comparison to commercially available standards, such as the 1 kb Plus DNA ladder (GIBCO BRL).

Gel purification

DNA fragments were excised from agarose gels and purified after electrophoresis using the Qiagen Gel Purification kit. Briefly, the agarose slice containing the required DNA fragment is dissolved in buffer QG (at 50°C) and added to a Qiagen column, which is spun at 13,000 rpm for 1 min. The column is then washed with buffer PE, spun twice as before and the DNA is eluted from the column with 30–50µl water.

Ligation

Ligation reactions were performed using T4 DNA ligase (Clontech) and the supplied buffer. Reactions were carried out in 20 µl and each reaction comprised 20–50 ng of vector and 20–200 ng of insert in a 1:2 vector:insert ratio (1:4 for blunt-ended ligations). 1 µl of ligase was added and the reaction was incubated at room temperature for 1–2 hours or at 14°C overnight. Usually, 8 µl of this reaction was used for ***transforming competent bacteria***.

PCR

Polymerase chain reaction (PCR) was performed using versions of the Thermus aquaticus DNA polymerase – AdvanTaq (Clontech) and Pfu (Stratagene) – as appropriate. Pfu polymerase was used preferentially when the PCR fragment was to be cloned, because of the higher fidelity of this polymerase. Reactions were carried out in 50 µl and contained 5 ng template DNA, dNTPs to a final concentration of 200 µM, sense and antisense primers to a final concentration of 400 nM, 2.5 U of polymerase and the appropriate buffer. Samples were overlain with mineral oil to prevent evaporation unless the PCR machine was equipped with a heated lid. Reactions were carried out using the Perkin Elmer Thermal Cycler, Stratagene Robocycler 40 and Eppendorf Mastercycler and conditions were as shown below; 25 cycles of amplification were usually carried out.

Denaturation	94°C	30 s (2 min on first cycle)
Annealing	45–65°C	30 s
(annealing temperature depends on primers)		
Polymerisation	72–75°C	1 min + 1 min/kb product
(polymerisation temperature depends on polymerase being used)		

RT-PCR

Reverse transcriptase-PCR (RT-PCR) was carried out in two stages: reverse transcription to produce cDNA from an RNA sample and then PCR to amplify a specific transcript.

Reverse transcription

The reaction was carried out with 1 μg of total RNA. cDNA synthesis was initiated using random primers, which were annealed by heating the sample to 70°C for 10 min and cooling on ice for 2 min. The first strand synthesis reaction contained 500 μM dNTPs, 10 mM DTT, 40 U RNasin, 200 U superscript II reverse transcriptase (GIBCO BRL) and buffer. The mixture was incubated at 42°C, first for 2 min before addition of enzyme and then for 1 hour.

PCR

1 μl of the above reaction containing first-strand cDNA was used as the template in a PCR reaction, essentially as described above (see PCR) using Pfu polymerase.

DNA Sequencing

DNA was sequenced by the dideoxy-mediated chain-termination method using the T7 Sequenase (version 2.0) kit from Amersham. 5 μg of template DNA was denatured by adding 0.1 volume of 2 M sodium hydroxide, incubating at 37°C for 5 min. The mixture was neutralised by adding 0.1 volume of 5 M ammonium acetate and the DNA precipitated by adding 4 volumes of ethanol, mixing, cooling on dry ice for 5 min and centrifuging at 4°C for 5 min. After washing the pellet with 70% ethanol, the DNA was resuspended in water. The specific sequencing primer was annealed to the denatured DNA by heating to 70°C and cooling to 37°C over 20–30 min.

The labelling reaction was carried out at room temperature for 2–5 min. The reaction contained the denatured DNA with the annealed primer, 1 mM DTT, 3.25 U T7 Sequenase polymerase and 0.37 MBq [^{35}S]-ATP (37 TBq/mmol). The mixture was then split into 4 and a quarter was added to each of the four wells, each containing one of the four dideoxy nucleotides, and incubated at 37°C for 5 min. The reaction was stopped by adding 4 µl of STOP solution (formamide loading buffer). The four reactions were then run on a 6% acrylamide/7 M urea gel until the xylene cyanol in the loading buffer (STOP solution) had travelled 75% of the gel length. The gel was then fixed in 10% methanol/10% acetic acid for 10 min, dried for 30 min and exposed to X-ray film at –80°C with an intensifier screen.

In vitro transcription

Synthetic capped mRNA for microinjection was produced by in vitro transcription reactions containing commercially available RNA polymerases (SP6, T3 and T7; Roche). Plasmid DNA encoding the specific gene was linearised with a restriction enzyme site at the 3' end of the gene (either blunt-ended or producing a 5' overhang), cleaned by phenol/chloroform extraction and precipitated to produce the template. The constructs used in this thesis are shown below (Table 2.1).

Reactions contained 5 µg of linearised template, 10 mM DTT, 100 U RNasin, 1 mM each of ATP, CTP and UTP, 100 µM GTP, 500 µM cap structure [m7G(5')ppp(5')G], 50 U of RNA polymerase plus 1 x transcription buffer. This was incubated for 30 min at 37°C, when more GTP was added to bring the concentration up to 500 µM and the reaction was incubated at 37°C for a further 1 hour. The mixture was then treated for 30 min at 37°C with 50 U RNase-free DNase I to remove the template, phenol/chloroform extracted and the supernatant cleaned further by the use of a CHROMASPIN-100 (Clontech) column to remove unincorporated nucleotides. The RNA was then concentrated, if necessary, by adding 0.5 volumes ammonium acetate, 2–3 volumes ethanol, cooling on dry ice for 5 min and centrifuging at 13,000 rpm to precipitate it.

Table 2.1. Constructs used as templates for in vitro transcription reactions

Gene	Plasmid	Linearisation site	Polymerase	Reference
Activin	pSP64T-mβA	*Sma* I	SP6	(Albano et al., 1993)
β -galactosidase	pSP6nucβGal	*Xho* I	SP6	(Smith and Harland, 1991)
derrière	pCS2^{+}-derrière	*Not* I	SP6	(Sun et al., 1999)
Eomesodermin	pSP64T-Eomes-HA	*Sal* I	SP6	(Conlon et al., 2001)
FAST1	pFTX9-XlFast1	*Xba* I	T7	(Howell et al., 1999)
VegT	pSP64T-VegT-HA	*Sal* I	SP6	(Conlon et al., 2001)
VegT-GR	pSP64TBX-VegT-GR	*Sal* I	SP6	This thesis
Xbra	pSP64TBX-Xbra-HA	*Sal* I	SP6	(Tada et al., 1997)
Xnr2	pXnr2	*Sma* I	SP6	(Jones et al., 1995)

In vitro translation

Proteins were produced by in vitro translation reactions, using rabbit reticulocyte lysate (Promega). Reactions contained 400 ng synthetic capped RNA, 8 µl reticulocyte lysate, 20 U RNasin, 50 µM amino acids (–Met), and 0.185 MBq [^{35}S]-Met (37 TBq/mmol). The reaction was incubated at 30°C for 70 min and then labelled proteins were separated on a 10% SDS-PAGE gel. Gels were run at 150 V for 45 min, fixed in 10% methanol/10% acetic acid, dried and exposed to X-ray film at room temperature.

Gel mix	*RESOLVING*	*STACKING*
30% acrylamide	3.3 ml	1.6 ml
Tris-HCl buffer	2.5 ml (1.5 M pH 8.8)	1.25 ml (1M pH 6.8)
10% SDS	0.1 ml	0.1 ml
10% APS	0.1 ml	0.1 ml
TEMED	0.004 ml	0.01 ml
H_20	4.0 ml	6.9 ml

Site-directed mutagenesis

All mutagenesis was performed using a two-step PCR-based approach. Oligonucleotides were designed and contained the mutations to be made and at least 15 base-pairs of sequence on either side (5' and 3') to ensure efficient hybridisation (Fig. 2.1 **P2** and **P3**). Two overlapping products were produced using these primers and primers outside the insert region (Fig. 2.1 **P1** and **P4**). These products were then pooled and a further round of PCR using just the most 5' and 3' primers (Fig. 2.1 **P1** and **P4**) was used to produce the final mutagenised insert (Fig. 2.1).

ΔTbs primers

M1 → GGA TGA GGG ACT GTT G**G**C A**T**C **G**CT CTC CTG ACT CCA G

M1 ← CTG GAG TCA GGA GAG **C**G**A** TG**C** CAA CAG TCC CTC ATC C

M2 → GTG GAT GAG AAC AGA GA**A** G**C**G T**A**T GAC ATA GGT GAT TG

M2 ← CAA TCA CCT ATG TCA **T**AC **G**C**T** TCT CTG TTC TCA TCC AC

Area spanning the T-box site is underlined. The consensus T-box site is the 8 base-pair sequence TCACACCT as defined by Conlon et al. (2001). Base changes are in **bold**.

ΔFAST primers

ΔF→ GGA ATG GGT CTA ATT AA**G** ATG CAC AT**C** TAT ATT CTG TGG ATG

ΔF← ATC CAC AGA ATA TA**G** ATG TGC AT**C** TTA ATT AGA CCC ATT

Fast sites are underlined. The Fast site is an 8 base-pair site with the consensus AATNNACA (Labbe et al., 1998; Zhou et al., 1998). Base changes are in **bold**.

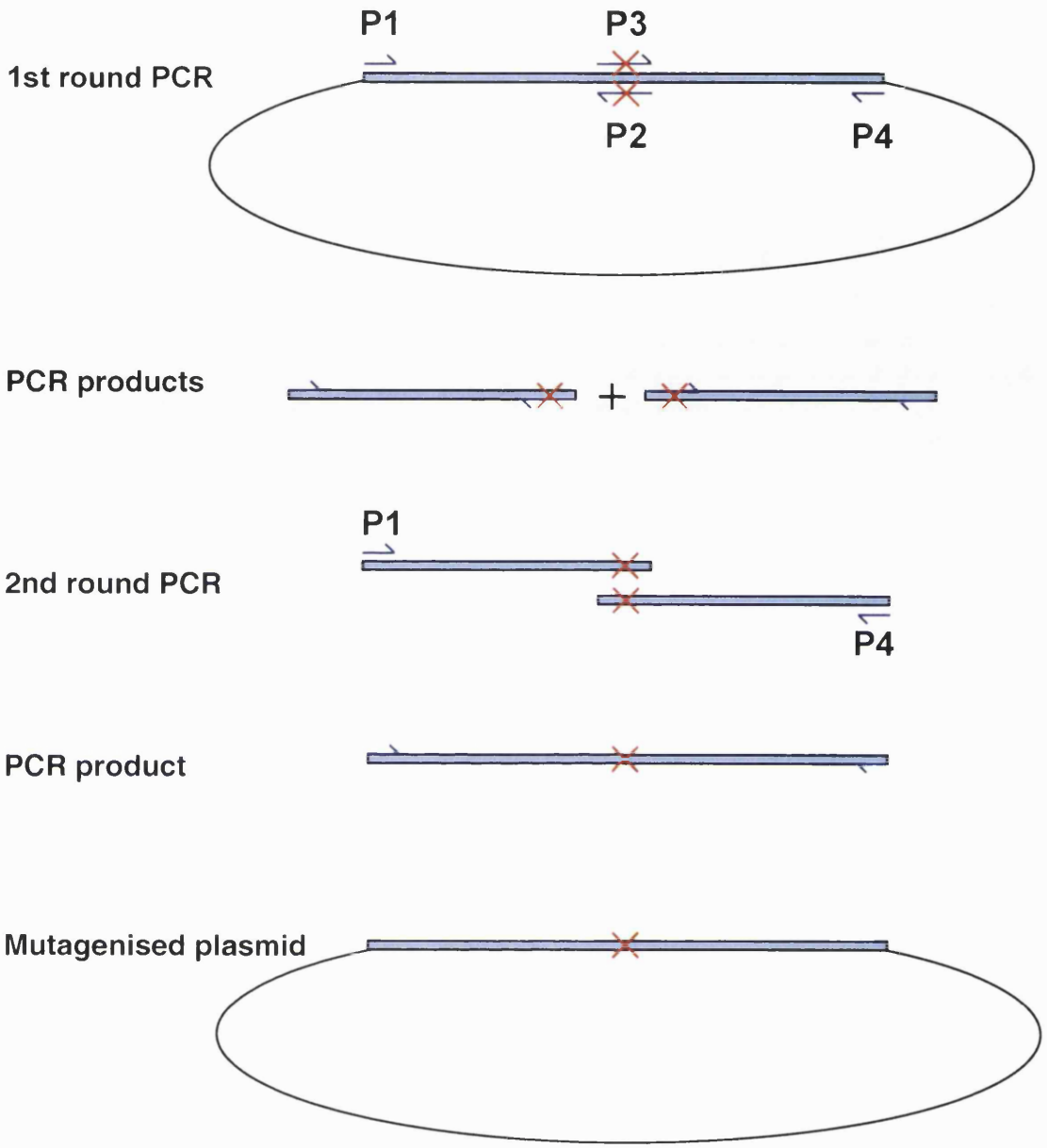

Fig. 2.1. Site-directed mutagenesis. Mutations were introduced into plasmids by a two-step PCR strategy. Primers were designed that contained the mutations to be introduced (red cross) and at least 15 base-pairs of sequence both on the 3' and 5' ends (**P2** and **P3**). The first round PCR creates two products, overlapping at the mutagenesis site, which are then used as the template for a subsequent PCR. This PCR, using the most 5' and 3' primers (**P1** and **P4**), creates the final mutagenised insert, which is then cloned back into an expression vector.

Library screening

A *Xenopus* genomic library in λFIXII (Stratagene) was screened using a probe consisting of the first 356 bp of the *derrière* ORF.

Probe labelling

A 356 bp *Hae* III fragment was labelled with [^{32}P]-dCTP (~110 TBq/mmol) by random priming with Klenow enzyme using the Megaprime labelling kit (Amersham). The fragment was gel-purified and 50 ng was annealed to random nonamer primers by heating to 95°C for 5 min and cooling to room temperature for 2 min. This was used in a 100 µl reaction containing 4 U Klenow enzyme, 1 x labelling buffer and 3.7 MBq [^{32}P]-dCTP, and was incubated at 37°C for 45–60 min. The probe was purified on a G-25 sephadex column and 1 µl was counted in a scintillation counter. 10^6 cpm/ml was used in the hybridisation reaction.

Hybridisation

Library filters were washed in 500 ml 6 x SSC for 10 min, which was replaced by 500 ml ***hybridisation buffer*** and incubated at 60°C for 2 hours before addition of probe. The filters were then incubated overnight at 60°C. After the overnight incubation, the filters were washed twice for 15 min at room temperature and twice for 30 min at 65°C in 500 ml 2 x SSC/0.1% SDS. After washing, the filters were wrapped in Saran-wrap and exposed to X-ray film at -80°C overnight. Positive plaques were picked into 500 µl SM buffer containing 20 µl $CHCl_3$.

Second-round and third-round screenings were carried out by plating dilution series of 'phage (10^{-1}–10^{-4}) with competent bacteria, selecting the plates where plaques were evenly spaced, preparing filters by transferring 'phage particles to nylon membranes and screening by hybridisation as above. Serial dilutions of 'phage were prepared, 10 µl of each was added to 700 µl competent MRA bacteria (see ***Preparation of DNA from bacteriophage*** below) and incubated for 20 min at 37°C. This mixture was then added to 7 ml molten top agar (0.7% agarose in 10 mM $MgSO_4$) and this was plated on top of a set LB-agar plate, allowed to set and then incubated at 37°C for 6–8 hours. Circular nylon membranes were then placed on the selected plates and incubated for 3 min to pick up 'phage particles.

The DNA was then denatured by treating the membrane with 0.5 N NaOH/1.5 M NaCl for 3 min followed by neutralisation with 1 M Tris-HCl (pH 7)/1.5 M NaCl for 3 min. The DNA was then UV-crosslinked to the membrane using a UV STRATALINKER 1800 (Stratagene) and left to dry. For each plate, duplicate filters were prepared to make identification of positive plaques easier. The filters were then screened as described above for the first round.

Four lambda clones (λd1–4) were selected, three of which contained the first exon and upstream sequence. Restriction digests and Southern blotting (see below) of λd1 identified a 2 kb *Xba* I fragment, which was sub-cloned into pBluescript II SK(-) (pBSK) to produce d1.2.1.

Hybridisation buffer

6 x SSC

50 mM NaPi

5 x Denhardt's solution

0.5% SDS

100 µg/ml Torula Yeast RNA

SM buffer

100 mM NaCl

16.7 mM $MgSO_4 \cdot 7H_20$

50 mM Tris-HCl (pH 7.5)

0.01% gelatin

Preparation of DNA from bacteriophage

DNA from lambda bacteriophage was prepared from liquid cultures using the Qiagen Mini Lambda DNA kit. Lambda 'phage were propagated in liquid culture using MRA-competent bacteria. The bacteria were made competent by culturing them at 37°C for 3 hours in LB containing 0.2% maltose and 10 mM $MgSO_4$ and then resuspending in 10 mM $MgSO_4$, diluting to give an OD $_{\lambda=550}$ = 0.5. A single plaque was added to 200 µl MRA competent bacteria and cultured for 10 min at room temperature. Then 10 ml LB containing 5 mM $CaCl_2$ and 10 mM $MgSO_4$ was added and cultured at 37°C for 6–8 hours.

To complete lysis, 100 ml chloroform was added and incubated at 37°C for 30 min. The culture was then centrifuged at 10,000 x *g* for 10 min to remove the bacterial debris. 30 µl buffer ***L1*** was added to the 10 ml supernatant and incubated at 37°C for 30 min to digest bacterial RNA and chromosomal DNA. Then, 2 ml ice-cold buffer ***L2*** was added, gently mixed and incubated on ice for 60 min.

The 'phage particles thus precipitated were centrifuged at 10,000 x *g* for 10 min and the pellet resuspended in 1 ml buffer ***L3***. The 'phage proteins were denatured by adding 1 ml buffer ***L4*** , mixing gently and incubating at 70°C for 10 min, and then precipitated by adding 1 ml buffer ***L5***, mixing and centrifuging twice at 15,000 x *g* for 30 min. The lambda DNA was then purified by passing it over a Qiagen anion-exchange resin and concentrated by isopropanol precipitation.

L1
300 mM NaCl
100 mM Tris-HCl (pH 7.5)
10 mM EDTA
0.2 mg/ml BSA
20 mg/ml RNase A
6 mg/ml DNase I

L2
30% polyethylene glycol
3 M NaCl

L3
100 mM NaCl
100 mM Tris-HCl (pH 7.5)
25 mM EDTA

L4
4% SDS

L5
3M NaOAc (pH 5.5)

Southern blotting

Lambda clones were analysed by restriction digestion and southern blotting. Restriction fragments were separated by agarose gel electrophoresis as described previously (***Agarose gels***). To prepare the DNA in the gel for blotting, the gel was first treated for 45 min with 0.5 N NaOH/1.5 M NaCl to denature the DNA. It was rinsed in deionised water and neutralised by washing twice (30 min and then 15 min) in 1 M Tris (pH 7.4)/1.5 M NaCl. The DNA was transferred to a nylon membrane by capillary transfer with the blotting apparatus set up as shown in Fig. 2.2.

The membrane was prepared by floating on deionised water until wet and it was then soaked in 10 x SSC for at least 5 min. The apparatus was set up as shown in Fig. 2.2. The gel was placed on Whatman 3 MM paper, which was in contact with the transfer buffer (10 x SSC). The membrane was placed on the gel and covered by 3 MM paper and paper towels with a weight to ensure a tight connection between the layers of material. It is important to ensure that there are no bubbles between any of the layers to guarantee the efficiency of transfer.

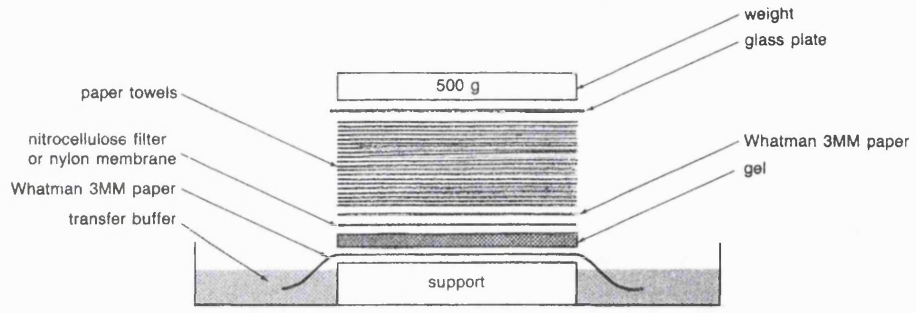

Fig. 2.2. Southern blotting apparatus. Southern blotting was carried out by capillary action. The gel was placed on Whatman 3 MM paper, which was in contact with the transfer buffer (10 x SSC). The membrane was placed on the gel and covered by 3 MM paper and paper towels with a weight to ensure a tight connection between the layers of material. It is important to ensure that there are no bubbles between any of the layers to guarantee the efficiency of transfer. Capillary action then draws transfer buffer through the gel and membrane, transferring the DNA to the nylon support. Taken from Sambrook et al. (1989)

The next day the apparatus was dismantled: the weight, 3 MM paper and paper towels were discarded, the positions of the wells were marked on the membrane, the bottom left-hand corner was cut off the membrane to allow orientation afterwards and the gel was discarded. The membrane was then soaked in 6 x SSC for 5 min, the DNA was cross-linked to the membrane using a UV STRATALINKER 1800 (Stratagene) and left to dry.

Hybridisation was carried out either using the method described above for ***Library screening*** or using ***CHURCH buffer***. Prehybridisation and hybridisation steps were carried out at 65°C using the same probe as that used for library screening. Filters were washed once at room temperature in 2 x SSC/1%SDS for 15 min and then twice at 65°C in 0.2 x SSC/1%SDS for 30 min. The membrane was wrapped in Saran-wrap and exposed to X-ray film for 30–180 min.

CHURCH Buffer

0.5 M $NaHPO_4$ (pH 7.2)

7% SDS

1 mM EDTA

1% BSA

100 µg/ml denatured salmon sperm DNA

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) were carried out as described by Trindade et al. (1999), with minor modifications as described below.

Production of protein

Proteins used in EMSAs were produced either by *in vitro* translation of synthetic RNA or by the production of an embryo extract. pSP64TBX–VegT–HA was linearised with *Sal* I and transcribed with SP6 RNA polymerase and pFTX9–XlFast1 (Howell et al., 1999) was linearised with *Xba* I and transcribed with T7 RNA polymerase. *In vitro* translation reactions were carried out as described previously, except that the [^{35}S]-Met was replaced by 1 mM amino acids (–Cys) and the reaction was scaled-up to include 2 μg of RNA.

To produce an embryo extract for EMSAs, embryos (either uninjected or injected with 200 pg activin and 1.5 ng XFast1) were taken at stage 10.5 and lysed on ice in ***E50*** (10 μl per embryo) by pipetting up and down repeatedly. The lysed embryos were centrifuged at 13,000 rpm at 4°C for 4 min and the supernatant was removed to a new tube with as little lipid as possible. 0.33 volumes of E50 containing 75% glycerol was then added, to make the extract contain 25% glycerol, and the extract was aliquoted and frozen at –80°C. This is the low-salt extract. To produce the high-salt extract, the pellet from the low-salt extract was washed by resuspension in E50 and centrifugation and then extracted by resuspension in ***E200*** followed by centrifugation as above. The high-salt extract was made 25% in glycerol by the addition of 0.33 volumes of E200 + 75% glycerol, aliquoted and frozen at –80°C.

E50/200

20 mM HEPES-KOH (pH 7.6)

50/200 mM KCl

10 mM sodium β-glycerophosphate

5 mM $MgCl_2$

0.1 mM EDTA

0.1 mM Spermine

Buffers are filter sterilised. 2 mM DTT and protease inhibitors (Roche, Complete tablets) are added just before use.

Probe labelling

Probes for EMSA were made by end-labeling annealed oligonucleotides with [^{32}P] dCTP. Oligonucleotides including sequence that spans the region of the binding site and an overhang were mixed in equimolar amounts and denatured for 10 min at 70°C, then annealed by cooling slowly to room temperature. Wild-type and mutant oligonucleotides for competition experiments were annealed in this way, but left unlabelled. Probe oligonucleotides were then labeled by 3' filling with [^{32}P] dCTP (~110 TBq/mmol) using the Klenow fragment (Promega). Reactions were carried out at room temperature for 25 min and the probe was then purified on a G-25 Sephadex column. The top strands of the probes used are shown below:

Tbs1 – GATCCGCCTCGACGCGGACTGTTGACACCTCTCTCG

TbsM1 – GATCCGCCTCGACGCGGACTGTTG**G**CA**T**C**G**CTCTCG

Tbs2 – GATCCTCGACCGTGCCAGAGAGGTGTCTGACATAG

TbsM2 – GATCCTCGACCGTGCCAGAGA**A**G**C**GT**A**TGACATAG

Area spanning the T-box site is underlined. Base changes are in bold.

FAST – GGTGGTCTAATTAACATGCACATGTATATTCTGTG

2MFAST – GGTGGTCTAATTAA**G**ATGCACAT**C**TATATTCTGTG

Areas spanning the FAST sites are underlined. Base changes are in bold.

Binding reactions

Binding reactions contained 2 µl of *in vitro* translated protein, 1 x binding buffer, 50 ng pBluescriptII plasmid, 1 µg poly-dIdC, 100,000 cpm [^{32}P]-dCTP-labelled probe and, when indicated, a 100-fold molar excess of unlabelled specific or mutant competitor, in a total volume of 12 µl. The 1x binding buffer contained 50 mM KCl, 1 mM EDTA, 100 µg/ml BSA, 1 mM DTT, 10% glycerol, 1 µg poly-dIdC and 20 mM Hepes pH 7.9.

Complexes were allowed to form at room temperature for 15 min after addition of probe. For antibody shift analyses, 1 µg of monoclonal anti-HA antibody (Roche), was added to the reactions and incubation was continued for an additional 10–15 min. Samples were then loaded onto a 6% acrylamide gel containing 0.5 x TBE and 1% glycerol and run for 2.5–3 hours at 150 V.

Binding reactions with embryo extracts contained approximately 10 μg extract (total protein), 2 μg poly-dIdC, 17.5% glycerol, 110 mM KCl, 10 mM HEPES-KOH, 5.5 mM $MgCl_2$, 5 mM sodium β-glycerophosphate, 0.05 mM EDTA, 0.05 mM Spermine, 100,000 cpm [^{32}P]-dCTP-labelled probe and, when indicated, a 100-fold molar excess of unlabelled specific or mutant competitor, in a total volume of 20 μl. Reactions were incubated at room temperature for 15 min and complexes were resolved on 1.5-mm-thick, 5% (40:1) acrylamide gels containing 2.5% glycerol and electrophoresed in 0.5 x TBE at 150 V for 3.25 h. For antibody shift analyses, 1 μl of anti-Fast1 (gift from Caroline Hill) was added to the reactions and incubation was continued for an additional 10–15 min.

Gels were fixed in 10% methanol/10% acetic acid, dried for 1–2 hours and exposed to X-ray film at –80°C.

Vectors and constructs

VegT–GR

The *VegT–GR* construct was generated by fusing the coding region of *VegT* to a fragment encoding the hormone-binding domain of the human glucocorticoid receptor (hGR) by PCR (Fig. 2.3). This fusion replaces the stop codon of *VegT* with two amino acids (Ala Ser) generated by the insertion of a *Nhe* I site (see below). Amino acids 1–437 of *VegT* were amplified from pCS2–*VegT* (Zhang and King, 1996) by PCR using the following primers:

Not I

Sense – 5' TAA AGC GGC CGC GGA ATG AGA AAC TGC TGT CGG 3'

Antisense – 5' G CCC GCT AGC CCA ACA GCT GTA GGG GAA GAG 3'

Nhe I

This fragment was cloned into the *Not* I and *EcoR*V sites of pBSK using the 5' *Not* I site introduced by the PCR primers (underlined) and the blunt 3' end to produce pBSK–*VegT*. hGR was amplified in a similar way using the following primers:

Nhe I

Sense – G CCC GCT AGC TCT GAA AAT CCT GGT AAC AAA

Antisense – C GGG CTC GAG TCA CTT TTG ATG AAA CAG AAG.

Xho I

This was then cloned into the *Nhe I* and *Xho I* sites of pBSK–*VegT*, producing pBSK–*VegT–GR*. The fragment encoding *VegT–GR* was then cloned into the *Not* I and *Xho* I sites of the vector pSP64TBX (a modified form of pSP64T, a gift from M. Tada) and designated pSP64TBX–*VegT–GR*.

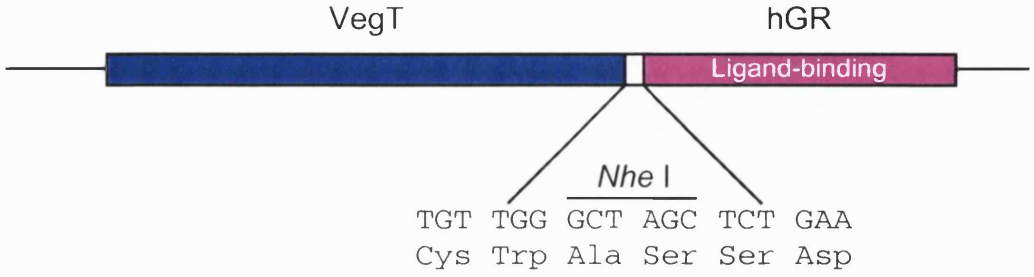

Fig. 2.3. Construction of *VegT–GR*. The hormone-binding domain of the human glucocorticoid receptor (hGR) amino acids 512-777 was fused to the carboxy-terminus of *VegT* (1-437) to generate VegT-GR. This fusion replaces the stop codon of VegT with two amino acids (Ala Ser) generated by the insertion of a *Nhe*I site (see Materials and methods). VegT is shown in blue. The hormone-binding domain of hGR is shown in magenta. The sequence of the joining region has been enlarged to show the addition of two amino acids (Ala Ser) to the fusion protein.

In situ hybridisation probes

All probes used were made from the plasmids shown below (Table 2.2).

Table 2.2. *in situ* hybridisation probes

Gene	Plasmid	Linearisation site	Polymerase	Details
derrière	pBSK-der	*Not* I	T7	*derrière* ORF in pBSK (this thesis)
VegT	pCS2$^+$-VegT	*BamH* I	T7	(Zhang and King, 1998)
Xbra	pXT1	*Bgl* II	T7	(Smith et al., 1991)
mgfp5	p BSK-GFP	*Nco* I	T7	(Zernicka-Goetz et al., 1997)

RNase protection probes

All probes used were made from the plasmids shown below (Table 2.3). The constructs for *derrière*, *PAPC*, *Xnr1*, and the *derrière* 5' region were made by PCR, using the primers shown, either from stage 10.5 embryo RNA or from already existing plasmids.

Table 2.3. RNase protection probes

Gene	Protected size (bp)	Linearisation site	Polymerase	Details
Bix4	336	*EcoR* V	T7	(Tada et al., 1998)
derrière	165	*Not* I	T7	→ AGT ACT CAT TCA TCA CCT C ← CCT TAG CAT TTC CGT CAG T
PAPC	200	*Not* I	T3	→ CTG GGA TAC CTT TTG TTG GAG ← CAA ATC TTG GGC TTG TAC CTC
Xnr1	373	*Hind* III	T3	→ CCA ACC ATA TTG GCT TTC CCA G ← CAA TGG ACA CTC CAC TCG TTC GG
Xnr2	248	*Hind* III	T3	248 bp Dpn I fragment
Xnr3	205	*Xho* I	T3	205 bp Hind III fragment
Xnr4	282	*Xho* I	T3	282 bp Acc I fragment
derrière 5'	121	*Nde* I	T7	→ T7 ← AGG AGC ATT CTT TCC TGG AAG
ODC	91	*Bgl* II	T7	(Isaacs et al., 1992)

Constructs for transgenesis

A green fluorescent protein (GFP) cDNA (Zernicka-Goetz et al., 1997) was cloned into the *Xho* I/*Xba* I sites of pGL3–promoter (Promega), which removes the luciferase gene and the SV40 minimal promoter, to produce pGL3–GFP (see Fig. 2.4). All the *derrière* promoter fragments created for luciferase assays were also cloned into pGL3–GFP.

Constructs for luciferase assays

Luciferase assays were carried out using the Dual Luciferase kit (Promega) using the supplied vectors. For firefly luciferase the pGL3-basic vector was used, which contains the firefly luciferase gene downstream of a multi-cloning site, and promoter fragments to be tested were cloned into this. The constructs used were pGL3–d851, pGL3–d441 and pGL3–d139 (which contain 851, 441 and 139 base-pairs, respectively, of the promoter upstream of the transcription start site) and pGL3–ΔTbs, which is pGl3–d851 with a deletion of 126 base-pairs that removes both the T-box sites and the Fast sites. Mutations in both the T-box sites and the Fast sites were generated as described above (see ***In vitro mutagenesis***) and subcloned back into pGL3–basic. For the internal Renilla luciferase control, both pRL–SV40 and pRL–TK were used.

Constructs for expression in tissue culture

For expression of VegT protein in tissue culture cells, pcDNA3.1–VegT was used (Conlon et al., 2001). This drives expression of VegT protein from a CMV promoter and contains both a V5 epitope tag and a 6 x His tag.

Construction of GFP–*derrière*

A GFP-tagged version of derrière was constructed in the manner described by Gonzalez-Gaitan et al. (1999). This was done by a PCR-based strategy, using chimeric oligonucleotides as primers to create overlapping sequences which were then fused together with a further round of PCR. See ***Chapter 6*** for details. The primers used are shown below.

P1 – 5′ TAAAGCGGCCGCAACATGGCAGAGTTGTGGCTATCACTTTCTTGC 3′

P2 – 5′ GCTCACCATCTCGAGGCTTGGGGTTGGAGGTGATG 3′

P3 – 5′ CCCCAAGCCTCGAGATGGTGAGCAAGGGCGAGGAGC 3′

P4 – 5′ GCAGATATTCTCGAGCTTGTACAGCTCGTCCATGCC 3′

P5 – 5′ CTGTACAAGCTCGAGAATATCTGCAAGAAAAGGAGATTG 3′

P6 – 5′ AACTGCAGAACTCACTTGCAACCACACTCATCCACTACCAT 3′

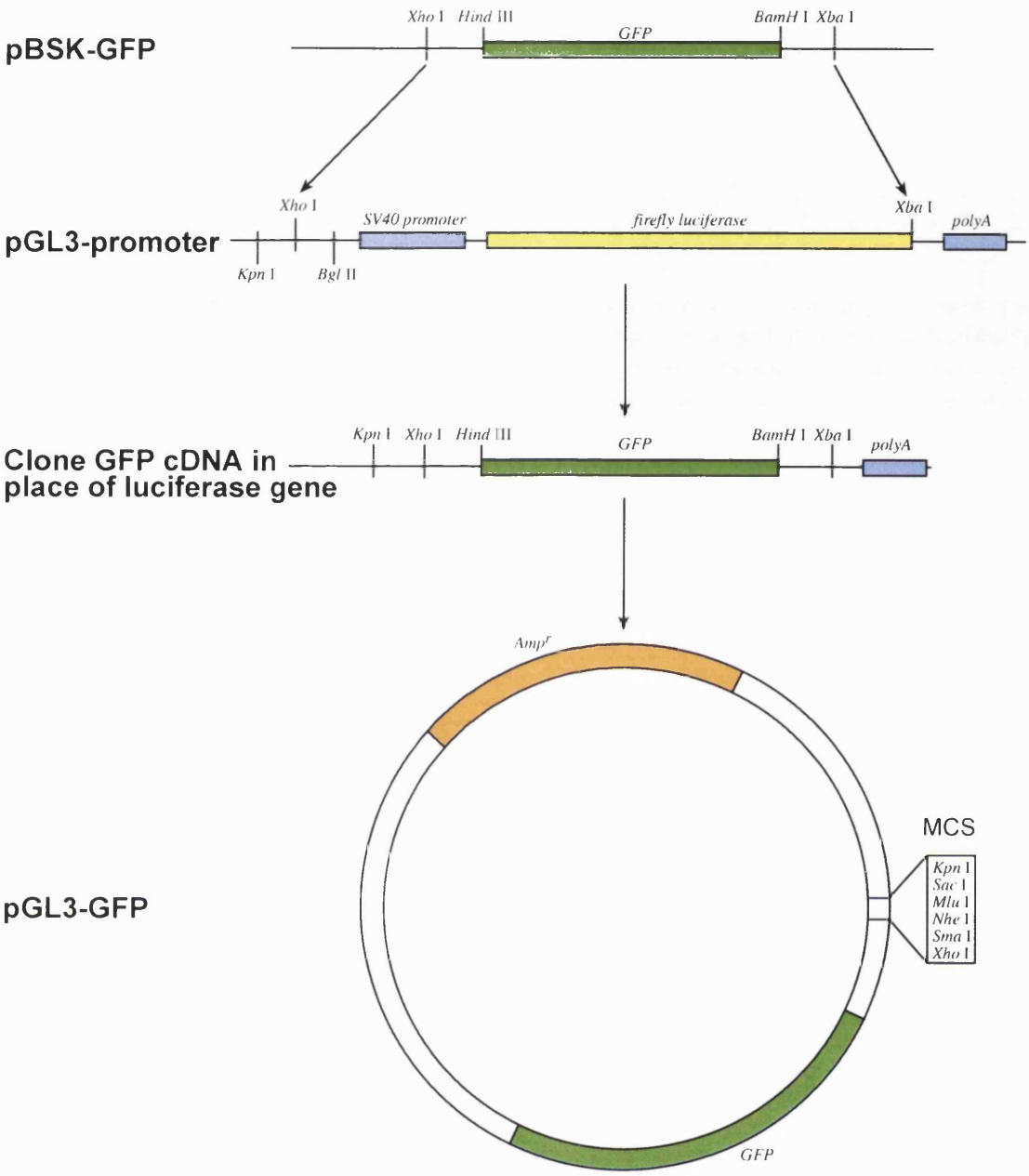

Fig. 2.4. Construction of pGL3–GFP. The GFP cDNA was cloned into pBluescript II. This was digested with Xho I and Xba I along with pGl3-promoter (Promega) to remove the firefly luciferase gene and the SV40 promoter. The GFP fragment was then cloned into the cut pGL3-promoter vector to produce pGL3-GFP. Promoter fragments used in transgenics were then cloned into the multi-cloning shown (MCS). GFP is shown in green, firefly luciferase is shown in yellow and the ampicillin resistance gene of pGL3-promoter is shown in orange.

Xenopus embryos and microinjection

Obtaining embryos

Xenopus embryos were obtained by *in vitro* fertilization (Smith and Slack, 1983). Briefly, adult *Xenopus* females were induced to ovulate by an injection of 150 units of Folligon (pregnant mares' serum), up to a week before collection, followed by an injection of 500 units of human chorionic gonadotrophin 12–16 hours before eggs were required. Eggs were collected from 1 x MMR solution into which the induced females were placed. The eggs were fertilised by rubbing macerated testis, dissected from a sacrificed male, over them. Males were sacrificed by injection of 3-aminobenzoic acid ethyl ester (MS-222) and subsequent destruction of the circulatory system. Testes were dissected and kept in 60% L-15 media (Sigma) at 4°C for up to a week. After fertilisation, the embryos were flooded with 10% normal amphibian medium (NAM; Slack, 1984). After 30 min the embryos were dejellied using 2% cysteine hydrochloride (pH 7.9–8.1) and staged according to Nieuwkoop and Faber (1967).

Micro-injection and culture of *Xenopus* embryos

After dejellying, embryos were transferred to 1% agarose coated dishes containing 4% Ficoll in 75% NAM. Embryos were injected using a Narishige IM 300 compressed nitrogen gas microinjector using needles made from glass capillaries and with the help of a micromanipulator (Micro Instruments). Embryos were injected at the one-cell to two-cell stage with RNA/DNA dissolved in water, as described by Smith (1993). Typical injection volumes were 5–10 nl, which was estimated by injecting a drop of water into oil and measuring the diameter using a graticule. By treating the drop as a sphere it is possible to calculate the volume of the drop (A sphere of diameter 267 μm has a volume of 10 nl).

Animal cap dissection and recombinations

For animal cap assays, animal pole explants (known as animal caps) were dissected from embryos at stage 8.5. The vitelline membrane was removed using number 5 watchmaker forceps and a square piece of tissue was dissected from the animal-most quarter of the embryo using the forceps as scissors. Explants were cultured in 75% NAM in 1% agarose coated dishes.

When dexamethasone (DEX) or cycloheximide (CHX) was included in the culture medium, explants were cultured in 75% NAM containing 0.1% bovine serum albumin (BSA). DEX (Sigma) was dissolved in ethanol at 2 mM and then diluted to a final concentration of 1 μM in 75% NAM + 0.1% BSA. CHX was applied at a concentration of 10 μM.

Recombination experiments were carried out as described by Jones et al. (1996). Animal caps were cut as described above and two individual caps were then juxtaposed – a "source" cap from an embryo injected with a test mRNA and an Fluorescein-injected "responding" cap – and allowed to heal for at least 5–10 min. The recombinants were cultured in 75% NAM until sibling embryos reached stage 10.5, at which point they were fixed in MEMFA and processed by *in situ* hybridisation for expression of *Xbra* RNA (***In situ hybridisation***).

Incorporation of [^{35}S]-Met

To assess the effectiveness of CHX treatment, the inhibition of protein synthesis was assayed by the incorporation of [^{35}S]-Met. Groups of 10 control animal caps were cultured in 75% NAM containing 0.1% BSA and 0.37 MBq/ml [^{35}S]-Met, either with or without CHX. These caps were collected and frozen at stage 10.5 in the same manner as sample caps. To assay the incorporation of radiolabel, the caps were disrupted in 200 μl ***extraction buffer*** by vigorous vortexing. 250 μl BSA (1 mg/ml) and 1 ml 10% ice-cold TCA were added, the mixture vortexed again and incubated on ice for 20 min.

The samples were then passed through a Whatman GF/C glass-fibre filter. The filter was washed five times with 2 ml 10% TCA and then three times with 3 ml ethanol. The filter was allowed to dry at room temperature for 20 min and was then placed in 1 ml of scintillation fluid and counted in a scintillation counter. The values for the two samples were then used to calculate a percentage inhibition of translation. Only experiments where inhibition was over 90% were used in RNase protection analyses.

Extraction buffer

20 mM Tris•HCl (pH 7.4)

100 mM NaCl

1 mM EDTA

0.5% SDS

In situ hybridisation

Whole-mount *in situ* hybridisation was performed as described by Harland (1991), except that BM purple was used as substrate and RNase treatment was omitted.

Probe labeling

Probes were labelled with digoxygenin (DIG)-11-UTP (Roche) in a standard *in vitro* transcription reaction using the appropriate RNA polymerase (see ***Vectors and constructs***). Reactions contained 2.5 µg/ml linearised DNA template, 400 mM DTT, 1 x DIG-labelling mix (Roche), 40 U RNasin, 50 U specific RNA polymerase and buffer. The reaction was incubated at 37°C for 2 hours, then treated with 20 U RNase-free DNase I at 37°C for 15 min to digest the template. The resulting labelled RNA was then purified over CHROMASPIN-100 columns to remove unincorporated nucleotides. Probes were used at 500 ng/ml in ***hybridisation buffer***.

Hybridisation

Embryos were processed in 5 ml glass vials. They were fixed at the appropriate stage in MEMFA for 1 hour at room temperature. Before fixation a hole was poked in the blastocoel to prevent non-specific trapping of probe or antibody. After fixation the embryos were washed twice in 100% ethanol or methanol and then stored at –20°C.

Embryos were first rehydrated by incubation for 2–5 min in solutions of decreasing percentage alcohol until they were completely rehydrated (75% EtOH, 50% EtOH, 25% EtOH). They were then washed three times in PBST (1 x PBS + 0.1% Tween-20) for 5 min each and incubated for 10 min in 5 µg/ml proteinase K in PBST. Embryos were then rinsed twice in 0.1 M triethanolamine (pH 7–8) to which acetic anhydride is also added twice (2.5 µl/ml triethanolamine) and incubated for 5 min each time. This treatment blocks charged groups within the tissue and decreases background (Hayashi et al., 1978). The embryos were then washed twice in PBST for 5 min, refixed in MEMFA for 20 min at room temperature and washed five times in PBST for 5 min. The PBST was then replaced by 500 µl hybridisation buffer and embryos were then incubated at 60°C for 2 hours to prehybridise. After 2 hours the DIG-labelled probe was added to hybridisation buffer at a concentration of 500 ng/ml and incubated at 60°C overnight.

Hybridisation buffer

50% formamide

5 x SSC

1 x Denhardt's solution

5 mM EDTA

100 µg/ml heparin

1 mg/ml Torula RNA

0.1% Tween-20

0.1% CHAPS

Store at –20°C

Washes

The embryos were then washed to remove any unbound probe. All washes were carried out at 60°C unless stated otherwise. Embryos were washed for 10 min in 3:1 (5 x SSC/50% Formamide):(2 x SSC) and then in 1:3 (5 x SSC/50% Formamide):(2 x SSC). Then they were washed twice in 2 x SSC/0.1% CHAPS for 30 min and twice in 0.2 x SSC/0.1% CHAPS for 30 min.

Antibody incubation and washes

Antibody incubations were performed in MABT (100 mM maleic acid, 150 mM NaCl, 0.1% Triton). Embryos were first washed twice in MABT for 5 min at room temperature and then blocked for 1 hour in MABT + 2% blocking reagent (Roche) + 20% heat-treated lamb serum. The blocking buffer was then replaced with the same solution containing a 1:4000 dilution of anti-digoxygenin antibody Fab fragments coupled to alkaline phosphatase (Roche) and incubated with shaking at 4°C overnight. Then the embryos were washed five times in MABT for 1 hour each at room temperature to remove any unbound antibody.

Chromogenic reaction

Embryos were rinsed twice for 5 min in alkaline phosphatase (AP) Buffer. The AP buffer was then replaced with BM Purple substrate (Roche) and the reaction was developed until the desired amount of staining was reached. The embryos were then fixed in MEMFA to stop the reaction and preserve the stain.

Alkaline phosphatase buffer

100 mM Tris (pH 9.5)

50 mM MgCl2

100 mM NaCl

0.1% Tween-20

5 mM levamisol

AP Buffer should be made fresh just before use, as levamisol is not stable.

RNase protection assays

Probe labeling

RNase protection probes were made by producing radiolabelled antisense RNA by *in vitro* transcription. Probes were used that are specific for *derrière*, *PAPC*, *Xnr1*, *Xnr2*, *Xnr3*, *Xnr4*, *derrière* 5' region and *ornithine decarboxylase* (*ODC*) (Isaacs et al., 1992) as described previously (see ***Vectors and constructs***). The reaction mixture is shown below:

DNA (1 μg/μl)	1 μl
5 x transcription buffer	4 μl
DTT (10mM)	2 μl
RNasin (40 U/μl)	1 μl
3 rNTP (ATP, GTP, UTP 5 mM each)	2 μl
200 μM UTP	1.2 μl
[^{32}P]-UTP	5 μl
RNA polymerase (T7 or T3)	1 μl
Water	2.8 μl

The reaction mix was incubated at 37°C for 1.5 hours. 10 U RNase-free DNase I was then added and incubated at 37°C for 20 min to digest the template. 20 μl formamide loading buffer was then added to the probe mixture, which was heated to 85°C for 5 min to denature the probe and then loaded on a 7M urea/6% polyacrylamide gel. The gel was exposed to X-ray film for 30–60 s and the resulting autoradiograph was used to facilitate cutting of the radiolabelled probe band from the gel.

The gel slice was then placed in 500 µl ***elution buffer***, macerated with a pipette tip and left to elute overnight. The eluate was kept and the probe was ethanol precipitated from this and resuspended in 50 µl water. 1 µl was taken for scintillation counting and 500,000 cpm was used per sample.

Elution buffer

0.5 M NH_4Ac

10 mM $Mg(Ac)_2$

0.1% SDS

1 mM EDTA

RNA isolation

RNA was extracted from animal caps by the acid guanidinium thio-cyanate/ phenol/ chloroform method (Chomczynski and Sacchi, 1987). 15 animal caps were disrupted by vortexing for 30 s in 500 µl ***solution D***. Then 50 µl 2 M NaOAc (pH 4), 500 µl phenol and 100 µl chloroform was added to the mixture, vortexing for 30 s after the addition of each solution. This was then incubated on ice for 15 min and centrifuged at 13,000 rpm at 4°C for 20 min. The aqueous layer was removed and precipitated with an equal volume of isopropanol (see ***Precipitation of nuclei acids***). The pellet was then resuspended in 10 µl water after washing with 70% ethanol.

Solution D

4 M guanidinium thio-cyanate

25 mM sodium citrate

0.5% sarkosyl

0.7% β-mercaptoethanol (added just before use)

RNase Protection procedure

RNase protection analysis was performed essentially as described by Jones et al. (1992). 20 µl hybridisation mix was added to each sample so that the final volume for hybridisation was 30 µl. The hybridisation mix contains 50% formamide, 1 x PIPES buffer and 500,000 cpm each of radiolabelled probes. Each reaction contains 500,000 cpm ODC loading control probe and 500,000 cpm probe for the gene being assayed. The mix was heated to 85°C for 5 min to denature the nucleic acid and then incubated at 55°C overnight to allow for hybridisation.

After this, single-stranded RNA was digested by adding 350 µl ***RNase digestion buffer*** and incubating for 30 min at room temperature. The RNase was then destroyed by adding 20 µl 10% SDS and 3 µl proteinase K (14 mg/ml; Roche) and incubating for 20 min at 37°C. RNase digestion was carried out using RNase T1 alone for all probes. The mixture was phenol/chloroform-extracted and ethanol-precipitated and resuspended in 5 µl formamide loading buffer.

The samples were then denatured at 85°C for 5 min and loaded onto a 7 M urea/6% polyacrylamide gel. This was run at a constant 50 W until the xylene cyanol in the formamide dye had run two-thirds of the length of the gel (approximately 2 hours). The gel was fixed in 10% methanol/10% acetic acid, dried for 30–60 min and exposed to X-ray film at –80°C with an intensifier screen.

RNase digestion buffer
10 mM Tris-HCl (pH 7.4)
5mM EDTA (pH 8.0)
300 mM NaCl
10 U/ml RNase T1 (added just before use)

10 x PIPES buffer
0.4 M PIPES (pH 6.4)
4 M NaCl
10 mM EDTA

Mapping transcription start site

To map the transcription start site of *derrière*, an RNase protection assay was used. The genomic sequence isolated was used to produce a probe beginning 92 base-pairs downstream of the start codon and therefore spanning the transcription start site. This probe was used on RNA prepared from embryos over-expressing VegT in a standard RNase protection assay (see above). At the same time a sequencing reaction was carried out on the genomic DNA, starting at the same point on the DNA as the RNase protection probe. The samples were run together on a 7M Urea/6% polyacrylamide gel. The size of the protected fragment can then be determined with reference to the sequencing reaction and this can be used to calculate the transcription start site.

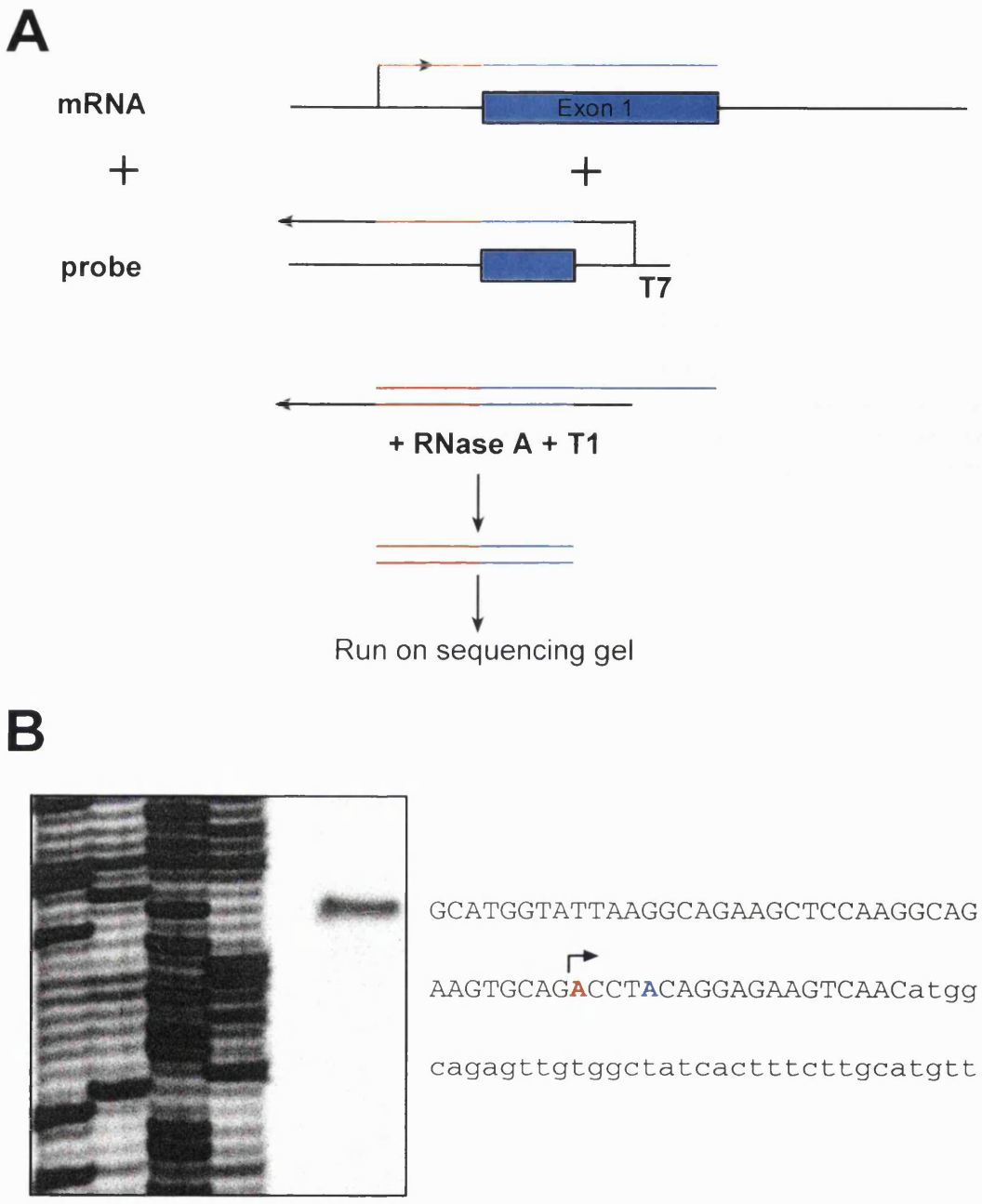

Fig. 2.5. Mapping transcription start site. (A) Experimental scheme. The genomic fragment d1.2.1 was used to make an antisense probe containing 92 base-pairs of exon and sequence further 5'. This was used in a RNase protection assay against stage 10.5 RNA. The protected fragment is run on a sequencing gel next to a sequencing reaction starting at the 3' end of the probe. (B) The size of the protected fragment is determined in comparison to the sequencing reaction and therefore the transcription start site is calculated. The base-pair in the sequence that corresponds to the weight of the protected fragment is shown in blue and the transcription start site in red

Sequencing primer contains 10 nucleotides not contained within the target sequence:

= 2 x dATP + 4 x dCTP + 3 x dGTP + 1 x dTTP

= (2 x 251.2) + (4 x 227.2) + (3 x 267.2) + (242.2)

= 2455

Sequence up to protected fragment contains 107 nucleotides:

= 33 x dATP + 19 x dCTP + 28 x dGTP + 27 x dTTP

= (33 x 251.2) + (19 x 227.2) + (28 x 267.2) + (27 x 242.2)

= 26627.4

$\Rightarrow$ Total weight of DNA = 26627.4 + 2455 = 29082.4

$\therefore$ Weight of protected fragment $\approx$ 29082.4

Weight of RNA with same sequence as DNA above:

= 33 x ATP + 19 x CTP + 28 x GTP + 27 x UTP

= (33 x 267.2) + (19 x 243.2) + (28 x 283.2) + (27 x 244.2)

= 27961.4

Add nucleotides until weight matches:

+ ATP = 27961.4 + 267.2 = 28228.6

+ GTP = 28228.6 + 283.2 = 28511.8

+ GTP = 28511.8 + 283.2 = 28795.0

+ UTP = 28795.0 + 244.2 = 29039.2

$\Rightarrow$ Transcription start site is located 19 base-pairs upstream of the start codon (see Fig. 2.5).

Luciferase assays

Luciferase assays used the Promega Dual Luciferase kit. *Xenopus* embryos were injected at the one-cell stage with 20 pg pRL-SV40/TK (Promega), 20 pg firefly reporter and 1 ng *VegT*, *Xbra*, *Eomes* or *β-galactosidase* (β-gal) RNA. Animal caps were dissected at stage 8.5 and cultured until sibling embryos reached stage 10.5. Samples were assayed in triplicate using 5 caps per replicate. Each sample of 5 caps was homogenised in 10 µl per cap of 1 x passive lysis buffer (Promega) and the luminescence of 10 µl was measured. For each sample, the firefly luciferase values were normalised by dividing by the corresponding renilla luciferase value.

Transgenesis

Transgenic *Xenopus* embryos were generated essentially as described by Sparrow et al. (2000), a simplified version of the protocol of Kroll and Amaya (1996). Sparrow et al. (2000) determined that typically, 1–15 copies of the reporter gene were integrated into the host genome at 1–6 sites, with 1–6 copies at a single site.

Preparation of Sperm

Testes were dissected from male *Xenopus laevis* and the external membrane of the testes was removed along with as much of the surrounding blood vessels as possible. The testes were washed twice in 1 x MMR and then once for 5 min in 1 x ***NPB***. To release the sperm, the testes were macerated with forceps in a fresh dry petri dish until there were no lumps remaining and the tissue was then resuspended in 2 ml 1 x NPB. This solution was then added to a cell strainer (FALCON) above a 15 ml FALCON tube, and then washed through with 3 ml and then 5 ml 1 x NPB. The resulting sperm solution was then centrifuged at 3000 rpm at 4°C for 10 min. The pellet was resuspended in 10 ml 1 x NPB and centrifuged again at 3000 rpm at 4°C for 10 min.

The pellet was resuspended in 1 ml 1 x NPB at room temperature, to which 50 µl digitonin (10 mg/ml in DMSO) was added, and it was then incubated at room temperature for 5 min. A solution containing 3 ml 10 % BSA, 5 ml 2 x NPB and 1 ml H_20 was then added to the 1 ml sperm solution so that the final concentrations are 3% BSA in 1 x NPB. The solution was centrifuged as before, the pellet resuspended in a 0.3% BSA solution in 1 x NPB and centrifuged again as before.

Finally, the pellet was resuspended in 250 µl sperm storage buffer (***SSB***), using a clipped pipette tip. 2 µl of the suspension was taken, diluted in 200 µl sperm dilution buffer (***SDB***) plus 1 µl HOECHST solution and the sperm density was counted in a haemocytometer (see below). The suspension was incubated at 4°C overnight and then aliquoted, snap-frozen in liquid N_2 and stored at –80°C.

The haemocytometer field measures 1 mm x 1 mm x 0.1 mm $= 0.1\ mm^3$
$\equiv 0.1\ \mu l$

If there is x sperm/ field (of a 1:100 dilution)
$\Rightarrow$ x sperm/ 0.1 µl (of a 1:100 dilution)
$\Rightarrow$ $100x$ sperm/ 0.1 µl
$\Rightarrow$ x sperm/nl $\equiv 1000x$ sperm/µl

Concentration of sperm required = 100 000 sperm/µl

$\because$ Dilute sperm to produce 100 sperm/haemocytometer field when diluted 1:100

2 x NPB
0.5 M sucrose
30 mM HEPES (pH 8.55)
1 mM spermidine
400 µM spermine
2 MM DTT
2 mM EDTA (pH 8.0)

SSB
1 x NPB
0.3% BSA
30% glycerol

SDB

250 mM sucrose

75 mM KCl

0.5 mM spermidine trihydrochloride

0.2 mM spermine tetrahydrochloride

pH to 7.3–7.5 with 0.1 N NaOH and store at –20°C in 0.5 ml aliquots

Transgenic procedure

The simplified transgenic procedure involves incubating sperm nuclei with linearised DNA and then fertilising eggs by microinjecting the nuclei directly into them. 250,000 sperm nuclei in 2.5 µl were mixed with 250 ng linearised DNA in 2.5 µl water. DNA for transgenesis was prepared by restriction enzyme digestion followed by gel purification. The sperm nuclei and DNA mixture was incubated at room temperature for 10–15 min, then 2.1 µl was diluted in 200 µl SDB, mixed by pipetting up and down with a clipped tip to ensure an even suspension of nuclei and injected into eggs (in 0.4 x MMR + 6% Ficoll) using a Harvard Instruments constant flow injector with a flow rate of 0.6 µl/min. Normally cleaving embryos were picked at the 2–4 cell stage and transferred into 0.1 x MMR + 6% Ficoll + gentamycin (50 µg/ml). At gastrulation stages, embryos were fixed for *in situ* hybridisation.

Tissue Culture

Handling of tissue culture cells

COS cells were stored at –80°C in DMEM containing 10% NBCS and 10% DMSO. Cells were defrosted at 37°C until almost thawed and then placed in 9 ml complete DMEM (Dulbecco's modified eagle medium + 10% NBCS + 50 µg/ml gentamycin) in a 15 ml FALCON tube. The tube was then centrifuged at 1000 rpm at 4°C for 5 min and the pellet was resuspended in 10 ml DMEM, which was added to a flask and cultured overnight at 37°C. Once cells reached 70% confluency, they were diluted between 1:2 and 1:10 in DMEM and re-plated.

To freeze cells, they were first trypsinised as above, centrifuged at 1000 rpm at 4°C for 5 min and the pellet was resuspended in 500 µl DMEM. To this, 500 µl of 2 x freezing media (DMEM containing 20% NBCS and 20% DMSO) was added and the cells were frozen slowly in a isopropanol cryochamber placed at –80°C overnight.

Lipofectamine-based transient transfection

To plate cells at the correct density for the assay, cells were trypsinised as above, centrifuged at 1000 rpm at 4°C for 5 min and the pellet was resuspended in 10 ml DMEM. The density of the cells was counted in a haemocytometer and the cells were diluted to 1.2 x 10^5 cells/ml. The diluted cells were then plated out by adding 2 ml of cell suspension into 6 well plates. These were incubated at 37°C overnight.

Transfection was carried out the next day. First, the cells were fed by removing the old medium, washing once in PBS and adding 2 ml new DMEM. Transfection mixes contained 0.1 µg pGL3 firefly luciferase reporter construct, 0.1 µg pRL-SV40 renilla luciferase reporter construct, 0.1–1 µg pcDNA3.1–VegT and was made up to 1.2 µg total DNA by addition of pCS2 as a carrier. 1.5 hrs after feeding the cells, 10 µl of the DNA mixes were mixed with 10 µl lipofectamine and 200 µl OPTIMEM. These mixtures were incubated at room temperature for 30 min.

To prepare cells for transfection they were washed twice in PBS and 800 µl OPTIMEM was added. To this, 200 µl transfection mix was added and, after incubation at 37°C for 4 hrs, 1 ml of OPTIMEM + 20 %NBCS was also added. The cultures were then incubated at 37°C overnight. The next day, the cells were fed as above and again incubated at 37°C overnight. The cells were then lysed for the assay. The medium was removed and the cells washed thoroughly in PBS. This was then replaced with 500 µl 1 x passive lysis buffer and the plates were placed on a horizontal shaker at room temperature for 15–30 min until lysis was complete. The cell lysate was transferred to 1.5 ml eppendorf tubes, which were centrifuged at 13,000 rpm for 30 s to pellet the cell debris. 20 µl of the lysate was then used for the luciferase assay.

Luciferase assays

Luciferase assays used the Promega Dual Luciferase kit. Samples were assayed in triplicate using 20 µl cell lysate per replicate. For each sample the firefly luciferase values were normalised by dividing by the corresponding renilla luciferase value.

Oocytes

Obtaining oocytes

Xenopus oocytes were obtained from sacrificed female frogs. Females were sacrificed by injection of 3-aminobenzoic acid ethyl ester (MS-222) and subsequent destruction of the circulatory system. The ovaries of the frogs were then dissected out and placed in 60% L-15 containing 50 μg/ml gentamycin. Oocytes were defolliculated by collagenase treatment. Oocytes were placed in a 2% collagenase solution for 30–60 min, after which they were placed in fresh media and sorted according to stage. Stage VI oocytes were selected for these assays and were left in fresh media overnight at 18°C.

Microinjections

Surviving oocytes were then injected in the vegetal pole to avoid the germinal vesicle with 4 ng VegT-HA RNA. The oocytes were then left at 18°C for 24 hours for the message to be translated. The oocytes were injected with DNA into the germinal vesicle. The DNA mixtures contained pRL-TK and either pGL3-d851, ΔTbs1, ΔTbs2 or ΔTbs1,2 at a concentration of 0.1 mg/ml.

Luciferase assays

Oocytes were collected after 24 hours and assayed. Each sample was measured in triplicate with approximately 10 oocytes per replicate. Oocytes were homogenised in 20 μl per oocyte of 1 x passive lysis buffer. Samples were centrifuged for 30 s at 13,000 rpm to pellet cell debris and then the luminescence of 20 μl was measured. As before, firefly values were normalised using the renilla values.

Statistics

Student's t-test

To assess the significance of differences between constructs found in luciferase assays, the Student's t-test was employed. Differences that are significant ($p < 0.05$) are marked on graphs with an asterisk (*).

Buffers and Media

2% Cysteine hydrochloride

4.4 g cysteine hydrochloride monohydrate

1.33–1.36 g NaOH

Make up to 200 ml with H_2O and adjust pH to 7.9–8.1 if necessary with 10 M NaOH

MEMFA

0.1 M MOPS (pH 7.4)

2 mM EGTA

1 mM $MgSO_4$

3.7% formaldehyde

10 x MMR (Marc's modified ringer)

1 M NaCl

20 mM KCl

10 mM $MgSO_4$

20 mM $CaCl_2$

50 mM HEPES (pH 7.5)

10 x NAM salts

1.1 M NaCl

20 mM KCl

10 mM $Ca(NO_3)_2$

10 mM $MgSO_4$

10% NAM

0.1 x NAM salts

2 mM sodium phosphate (pH 7.4)

50 µg/ml gentamycin

75% NAM

0.75 x NAM salts

2 mM sodium phosphate (pH 7.4)

50 µg/ml gentamycin

1 mM $NaHCO_3$

10 x PBS

80 g NaCl

2 g KCl

14.4 g Na_2HPO_4

2.4 g KH_2PO_4

Make up to 1 litre with H_2O and pH to 7.4 with 10 M NaOH

20 x SSC

3 M NaCl

0.3 M sodium citrate

50 x TAE

2 M Tris-acetate

50 mM EDTA

10 x TBE

900 mM Tris-borate

10 mM EDTA

6 x Loading buffer

6 x TAE

30% glycerol

0.25% Bromophenol blue

0.25% Xylene cyanol

Formamide loading buffer

90% formamide

10 mM EDTA

0.1% Bromophenol blue

0.1% Xylene cyanol

2 x SDS loading buffer

100 mM Tris-HCl (pH 6.8)

200 mM DTT

4% SDS

0.2% Bromophenol blue

20% glycerol

LB Medium

10 g bacto-tryptone

5 g bacto-yeast extract

10 g NaCl

Make up to 1 litre with H_2O and adjust to pH 7.0 with 5 N NaCl

L-agar

Prepare media as LB (above)

Add 15 g bacto-agar

2 x TY

16 g bacto-tryptone

10 g bacto-yeast extract

5 g NaCl

Make up to 1 litre with H_2O and adjust to pH 7.0 with 5 N NaCl

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Chapter 3. Functional analysis of *derrière*

INTRODUCTION

In this chapter, I shall focus on the function of derrière. Sun et al. (1999) showed that over-expression of derrière in prospective ectodermal cells induces both mesodermal and endodermal marker genes in a dose-dependent manner. Interestingly, in contrast to activin, derrière induces *Xsox17α* at lower concentrations than are required to induce expression of *Xbra* and *muscle actin*. Over-expression of derrière in whole embryos leads to an incomplete secondary axis when expressed ventrally and to loss of the most anterior structures when expressed dorsally, suggesting a role in the development of trunk and tail.

To assess the requirement for derrière during embryogenesis, Sun et al. (1999) constructed a cleavage-mutant version of derrière, in which the consensus furin cleavage site is mutated. This is predicted to act in a dominant-negative manner by dimerising with endogenous derrière molecules and preventing correct processing and secretion. This construct interferes with over-expressed derrière in an animal cap assay and also slightly affects the action of both *Xnr1* and *Xnr3* (Sun et al., 1999). When over-expressed in whole embryos the cleavage mutant causes loss of posterior structures. At gastrulation stages a blastopore lip fails to form in the injected region and at tailbud stages and onwards somites and tail fail to form. All of these observations are consistent with the suggestion that derrière is involved in the development of the posterior of the embryo (Sun et al., 1999).

This conclusion is confirmed by an experiment attempting to rescue the phenotype of VegT-depleted embryos by over-expression of signalling molecules. Injection of VegT-depleted embryos with increasing amounts of derrière RNA lead to the rescue of trunk and tail but not head structures (Kofron et al., 1999).

I have carried out additional experiments, to address the function of derrière and its mode of action. In this chapter I show that derrière is able to act at a distance of several cell diameters and have constructed a GFP-tagged form of derrière which should be useful in the future for studies on the distribution and movement of derrière protein in the embryo. I have also used a morpholino oligonucleotide to 'knock-down' endogenous derrière protein in an attempt to define the function of derrière in embryonic development.

RESULTS

Derrière is able to act at a distance

To investigate the question of the range of action and activity of derrière, I performed an assay first used by Jones et al. (1996) to study the effective range of signalling molecules. The experimental design is shown in Fig. 3.1A. Animal cap explants are juxtaposed to create a recombinant in which there is a border between cells expressing a signalling molecule and those not. One explant acts as a source of the signalling molecule to be tested whereas the other acts as a responding tissue. The 'source' cap is explanted from an embryo that has been injected with RNA encoding the particular molecule being tested and the 'responding' cap comes from an embryo injected with Fluorescein Lysinated-Dextran as a lineage marker. The caps are combined immediately after being explanted and are then cultured until sibling embryos reach early gastrula stage (stage 10.5). At this point the recombinants are fixed and processed by *in situ* hybridisation for expression of the mesodermal marker gene *Xbra*. Activin, which is able to activate *Xbra* in the responding cap, was used as a positive control (Fig. 3.1D–E). Xnr2, which in this assay acts essentially cell-autonomously, was used as a negative control (Fig. 3.1F–G; Jones et al., 1996).

When this assay is performed with derrière as the test molecule, derrière is able to activate *Xbra* in the responding cap, showing that it can act over several cell diameters (Fig. 3.1H–I). It is impossible to say from this assay how derrière actually produces this effect, but one possibility is that the protein is actually present several cell diameters away from the source tissue/responding tissue boundary and directly induces *Xbra* where it is present. The extent of *Xbra* expression would then be dependent on the spread of derrière protein through the tissue. However, due to the timing and design of the assay, it is also possible that derrière initiates a relay mechanism at the border between the source and responding cap and Xnr2 is unable to do this.

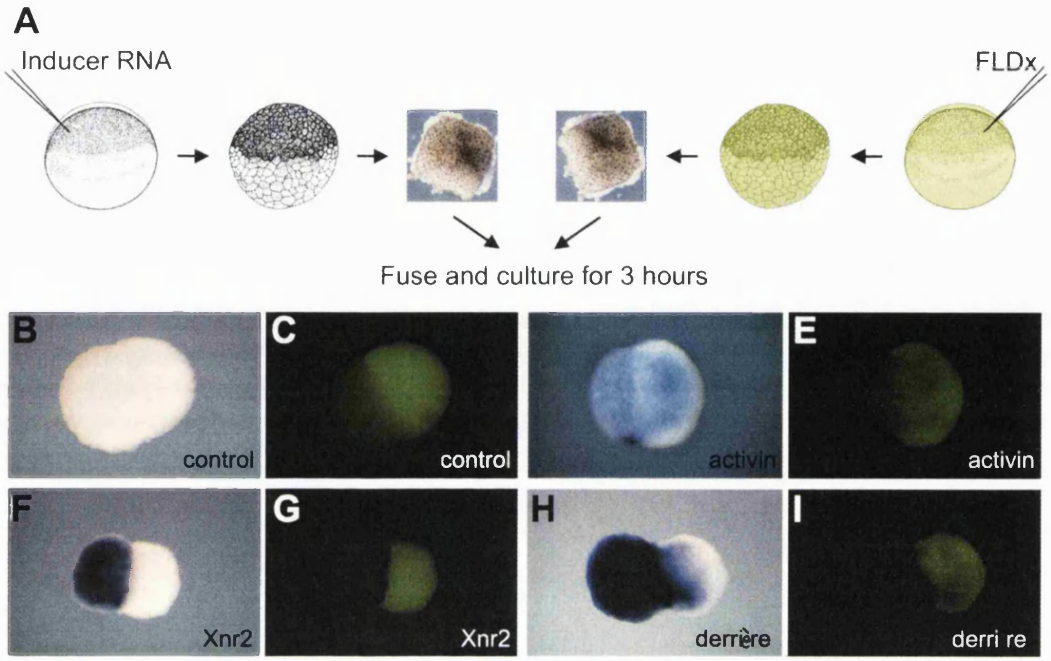

Fig. 3.1. Derrière is able to act at a distance. (A) Experimental scheme. Two explants are combined to make each recombinant. One is explanted from an embryo that has been injected with RNA encoding the particular molecule being tested (uninjected, activin, Xnr2 or derrière) and the other comes from an embryo injected with Fluorescein Lysinated-Dextran as a lineage marker (shown in green in the diagram). The caps are combined immediately after being explanted and are then cultured until sibling embryos reach stage 10.5. They were examined by whole-mount *in situ* hybridisation for expression of *Xbra* (B, D, F and H) or by flourescence microscopy for detection of FLDx (C, E, G and I). (B–C) uninjected caps do not induce expression of *Xbra*. (D–E) Activin (10 pg) is able to activate *Xbra* in the FLDx-labelled tissue. (F–G) Xnr2 (500 pg) activates expression of *Xbra* only in the unlabelled explant. (H–I) Derrière (500 pg) induces expression of *Xbra* in both unlabelled and FLDx-labelled tissue.

Construction of a derrière–GFP fusion protein

One way to investigate further the way in which derrière gives rise to this action at a distance is to study directly the movement of derrière through tissues. To do this, a GFP-tagged version of derrière was created. This construct was based on the design of a GFP-tagged TGF-β molecule made by Gonzalez-Gaitan et al. (1999). They constructed a GFP-tagged form of *decapentaplegic*, a TGF-β involved in imaginal disc patterning in *Drosophila melanogaster*. This fusion protein (dpp–GFP) was then used to study the movement of dpp through cells in the imaginal disc.

Because TGF-β molecules are produced as large precursor pro-proteins that are cleaved to give the mature signalling molecule, the GFP open reading frame (ORF) was fused in frame after the consensus furin cleavage site, so that the GFP remains with the mature region after cleavage. This was done by a PCR-based strategy, using chimeric oligonucleotides as primers. Each primer contained 21 base-pairs of the particular fragment being amplified at its 3' end and 15 base-pairs from the end of the fragment it was to be fused to (see Fig. 3.2). This means that each of the three fragments amplified (pro-region, GFP ORF and mature region) contained sequence overlapping with the fragment it was to be fused to (see Fig. 3.2). The fragments were then fused together by using them as the template in another PCR reaction containing only the most 5' and 3' primers.

Derrière–GFP is a functional TGF-β ligand

It is important to establish that derrière–GFP is still a functional TGF-β ligand. Gonzalez-Gaitan et al. (1999) showed this by rescuing dpp -/- embryos with their fusion protein. This is not possible in *Xenopus*, as there is no *derrière* mutant. However, it is possible to compare the effects of over-expressing the two ligands, to see if they behave similarly and produce a similar phenotype and molecular effects.

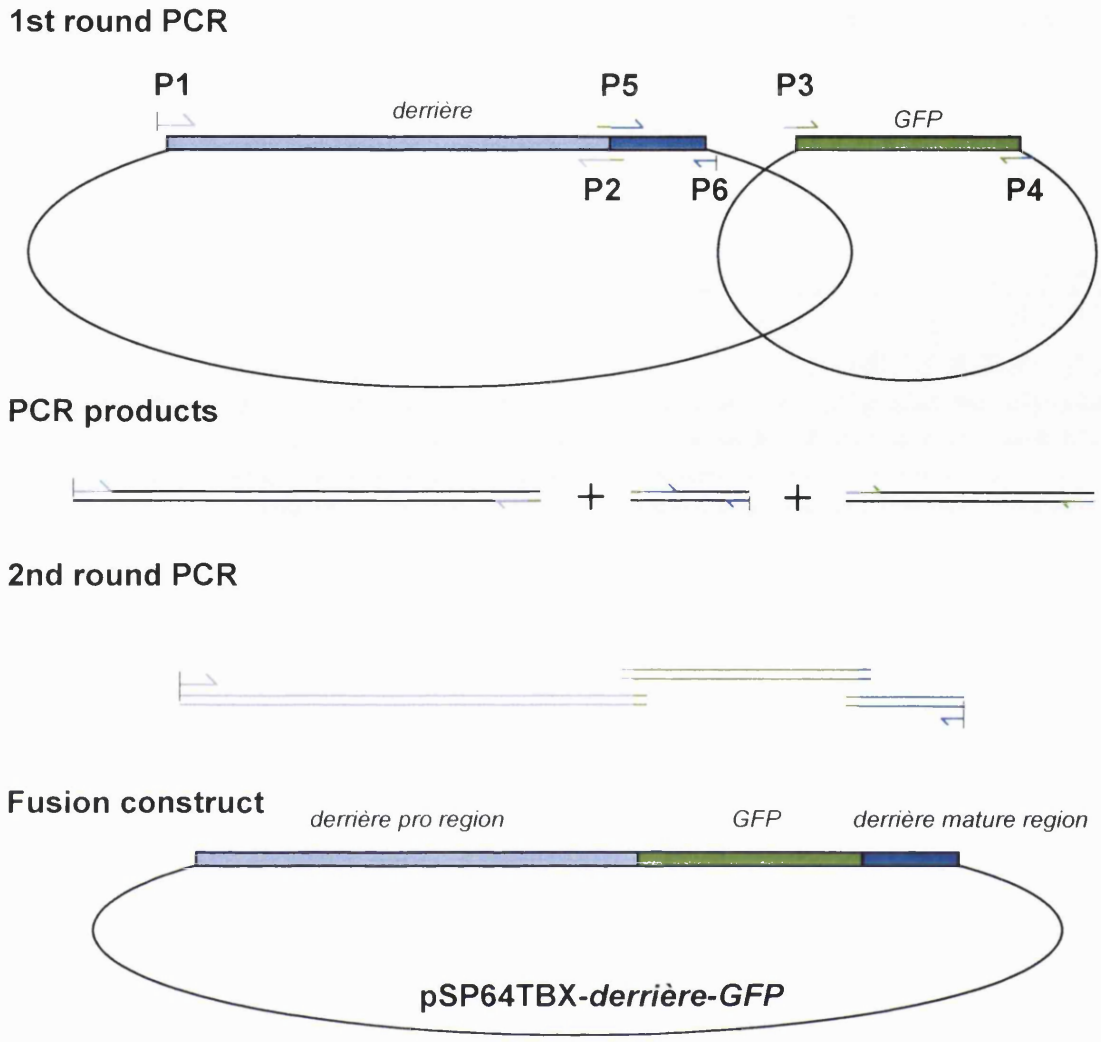

Fig. 3.2. Construction of derrière–GFP. Derrière–GFP was constructed by a PCR-based strategy. Oligonucleotide primers were designed to create overlapping sequences which could then be fused together in a second round of PCR. Primers at the join regions (**P2**–**P5**) contained at least 21 base-pairs from the fragment being amplified and 15 base-pairs from the sequence it was to be fused to. For example, **P2** contained 15 base-pairs from the 3' end of the derrière mature region at its 3' end, then 15 base-pairs from the bottom strand of the 5' end of GFP. This created 30 base-pairs of overlap between each product and the section it is to be fused to. These three products were then used as the template in a second PCR reaction containing primers **P1** and **P6**. This created the three-part fusion construct which was then cloned into pSP64TBX. *Derrière* is shown in blue and GFP in green.

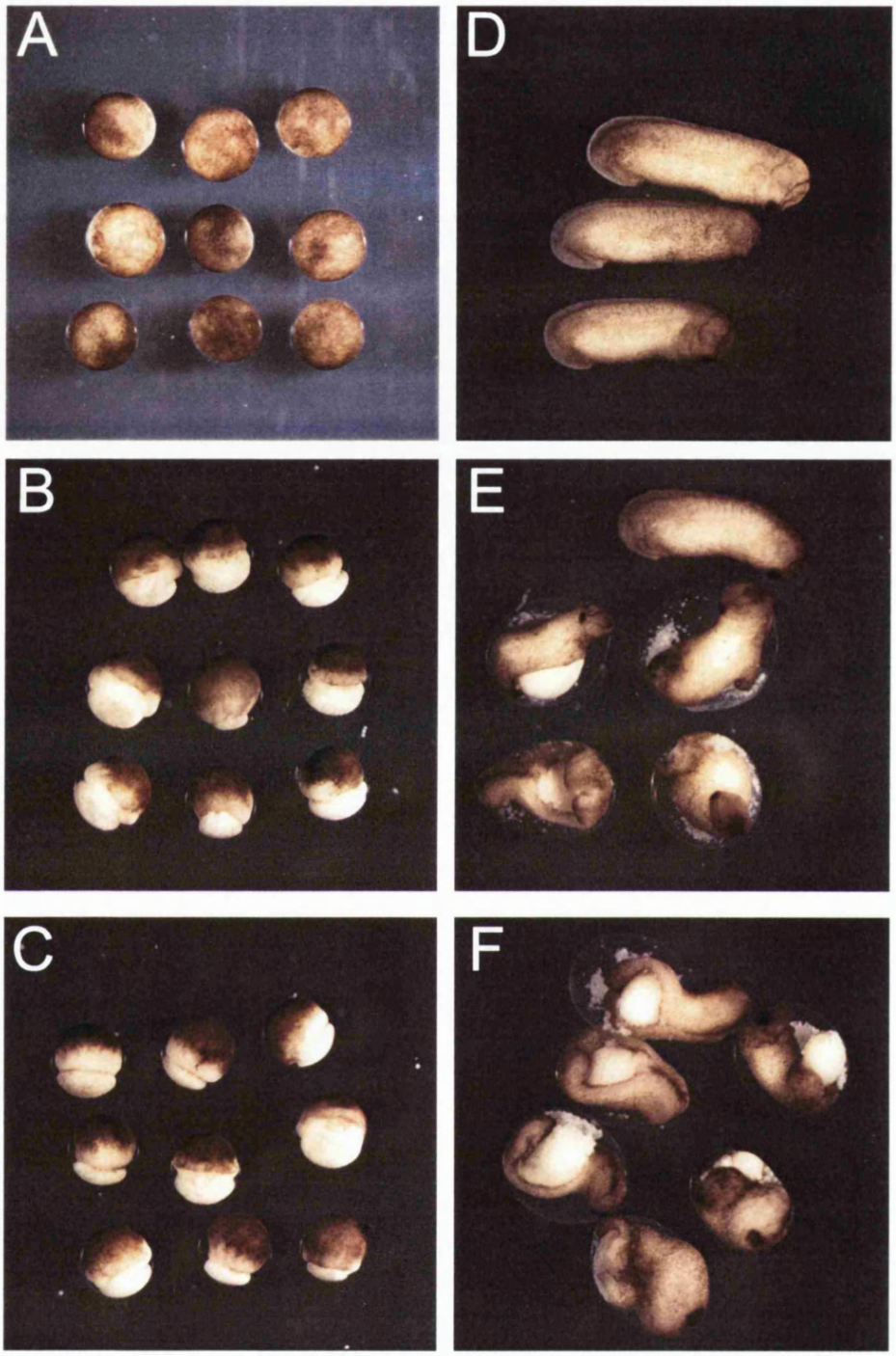

Fig. 3.3. Derriere–GFP is a functional TGF-β ligand. Whole embryo phenotype of over-expressing derrière and derrière–GFP. (A–C) Stage 12 embryos. (D–F) Stage 26 embryos, lateral view. (A & D) Uninjected embryos develop normally. (B & E) Embryos injected with 10 pg derrière RNA fail to gastrulate properly and this leads to a failure of the dorsal midline closure. (C & F) Over-expression of 10 pg derrière–GFP RNA exhibit a similar phenotype. This experiment was carried out by Huw Williams.

A series of over-expression experiments was carried out by Huw Williams comparing the effects of over-expressing derrière with derrière–GFP. Preliminary results are shown in Fig. 3.3.

Xenopus embryos were injected into the animal pole at the one-cell stage with 10 pg of either *derrière* or *derrière–GFP* RNA. Embryos injected with either *derrière* or *derrière–GFP* show a consistent gastrulation defect, where involution does not occur properly (Fig. 3.3B–C) and there is a failure of the blastopore to close. This produces embryos with posterior defects in which the dorsal midline has failed to close (Fig. 3.3, derrière 10 pg panel E; 42%; n=12, derrière–GFP panel F; 95%; n=12). Thus, over-expression of *derrière–GFP* produces similar morphological defects as over-expression of *derrière*. The inducing properties of the two molecules are currently being tested using molecular markers.

An antisense morpholino oligonucleotide directed against the 5' UTR of *derrière* leads to a defect in posterior structures

One way to perform a loss-of-function experiment without the use of mutations is to use antisense oligonucleotides. In this case, I have used a morpholino oligonucleotide, which is very stable and acts by preventing translation of the target RNA (Heasman, 2002; Heasman et al., 2000; Nutt et al., 2001). To do this effectively it must be designed to recognise sequence either in the 5' untranslated region (UTR) or sequence no more than 25 bp downstream of the translation start site. The design of the *derrière* morpholino is shown in Fig. 3.4.

To test that this morpholino is acting in the correct manner (i.e. preventing the translation of derrière) would require an antibody raised against derrière in order to compare the amount of endogenous derrière protein in injected and uninjected embryos and we do not currently have such an antibody. As an alternative one can show that translation of RNA for a tagged form of derrière can be blocked by the morpholino. For this experiment, I used the GFP-tagged derrière as shown in Fig. 3.4. Over-expression of GFP–derrière in the animal pole at the one-cell stage leads to production of fluorescent protein in the animal cap at blastula stages (Fig. 3.4). Co-expression of the morpholino directed against derrière leads to loss of fluorescence, showing that translation of the over-expressed RNA has been prevented.

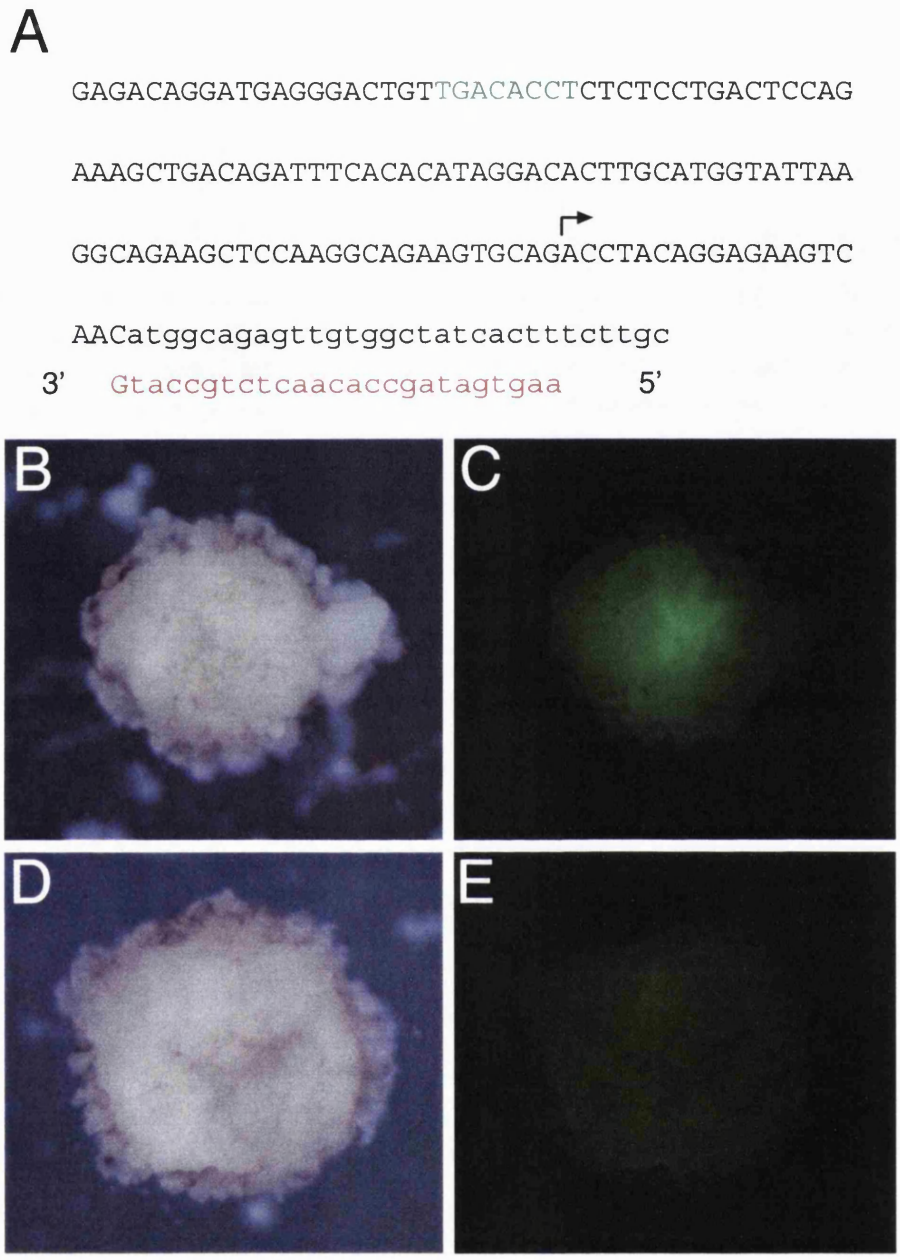

Fig. 3.4. Design and function of derriere morpholino. (A) Sequence of the 5' region of *derrière* showing the design of the morpholino. The transcription start site is indicated by an arrow and the beginning of the protein sequence is shown in lower case. The morpholino (shown in red) covers one base-pair 5' of the translation start site and the 24 base-pairs downstream. (B–E) The morpholino is able to block translation of over-expressed derrière–GFP. *Xenopus* embryos were injected with derrière–GFP RNA and either the control morpholino or the derrière morpholino. Animal caps were explanted at blastula stage and photographed under both light (B and D) and fluorescence microscopy (C and E). (B–C) Over-expressed derrière–GFP RNA produces a fluorescent protein (D–E) Co-injection of 40 ng *derrière* morpholino blocks fluorescence.

When this antisense morpholino oligonucleotide is injected into embryos at the one-cell stage, it produces a defect in the posterior of the embryo. Embryos are slightly shorter than normal, the body axis is bent slightly ventrally in the trunk and the tail is shorter and bent dorsally. The head appears normal. This phenotype becomes stronger and more frequent as the concentration of injected morpholino increases (see Fig. 3.5 and Table 3.1). However, even at its strongest, this phenotype is not as severe as that produced by the dominant negative cm-derrière.

Due to technical problems this experiment has only been performed once and is therefore only preliminary. Therefore, it is essential to repeat the experiment to be sure that the result is not artefactual and then to elucidate the cause of the defect using histology and assaying its effect on molecular markers. It is also important to show that the endogenous protein is actually being affected.

Table 3.1. Results of morpholino injection in embryos

Amount of morpholino injected	Number showing defect	Total number of embryos	% with defect
uninjected (0 ng)	4	21	19.1 %
5 ng	9	43	20.9 %
10 ng	4	7	57.1 %
20 ng	22	24	91.7 %
40 ng	42	42	100 %

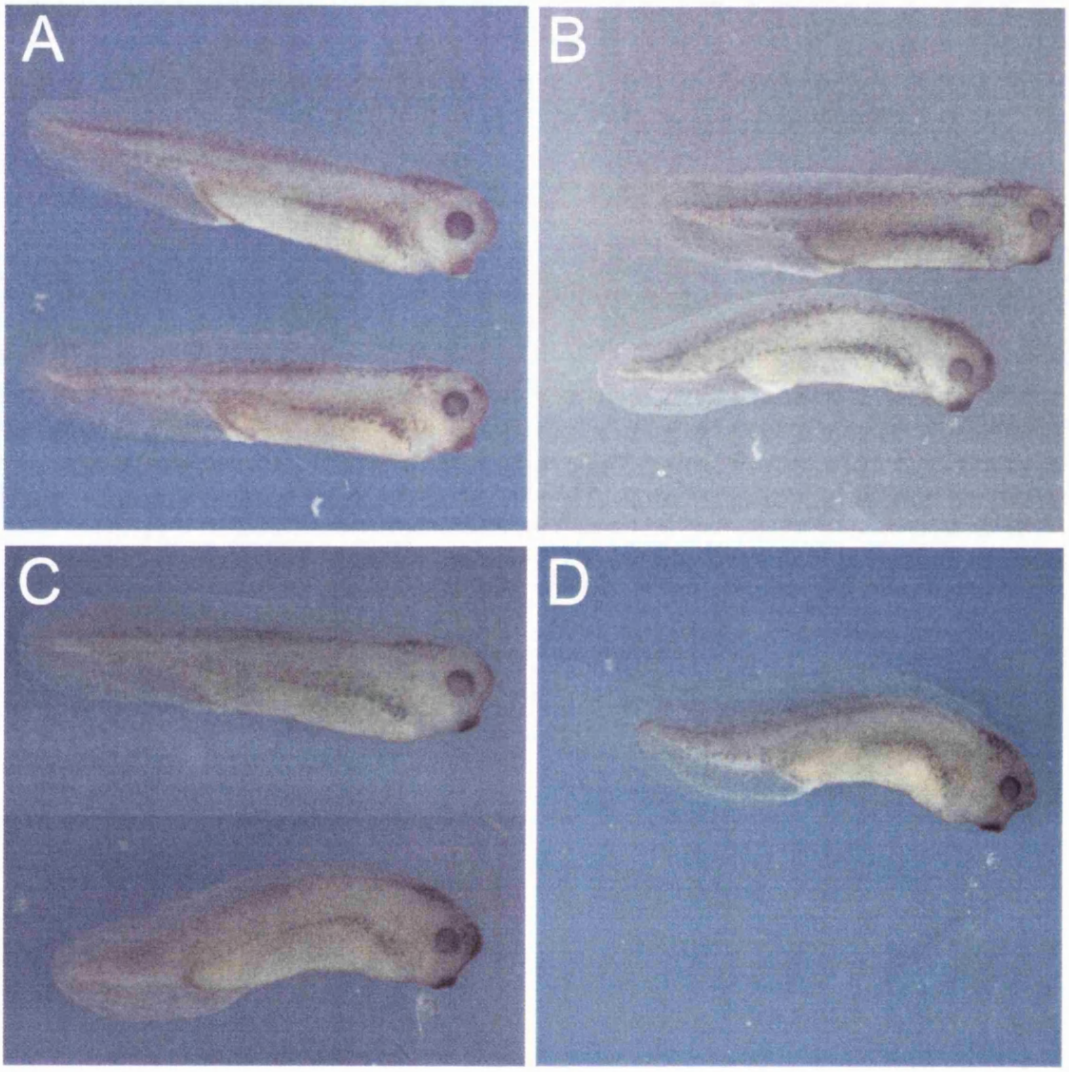

Fig. 3.5. *Derrière* is required for formation of posterior structures. Embryos were injected at the one-cell stage with increasing amounts of *derrière* morpholino and fixed at stage 35 for morphological analysis. Embryos show an increasing frequency of posterior defects with increasing concentration of morpholino (see Table 3.1). They are shorter than wild-type, the trunk section is bent ventrally, whereas the tail is bent dorsally and shorter. (A) uninjected embryos (B) 10 ng morpholino (C) 20 ng morpholino (D) 40 ng morpholino.

DISCUSSION

The results described in this chapter outline the importance of *derrière* in the context of the whole embryo and make it an excellent candidate for one of the endogenous mesoderm-inducing signals downstream of *VegT*. Thus, derrière is able to act over a distance of several cell diameters to induce the expression of *Xbra* and preliminary experiments suggests it is required for the formation of posterior mesodermal structures.

Action of *derrière* at a distance

The results of this assay show that derrière is able to act over a range of several cell diameters to induce *Xbra*. One interpretation of this observation is that derrière protein is actually present several cell diameters away from the source tissue/responding tissue boundary and directly induces *Xbra* wherever it is present. However, the final readout of this assay (expression of *Xbra*) is 3-3.5 hrs from conjugation and is therefore too far downstream to make any statements about the distribution of derrière protein. Another scenario equally consistent with these results is that derrière, but not Xnr2 is able to activate some sort of relay at the boundary between the source and responding cap. One interesting point is that derrière protein, when used to treat animal caps, is unable to induce expression of *Xbra* in the presence of CHX (Personal comm. B.Sun). The reason for this may be that derrière function requires XFast3 (Howell et al., 2002), which is not expressed until gastrulation, and therefore would not have been translated in this experiment. Another possibility is that expression of *Xbra* was induced but not maintained as the assay was carried out at stage 11.

Derrière–GFP

In order to test whether derrière protein is actually present at the furthest extent of *Xbra* expression in these recombinants, the GFP-tagged form of derrière could be used to study the production, secretion and transport/diffusion of derrière. These experiments are currently under way.

The GFP-tagged derrière described here appears to be a functional TGF-β ligand. It will also be a very useful tool to investigate questions concerning the distribution of TGF-β molecules in the embryo. Very little direct evidence currently exists that TGF-β proteins are able to freely diffuse within the extracellular space as they have been very difficult to visualise in real time. Other hypotheses exist as to how the distribution of signalling molecules is achieved including a model of transcytosis, where the protein is taken up by a cell and re-secreted on the opposite side of the cell leading to directed transport (Pfeiffer and Vincent, 1999; Strigini and Cohen, 1999). This reagent would allow one to follow the progress of derrière protein as it is secreted and to investigate which of the current hypotheses best explain the data.

However derrière achieves this action at a distance, it is able to influence the fate of cells several cell diameters away, making it an excellent candidate for a component of the endogenous mesoderm-inducing signal. To test which aspect of mesoderm development it is involved in, I attempted to remove the endogenous protein using an antisense morpholino oligonucleotide.

Derrière is required for posterior development

The results from this preliminary morpholino injection experiment are consistent with the dominant negative cm-derrière experiments performed by Sun et al. (1999) and suggest that *derrière* is involved in the development of posterior structures. However, the phenotype shown here is much weaker than that produced by over-expression of cm-derrière.

A combination of two things could produce this effect. First, because *Xenopus laevis* are tetraploid (Bisbee et al., 1977), they possess four copies of each gene organised as two pairs of pseudoalleles. These two sets of each gene usually differ in sequence by 5–10% in coding regions and more in untranslated regions due to the accu mulation of polymorphisms. However, they are often similar enough at the protein level that they can functionally substitute for one another. Thus, it is possible that there exists another copy of *derrière* with a sufficiently divergent sequence that the morpholino does not recognise it. The phenotype would then represent a 'knock-down' rather than a total loss-of-function. Second, it has been shown recently that cm-derrière affects other TGF-β molecules , including Xnr2 and BMP4, by directly dimerising with them and preventing their activity (Eimon and Harland, 2002) and this would therefore be expected to give a more severe phenotype.

More experiments are needed, first to confirm the result itself (to make sure it is not an injection artefact) and second to find the cause of the defect. In situ hybridisation and immuno-cytohistochemistry could be used to investigate the patterning of the defective embryos and to ascertain which tissues and structures are affected.

In this chapter, I have shown some preliminary data on the subject of how derrière functions in the embryo, using an antisense morpholino to knock-down the endogenous protein levels and the construction of a GFP-tagged form of derrière to attempt to monitor the secretion and movement of the protein.

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Chapter 4. A screen for direct targets of *VegT*

INTRODUCTION

The T-box transcription factor VegT is required for germ layer specification (Kofron et al., 1999; Zhang et al., 1998). It is also necessary for the production of the mesoderm-inducing signal that is released from vegetal explants (Zhang et al., 1998). In order to understand the mode of action of *VegT* – how it generates both endoderm and mesoderm by the activation of a transcriptional network – it is necessary to know which target genes it activates and most importantly, which direct targets. Although several genes have been shown to be downstream of *VegT*, very few direct *VegT* target genes are currently known. To find direct target genes of *VegT* there are two potential strategies:

1) Subtractive hybridisation screen

A subtractive hybridisation screen involves preparing RNA from animal caps which are either over-expressing the inducer being tested (VegT in this case) or not. The two pools are then hybridised together and suppression PCR is used to amplify transcripts found uniquely in the induced pool while eliminating transcripts common to both pools. Screens of this nature have already been carried out for the transcription factors *Xbra* and *Pintallavis* (Saka et al., 2000; Tada et al., 1998), using hormone-inducible versions of these genes. Interestingly, the first of these screens using *Xbra–GR* (Tada et al., 1998) identified genes that are *VegT* targets as well as *Xbra* targets.

2) Candidate gene approach

This approach relies on the existence of already known genes where there is evidence that they are likely to be downstream of the inducer (VegT). The properties of good candidates for being direct targets of VegT are listed below:

Expression pattern – because VegT is a transcription factor, direct target genes will be activated cell autonomously and therefore should be expressed in the same pattern as *VegT* (or at least a subset of their expression pattern should overlap with that of *VegT*).

Activity – because *VegT* is involved in germ layer specification, candidate target genes should have some role in either endoderm or mesoderm formation

Reliance on VegT – any candidate gene should be inducible by VegT and should be reduced in VegT-depleted embryos.

In this study, there were several genes (many of them involved in mesoderm formation) that had already been identified as being downstream of *VegT* and so it was decided to take a candidate approach and simply test candidate genes for direct induction by *VegT*. This was done by testing if the candidate gene could be induced by VegT in the presence of cycloheximide (CHX). Several known genes were tested for direct *VegT*-responsiveness using the hormone-inducible construct *VegT–GR* and the results are discussed in this chapter.

RESULTS

VegT–GR is a hormone-inducible version of *VegT*

In order to control accurately the timing of activation of *VegT* target genes, I constructed an inducible form of *VegT* as described in ***Materials & Methods***. This consists of the entire coding region of *VegT* fused in-frame to the C-terminal ligand-binding domain of the human glucocorticoid receptor (hGR) (Fig. 4.1A). The glucocorticoid receptor is a nuclear steroid hormone receptor. It is normally present as a latent complex in the cytoplasm bound to heat shock protein 90 (hsp90; Cadepond et al., 1991; Pratt et al., 1988). When the particular steroid that the receptor responds to diffuses into the cell, it is bound by the ligand-binding domain of the receptor, which leads to a conformational change, releasing the receptor from hsp90 and allowing it to translocate to the nucleus where it can affect transcription. When linked to a transcription factor, the hGR ligand-binding domain confers hormone sensitivity on the resultant fusion protein.

Thus, when synthetic mRNA for the *VegT–GR* construct is injected into embryos it will be translated, but the protein will be bound by the heat-shock apparatus of the cell and remain inactive unless the ligand, dexamethasone (DEX), is added. When DEX is added it binds to the hGR ligand-binding domain, freeing the fusion protein and allowing it to translocate to the nucleus. It can then activate transcription of downstream target genes (Fig. 4.1B). Because the only DNA-binding activity in the fusion protein comes from VegT, specificity of the construct is maintained.

This construct allows for the timing of activation of target genes to be regulated in concert with the inhibition of protein synthesis, allowing one to ask whether genes can be activated in the absence of protein synthesis. Constructs of this nature have been used successfully to identify target genes of the transcription factors *Xbra* and *Pintallavis* (Saka et al., 2000; Tada et al., 1998).

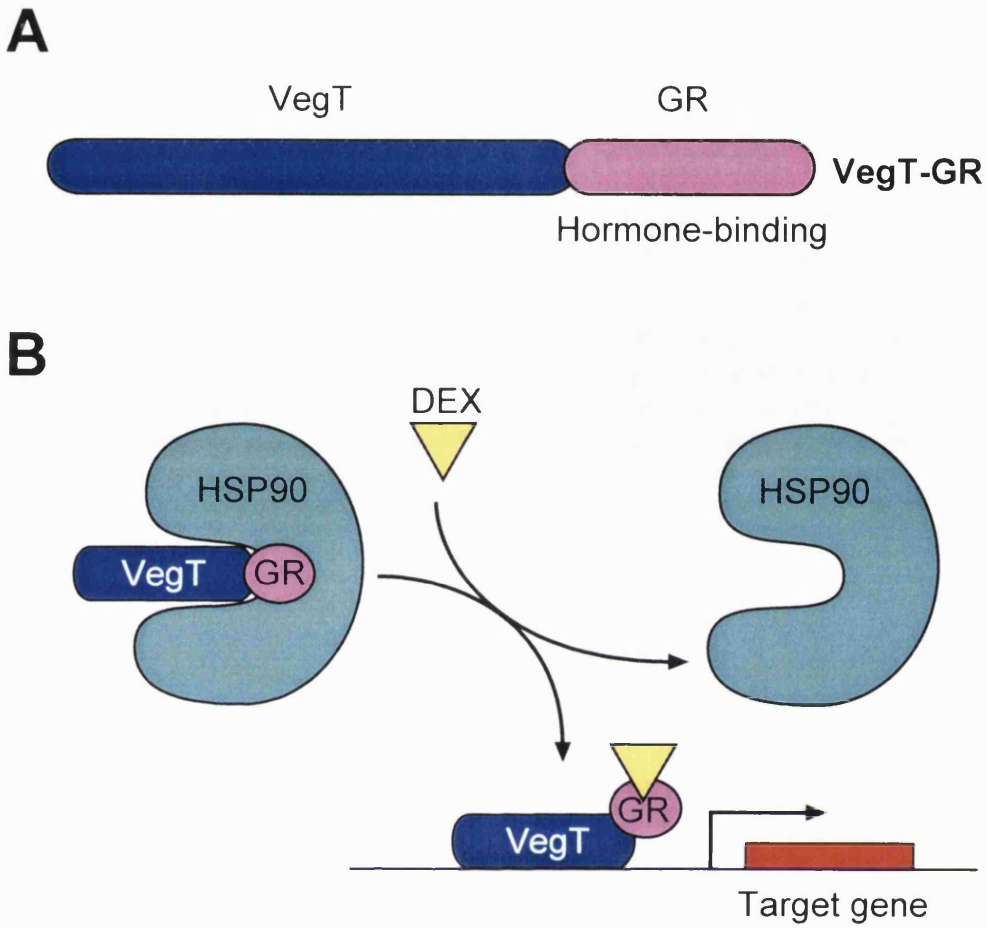

Fig. 4.1. Mode of action of VegT–GR. (A) VegT–GR consists of the hormone-binding domain of the human glucocorticoid receptor (hGR) fused in frame to the carboxy-terminus of VegT. (B) Mode of action. The hGR domain confers hormone-responsiveness on VegT. When synthetic VegT–GR RNA is injected in *Xenopus* embryos it is translated but remains inactive. The translated *VegT-GR* protein is bound by the heat-shock apparatus of the cell. On addition of the hormone, dexamethasone, the protein is released and is able to activate transcription of VegT target genes. VegT is shown in blue, the hGR domain in magenta.

Bix4 is a direct target of *VegT*

To test the action of the *VegT–GR* construct, *Bix4* was used as a positive control. *Bix4* was isolated in a screen for genes that respond to *Xbra* (Tada et al., 1998) and was subsequently found also to be a direct target of *VegT* (Casey et al., 1999). It was used to assay the efficacy of the *VegT–GR* construct. The construct was tested by injection of increasing amounts of *VegT–GR* RNA and in this way the optimal dose of injected RNA was determined to be 200 pg. At this dose of injected *VegT–GR* RNA, a robust induction of target genes is produced, but only in the presence of DEX. The construct does not activate transcription in the absence of DEX.

The experiment used to test prospective candidate genes is shown in Fig. 4.2A. *Xenopus* embryos were injected at the one-cell stage with synthetic *VegT–GR* RNA. These embryos were left to develop until the early blastula stage (stage 8.5) during which time *VegT–GR* RNA is translated, but remains inactive due to the presence of the GR domain. At stage 8.5 animal caps were explanted and then cultured in various combinations of DEX, which will activate the latent VegT–GR protein, and/or CHX, which will inhibit protein synthesis. The caps were incubated to the equivalent of early gastrula stage (stage 10.5), then frozen and assayed by RNase protection for induction of the gene of interest. Candidate genes that are induced by the addition of DEX even in the absence of intermediate protein synthesis are considered to be direct targets of *VegT*.

This experiment was carried out using *Bix4* as a positive control. Both the *VegT–GR* construct and *Bix4* behaved as expected. As shown in Fig. 4.2B, *Bix4* is induced on addition of DEX even in the presence of CHX.

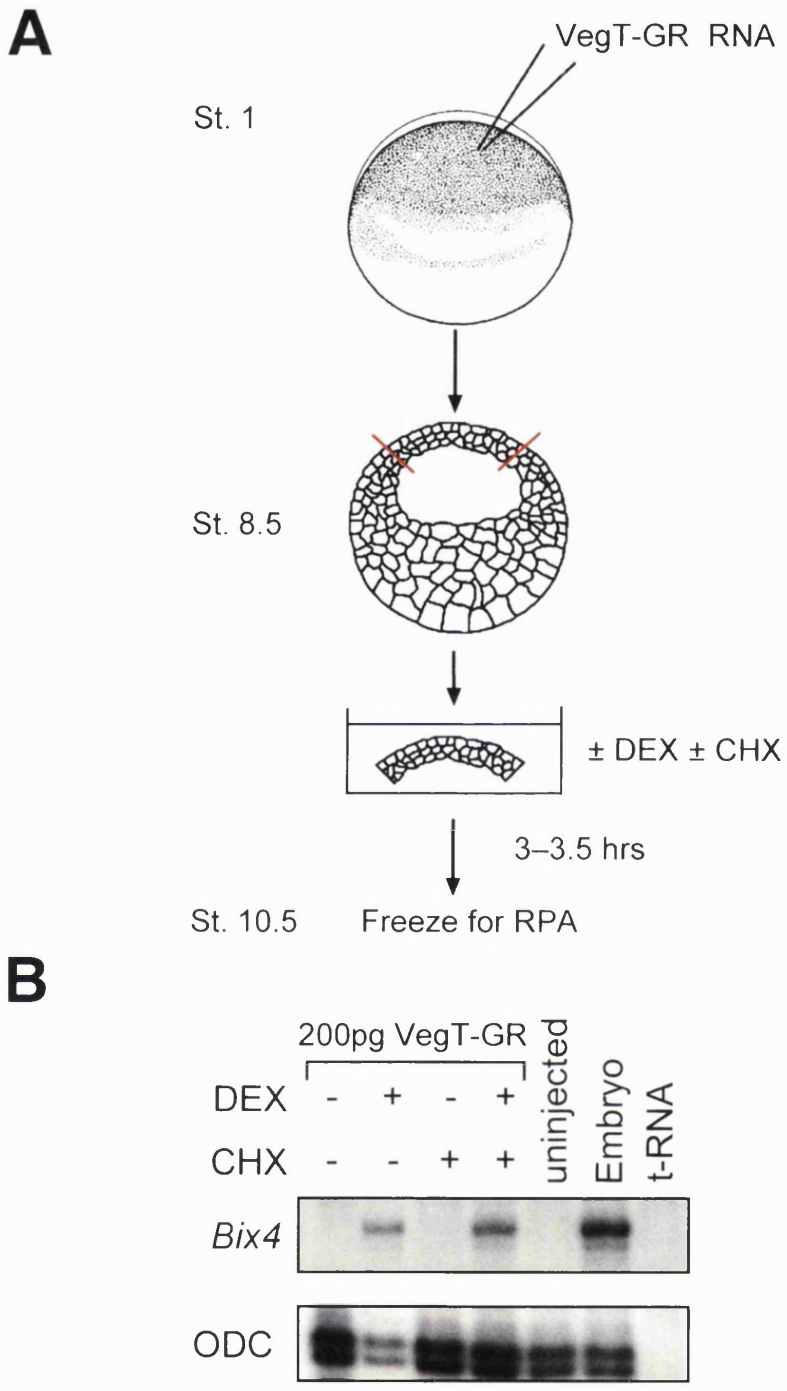

Fig. 4.2. Assay for direct targets of *VegT*. (A) *VegT-GR* RNA is injected into 1-cell embryos. Animal caps are explanted at blastula stage and placed in all combinations of dexamethasone (DEX) and cycloheximide (CHX) until sibling embryos reach stage 10.5. These caps are then frozen and assayed by RNase protection. (B) *Bix 4* is a direct target of *VegT*. Injection of 200 pg *VegT-GR* RNA is sufficient to induce expression of *Bix 4*, a known target of *VegT*, on addition of DEX even in the presence of CHX.

A candidate screen for targets of *VegT*

Candidates genes were tested using the animal cap assay described above. The genes selected for screening have functions related to mesoderm induction or patterning and their expression patterns resemble that of *VegT*. The results are discussed below.

Paraxial Protocadherin

Paraxial Protocadherin (*PAPC*) was identified in a differential screen for genes expressed in the dorsal blastopore lip (Bouwmeester et al., 1996; Kim et al., 1998). It is a transmembrane protein that falls into a family of molecules related to classical cadherins (Sano et al., 1993). It is also very closely related to *Axial Protocadherin* (AXPC; Kim et al., 1998). In *Xenopus*, *PAPC* is initially expressed in the marginal zone from late blastula stages and later is excluded from the dorsal midline while continuing to be expressed in the involuted pre-somitic paraxial mesoderm (Kim et al., 1998). In tissue culture, *PAPC* exhibits homotypic adhesion properties (Obata et al., 1995; Sano et al., 1993) and in *Xenopus* cell dissociation and reaggregation studies it has a cell adhesion activity that is mutually exclusive to *AXPC* and mediates cell sorting (Kim et al., 1998). Furthermore, a dominant-negative form of *PAPC* interferes with normal gastrulation movements following injection into *Xenopus* embryos (Kim et al., 1998).

PAPC appears to be a good candidate for a target of *VegT* for two reasons. First, its expression pattern is very similar to that of *Apod* (the zygotic form of *VegT*) and second, the zebrafish homologue, *zPAPC*, appears to be downstream of *spadetail* (*spt*) which is a likely orthologue of *VegT* (Griffin et al., 1998). In *spt* -/- embryos the expression of *zPAPC* is dramatically reduced, suggesting that *spt* is required for *zPAPC* expression (Yamamoto et al., 1998).

When *PAPC* was tested in the animal cap assay for its response to *VegT–GR* in the presence of DEX and/or CHX, it was not possible to show induction by *VegT* (Fig. 4.3). This suggests that *PAPC* is not downstream of *VegT* and that the zebrafish data cannot be extrapolated to *Xenopus*. However, Kim et al. (1998) have shown that injection of ectopic *VegT* mRNA can lead to ectopic *PAPC* expression in the *Xenopus* dorsal midline at stage 11.5 when it has normally been downregulated.

Therefore, there are two possibilities. First, *PAPC* may be downstream of *VegT*, but not directly, and there is insufficient time during the DEX treatment in the animal cap assay for induction of *PAPC* to be seen. Second, it may be that *VegT* is necessary but not sufficient for activation of *PAPC*. For example, there may be another factor, not present in animal caps but present in the normal expression domain of *PAPC*, that is also required and acts in concert with *VegT* to induce expression. In principle, these possibilities could be tested by repeating the CHX experiment and incubating the samples for longer or by repeating it in whole embryos.

Xenopus nodal related 1–6

Xenopus nodal related 1–6 (*Xnr1–6*) are members of the TGF-β superfamily and are most related to mouse *nodal*. Only *Xnr1–4* were studied in this thesis, as the cloning of *Xnr5* and *Xnr6* had not been reported when these experiments were carried out. *Xnr1* and *Xnr2* are excellent candidates for molecules downstream of *VegT*, as they are expressed in the same areas as *VegT* and they possess mesoderm-inducing activity (Jones et al., 1995). *Xnr4* is also a good candidate target gene as it is expressed in the marginal zone (albeit only a subset) and has mesoderm-inducing activity (Joseph and Melton, 1997). By contrast, *Xnr3* has neural-inducing properties and is expressed in the superficial layer of Spemann's organiser (Ecochard et al., 1995; Smith et al., 1995).

On the basis of this evidence, it was predicted that *Xnr1*, *Xnr2* and *Xnr4* might be responsive to *VegT* and that *Xnr3* might not. As shown in Fig. 4.3, this is exactly the result obtained. These results are consistent with recent reports, which also show that *Xnr1* is induced by *VegT* and that the gene contains potential T-box sites within its promoter (Agius et al., 2000; Hyde and Old, 2000; Kofron et al., 1999). In contrast, the *Xnr3* promoter does not appear to contain any T-box sites and is correspondingly unresponsive to *VegT* (Hyde and Old, 2000).

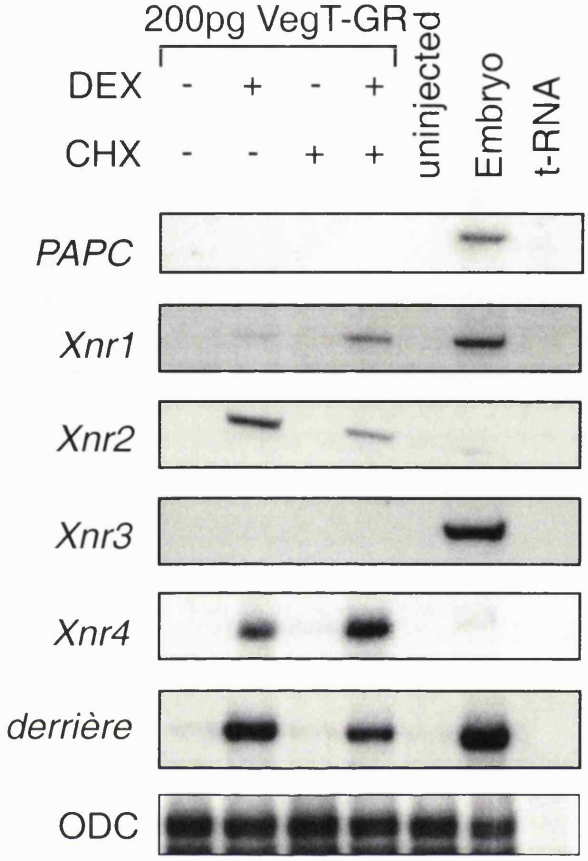

Fig. 4.3. Results of search for direct target genes of *VegT*. To test direct induction by VegT, VegT–GR was used in combination with DEX and CHX as shown in Fig. 4.2. ***PAPC***. *PAPC* is never induced in response to *VegT-GR* and DEX. ***Xnr1-4***. The mesoderm inducers *Xnr1*, *2* & *4* are induced by *VegT-GR* + DEX + CHX, whereas the neural inducer, *Xnr3*, is not. ***Derrière***. Injection of 200 pg VegT-GR is sufficient to induce expression of *derrière* on addition of DEX even in the presence of CHX.

The two untested members of the family; *Xnr5* and *Xnr6* (Takahashi et al., 2000), would be excellent candidates to be direct targets of *VegT*. Both these molecules are mesoderm inducers and have been shown to be downstream of *VegT* (Takahashi et al., 2000). Given their early onset of expression (stage 8.5–9), the prediction would be that they are directly downstream of *VegT*. Interestingly, while *Xnr1*, *Xnr2* and *Xnr4* are inducible by activin-like signalling, *Xnr5* and *Xnr6* are not, but they are induced by *VegT* (Clements et al., 1999; Jones et al., 1995; Takahashi et al., 2000). This would suggest a model in which *Xnr5* and *Xnr6* are the first genes of a regulatory cascade activated downstream of *VegT*.

Derrière

Derrière, another member of the TGF-β superfamily, has been shown to be an inducer of posterior mesoderm (Sun et al., 1999) and is an excellent candidate for a mesoderrm-inducing signal downstream of *VegT*.

- ***Expression pattern*** – the expression pattern of *derrière* parallels almost exactly the expression domain of *VegT* and *Apod* (compare Fig. 1.5 and 1.7).
- ***Activity*** – over-expression of *derrière* leads to the induction of a similar set of genes to those induced by *VegT* (Lustig et al., 1996; Stennard et al., 1996; Sun et al., 1999). Also, both have the ability to produce a secondary axis when injected into ventral vegetal blastomeres.
- ***Reliance on VegT*** – it has already been shown that *derrière* is induced by over-expression of *VegT* RNA (Clements et al., 1999; Sun et al., 1999; Yasuo and Lemaire, 1999) and that depletion of maternal *VegT* transcripts leads to a complete loss of *derrière* expression (Kofron et al., 1999).

These observations indicate that *derrière* is a gene that fulfils the requirements for a candidate direct target of *VegT*. When tested in the animal cap assay, *derrière* is induced in the presence of both DEX and CHX suggesting that it is a direct target of *VegT* (Fig. 4.3).

DISCUSSION

VegT–GR allows precise control of activation

An important issue to consider when using a hormone-inducible approach to investigate regulatory responses to a transcription factor is whether and to what extent the hGR ligand-binding domain affects the outcome of the assay (i.e. do genes respond any differently to *VegT–GR* than they do to *VegT*?). Simply adding another domain to the protein could affect its action either by changing the structure/folding of the protein or by masking a critical region. From previous experience this does not generally appear to be the case (Hollenberg et al., 1993; Saka et al., 2000; Smith et al., 2000; Tada et al., 1997), although it is still possible in specific instances.

The hGR domain used in these experiments has transcriptional activation function, which might alter the activity of the fusion protein, but because VegT has only ever been shown to act as a transcriptional activator, this is not critical in this context. Also, a mutated version of the hGR domain, which lacks this activity, is available and using it would remove this potential problem.

Another possible problem is that the hGR domain subtly affects the protein, such that its activation properties are not critically changed but specificity of target genes is affected. In this instance, the fusion protein might induce genes that contain T-box binding sites in their promoter but would not be activated by VegT under normal circumstances. In this way, it would be possible to generate false positives and so it is important to test whether unmodified VegT is able to induce any identified target gene.

It is important to remember that because a factor is sufficient to activate transcription of a gene directly in an animal cap assay, it does not mean that it is required to do so *in vivo*. For example, *Bix4* was originally identified as being a target of *Xbra*, although the expression pattern clearly shows that *Xbra* cannot be responsible for its entire expression pattern. This type of experiment only shows that a factor is capable of regulating the expression of a particular gene, not that it is the only factor capable of having this effect nor that it is even required. However, this caveat equally applies to any over-expression experiment. In summary, it is important to be aware of potential problems when using this approach, but the available data indicate that VegT–GR acts just like wild-type VegT with the added advantage of hormone-inducibility, allowing temporal control of action.

A search for direct target genes of *VegT*

The results of the candidate screen for targets of *VegT* are shown below (Table 4.1).

Table 4.1. Summary of results from the screen for direct targets of VegT

Gene	Induced by DEX	Induced by DEX + CHX
Bix4	✓	✓
PAPC	✗	✗
Xnr1	✓	✓
Xnr2	✓	✓
Xnr3	✗	✗
Xnr4	✓	✓
derrière	✓	✓

✓ = Induced by VegT as assayed by RNase protection
✗ = Not induced

I have identified four known genes as direct targets of *VegT*. I have also shown that two genes (*PAPC* and *Xnr3*) are not directly regulated by *VegT* and, indeed, within the time-frame of this assay they are not induced by *VegT* at all. As discussed above, these results are consistent with existing ideas about the regulation of various genes involved in mesoderm formation.

PAPC

One unexpected result was that *PAPC* was not induced by VegT-GR in this assay, even though it has previously been shown to be induced by *VegT*. The assays used were slightly different, however, in that Kim et al. (1998) showed ectopic expression of *PAPC* in response to over-expression of *VegT* in the marginal zone rather than the animal cap. Of the two explanations I offered earlier for this difference, it is less likely to be due to insufficient time for induction, as *PAPC* is initially expressed at stage 9.5 and the animal caps in the assay were cultured until stage 10.5 (an extra 1.5 hours). It is more likely that activation of *PAPC* requires a co-factor present in the marginal zone and not present in the animal cap. This could be tested by using VegT–GR in whole embryos.

Xnr4

One interesting question posed by these results is how the expression of *Xnr4* is restricted to the deep cells of the organiser. In the animal cap assay, *Xnr4* appears to respond to *VegT* just as well as *Xnr1* and *Xnr2* and yet, whereas all three genes are expressed throughout the vegetal hemisphere, *Xnr1* and *Xnr2* are expressed in the entire marginal zone and *Xnr4* is only expressed in a very small region of the margin. There are two explanations for this. First, some factor might repress the expression of *Xnr4* in the rest of the marginal zone. For example, genes expressed outside of the organiser might compete with *VegT* for binding sites in the promoter or *VegT* might be converted from an activator to a repressor by association with other factors expressed outside of the organiser region. Second, another factor, expressed on the dorsal side, may also be required for the expression of *Xnr4*. It is possible that this interaction is preserved in the animal cap assay. The induced expression of *Xnr4* would then only occur on the dorsal side of the cap. This spatial arrangement of expression would not be detected in an RNase protection assay. This dorsal factor might involve the β-catenin pathway. It has previously been shown that the organiser-specific genes *siamois* and *Xnr3* are activated by the β-catenin pathway (Brannon et al., 1997; McKendry et al., 1997) and that *Xnr1* can be activated synergistically by VegT and β-catenin to give elevated levels of expression within the organiser region (Agius et al., 2000).

Derrière is a direct target of *VegT*

The DEX/CHX animal cap experiment shows that *derrière* is a direct target of *VegT*, in that it can be induced even in the absence of protein synthesis. However, the RNase protection analysis (Fig. 4.3) shows that CHX treatment does reduce the level of induction of *derrière* by approximately 25%. This implies that at least some of the induction in response to DEX alone is mediated indirectly, requiring intermediate protein synthesis. Thus, although *VegT* is required for the expression of *derrière*, this effect may not occur solely through direct activation.

In the next chapter, I investigate and discuss the regulation of *derrière*.

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Chapter 5. Regulation of the *derrière* promoter

INTRODUCTION

VegT is an essential component of the developmental programme of *Xenopus* embryos (Kofron et al., 1999; Zhang et al., 1998). The results outlined in the previous chapter suggest that *derrière* is a direct target of *VegT* and a good candidate for an endogenous mesoderm-inducing factor; expression of *derrière* requires *VegT* (Kofron et al., 1999) and the CHX experiment in the previous chapter shows that induction of *derriere* by *VegT* must, at least in part, be direct. In order to investigate the regulation of *derrière*, I decided to study the *derrière* promoter. In this way, it will be possible to test the direct effects of VegT on the promoter both *in vitro* using reporter gene assays and *in vivo* using transgenic embryos.

Several T-box target genes have already been investigated. The first direct target gene to be identified was embryonic fibroblast growth factor (*eFGF*), a target of *Xbra*. Expression of *eFGF* is induced by *Xbra* by its direct action on the two T-box binding sites present in its promoter. Deletion of either T-box binding site leads to a dramatic decrease in induction by *Xbra* (Casey et al., 1998).

The first known direct target of *VegT* was *Bix4*, which requires maternal *VegT* RNA for its expression (Casey et al., 1999). The *Bix4* promoter contains three consensus T-box binding sites. Mutation of two of these sites leads to a complete loss of reporter gene expression in transgenic embryos (Casey et al., 1999).

At present, the only other direct T-box target in *Xenopus* whose promoter has been investigated is *Xnr1*. Upstream regions of this gene were isolated by two groups (Hyde and Old, 2000; Kofron et al., 1999) and putative T-box binding sites were identified. These promoter fragments respond to VegT in luciferase assays (Hyde and Old, 2000; Kofron et al., 1999), but Hyde and Old (2000) showed that this fragment drives expression only in the dorsal marginal zone in transgenic embryos, whereas endogenous expression occurs throughout the vegetal and marginal regions. Furthermore, mutation of these T-box sites has no effect on expression in transgenic embryos, an unexpected finding given that VegT is required for Xnr1 expression (Kofron et al., 1999).

As this brief summary shows, we are only beginning to understand how T-box genes regulate their target genes. We need more information in the form of examples of T-box targets and their promoters before we can attempt to make any generalisations about the mode of regulation of genes by T-box proteins.

Binding site specificity

Another question that can be investigated by the isolation of promoters is that of sequence specificity. Do different T-box proteins recognise different sequences? Binding site selection studies have been undertaken for some of the known T-box genes. These studies have shown that all the T-box proteins so far investigated bind a similar consensus sequence. For example, Conlon et al. (2001) undertook a comparative binding site selection study on Xbra, VegT and Eomes (*Xenopus* T-box proteins with overlapping expression patterns, but different effects on transcription). This showed that, although there are subtle differences in preference for nucleotides outside the consensus sequence, all three proteins selected the core sequence TCACACCT. And yet these T-box proteins have different effects *in vivo*.

One thing that was noticeably different between the consensus sites for the three proteins was the orientation and spacing of any double sites selected. Sites selected by Xbra are most often palindromic (designated →←) with no intervening nucleotides, whereas those selected by VegT are mostly palindromic but in the opposite orientations (← →) and almost always with a spacing of four nucleotides. Double sites selected by Eomes have two configurations (→ ← and → →)with 3–5 nucleotides between them.

These results suggest that the context of the binding site within a particular promoter (i.e. the sequence surrounding it) is probably important for defining specificity. For example, it is possible that particular co-factors are required (if there is a specific binding sequence near the site) or that the number, spacing and orientation of sites within a promoter influence the affinity with which particular T-box proteins bind to it.

Evidence for one of these possibilities has recently been reported (Bruneau et al., 2001; Hiroi et al., 2001). Nkx2.5, a homeobox gene involved in heart development, has been shown to interact with Tbx5 to promote expression of cardiac-specific genes (Bruneau et al., 2001; Hiroi et al., 2001). Binding sites for both proteins are found in three cardiac-specific genes – *connexin 40*, *atrial natriuretic factor* (ANF; Hiroi et al., 2001) and *natriuretic peptide precursor type A* (Nppa; Bruneau et al., 2001) – and *Tbx5* and *Nkx2.5* activate all three in a synergistic manner. All the T-box and Nkx binding sites in the *ANF* promoter are necessary for full activation of a reporter gene (Hiroi et al., 2001).

As a first step towards answering these question, I have cloned the *derrière* promoter. In this chapter, I have analysed the *derrière* promoter sequence for functional T-box binding sites and shown that this promoter fragment responds to VegT and is capable of driving appropriate expression of a reporter gene in transgenic embryos.

RESULTS

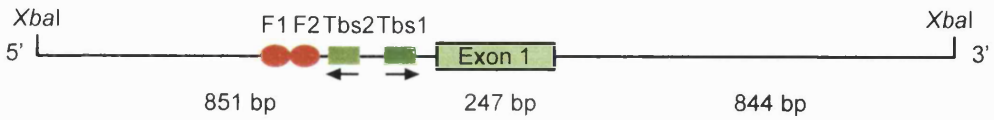
Isolation and investigation of the wild-type promoter

In order to study *derrière* and its regulation by *VegT* further, a *Xenopus* genomic library (Stratagene) was screened with a probe corresponding to the first 356 base-pairs of the *derrière* open reading frame (ORF). Four positive clones were picked, three of which were found to contain the first exon of *derrière*. Restriction digestion and Southern blotting identified a 2 kb *Xba* I fragment, containing exon1, which was sub-cloned into pBluescriptII (SK-) and sequenced. The 2 kb fragment (d1.2.1) consists of the 247 base-pair *derrière* exon 1 flanked by 850 base-pairs of upstream sequence and 844 base-pairs of intronic sequence (Fig. 5.1A). The sequence upstream of exon 1 is shown in Fig. 5.1B. The transcription start site was mapped by RNase protection (see ***Materials & Methods***) and is located approximately 30 base-pairs downstream of a putative TATA-box.

The *derrière* promoter contains potential T-box binding sites

Analysis of the upstream sequence identified two close matches to the consensus T-box binding site as defined by binding-site selection (Conlon et al., 2001). These sites also match half of the consensus *Xbra* binding site originally defined by Kispert and Herrmann (1993). The sites lie between 50 and 200 base-pairs upstream of the transcription start site and have been designated **Tbs1** (7/8 match) and **Tbs2** (6/8 match), as shown in Fig. 5.1B. A comparison with the consensus T-box binding site (Conlon et al., 2001) is shown in Fig. 5.1C.

A



B

F1 F2

GCCACTCAATGGAGGAATGGGTCTAATTAACATGCACATGTATATTCTGT

Tbs2

GGATGAGAACAGAGAGGTGTCTGACATAGGTGATTGTTTCATAGAAAGTG

Tbs1

GGGATTTAAATTAGAAAGAGAAGAGACAGGATGAGGGACTGTTGACACCT

CTCTCCTGACTCCAGAAAGCTGACAGATTTCACACATAGGACACTTGCAT

GGTATTAAGGCAGAAGCTCCAAGGCAGAAGTGCAGACCTACAGGAGAAGT

CAACatggcagagttgtggctatcactttcttgcatgttc

C

Consensus	TCACACCT
Tbs1	TGACACCT
Tbs2	AGACACCT

Fig. 5.1. Binding sites in the *derrière* promoter. (A) Diagram of the d1.2.1. fragment. The fragment consists of 851 base-pairs of sequence 5' to the start codon, the 244 base-pair first exon and 844 base-pairs of the first intron. (B) Partial sequence of the *derrière* promoter region. The transcription start site is indicated by an arrow and the beginning of the protein sequence is shown in lower case. Putative 8 base-pair binding sites are underlined. T-box sites (Tbs1 and Tbs2) are shown in green and Fast sites (F1 and F2) are shown in red. (C) Comparison of Tbs1 & 2 and T-box consensus binding site as defined by Conlon et al. (2001). Bases that differ from the consensus sequence are shown in red.

VegT binds Tbs1 *in vitro*

To ask whether VegT can bind either of these two sites, I performed electrophoretic mobility shift assays (EMSA) with probes corresponding to either Tbs1 or Tbs2. As shown in Fig. 5.2, VegT is able to bind Tbs1 *in vitro*. In this assay, I was not able to show binding of VegT to Tbs2 (data not shown).

Radiolabelled oligonucleotide probes were run on native polyacrylamide gels with or without the addition of *in vitro* translated HA-tagged VegT protein (VegT–HA). Addition of VegT–HA to the labelled Tbs1 probe results in a mobility shift indicating the formation of a VegT–HA–probe complex (Lane 4). This complex is not observed when using unprogrammed reticulocyte lysate to control for the presence of background complexes (Lanes 1–3). The binding is specific because it is competed out by a 100-fold excess of unlabelled probe, but not by an excess of an unlabelled mutant probe (Lanes 5 and 6). The mutant probe contains three base-pair changes in the 10 base-pair binding site, which have been shown previously to disrupt binding (Casey et al., 1998). The addition of an anti-HA antibody (Roche) results in a further shift of the VegT–HA–probe complex, showing that the complex contains the VegT–HA protein (Lanes 7–9).

These results show that, *in vitro*, VegT protein is able to bind Tbs1 but not Tbs2. Therefore, Tbs1 at least has the potential to act as a T-box binding site *in vivo*. Two possibilities exist for Tbs2. First, these eight base-pairs may have no role whatsoever to play in the regulation of *derrière*. Second, it is possible that although the sequence of Tbs2 has insufficient affinity for VegT alone, it may well function as a T-box binding site *in vivo*, in combination with other DNA binding proteins. VegT could dimerise with itself or another T-box protein bound to Tbs1 or it could bind co-operatively with another transcriptional complex such as activin response factor 2 (ARF2; comprised of Smad2, Smad4 and XFast3).

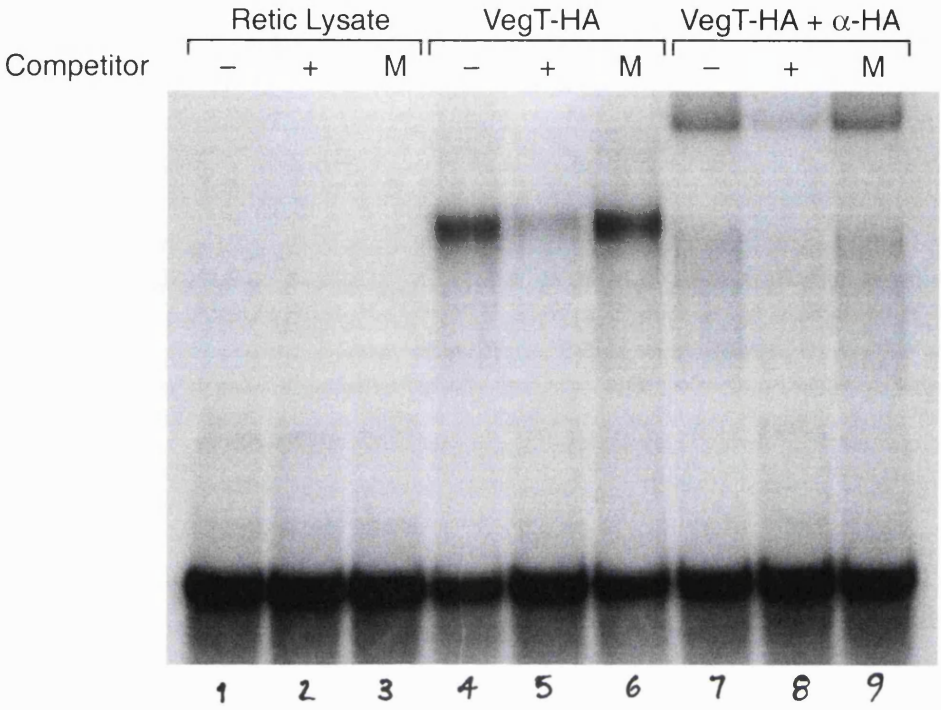


Fig. 5.2. VegT binds to Tbs1 *in vitro*. Electrophoretic mobility shift assay demonstrating that VegT protein binds to Tbs1. A [^{32}P]-labelled 36 base-pair probe containing Tbs1 was incubated with VegT–HA. (Lanes 1-3) Retic lysate incubated with probe to test for non-specific binding. (Lanes 4-6) VegT–HA + probe. Competition experiments were carried out by incubating protein with a 100-fold molar excess of unlabelled probe, including a mutated version of the site (M), prior to addition of probe. (Lanes 7-9) VegT–HA + probe + α–HA antibody. Super shift assays were carried out by incubating DNA–protein complexes with a rat monoclonal anti-HA antibody for 10 minutes on ice.

d851 is induced by both *VegT* and *Xbra in vitro*

To investigate whether Tbs1 and Tbs2 are able to mediate induction of transcription by *VegT*, I used a reporter construct in an animal cap assay. *Xenopus* embryos were injected at the one-cell stage with DNA for two reporter genes plus synthetic RNA for the inducer being tested. In this case, the two reporter genes were firefly luciferase and renilla luciferase and the inducing molecule was VegT. The firefly luciferase is driven by the promoter being tested, which in this case was 851 base-pairs of upstream sequence from the *derrière* promoter (pGL3-d851). The renilla luciferase gene is driven by a constitutive promoter (SV40 or HSV-TK) and acts as an internal control for the amount of DNA injected and the efficiency of transcription and translation. Animal caps were dissected at stage 8.5 and cultured until sibling embryos reached stage 10.5 (approximately 3–3.5 hours). The samples were then lysed and the activity of the reporter genes was assayed. The activities of both reporter proteins can be measured in a simple assay, which gives a measure of the activity of the promoter in response to the inducer.

As shown in Fig. 5.3A, the pGL3–d851 construct is induced equally strongly by *VegT* and *Xbra*, whereas *Eomes* is less effective. The induction produced by *VegT* is 4.8 times the background expression level seen with co-injection of *β-galactosidase* (+ lacZ) RNA. To investigate how accurately this reflects the *in vivo* situation, RNase protection analysis was carried out on animal caps injected with 1 ng of *VegT*, *Xbra* or *Eomes*. As shown in Fig. 5.3B, endogenous *derrière* is induced strongly by *VegT* and less strongly by *Xbra* and *Eomes*. Quantification of the results shows that *VegT* gives a mean induction of 15-fold, whereas induction by *Xbra* and *Eomes* is only 4-fold above background (+ lacZ). The caveat to this experiment is that the quantification is based on the assumption that all three RNAs are translated equally.

Overall, the data from the EMSAs and luciferase assays together suggest that VegT can induce transcription from the d851 promoter by direct binding to Tbs1.

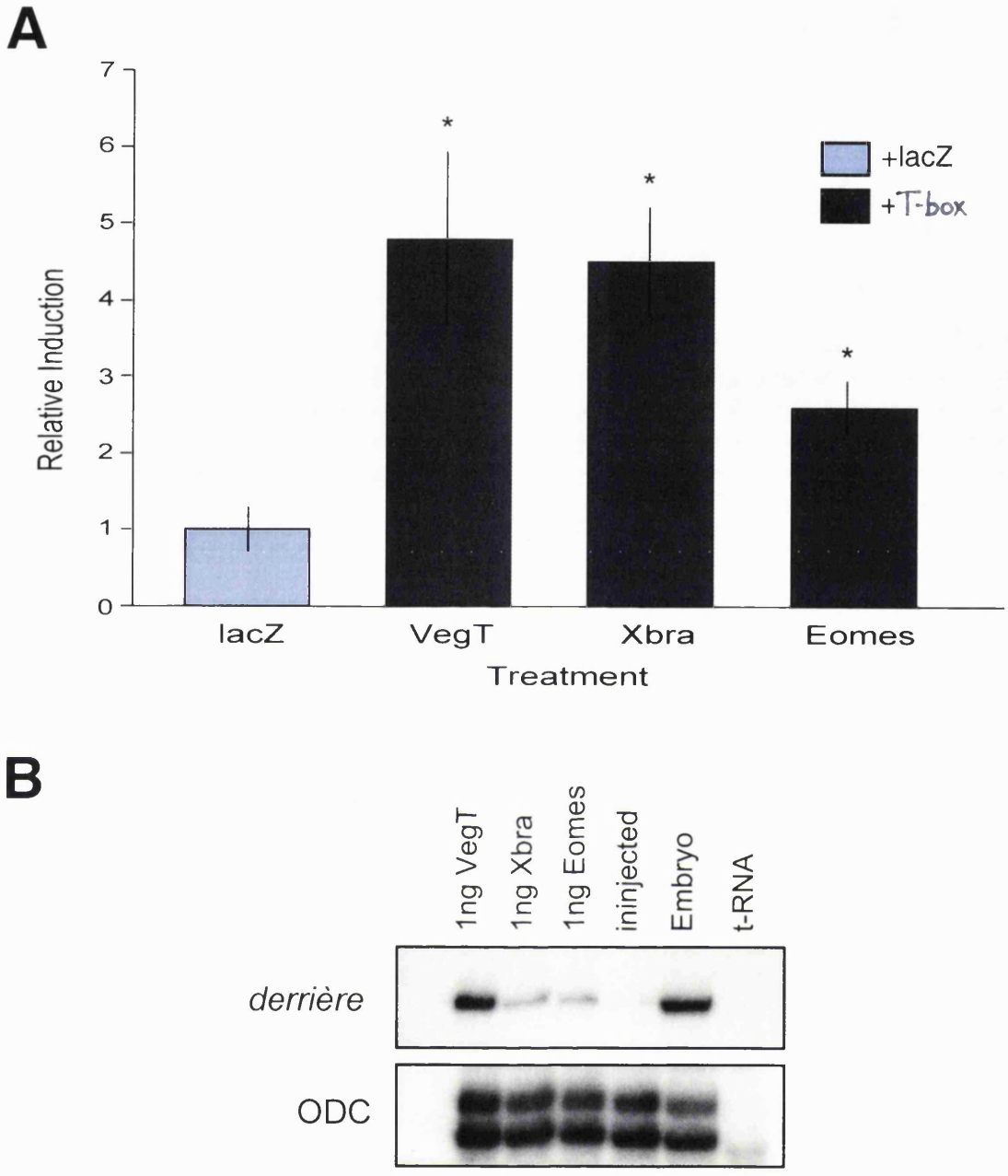


Fig. 5.3. *VegT* and *Xbra* induce expression of endogenous *derrière* and of *derrière* reporter constructs. (A) Both *VegT* and *Xbra* activate expression of the d851 reporter construct. Embryos were injected at the one-cell stage with DNA for reporter and reference plasmids and RNA for the test inducer. Animal caps were dissected at stage 8.5 and groups of five were assayed in triplicate for firefly and renilla luciferase activities 3.5 hours later. Embryos were injected with 20 pg pGl3-d851, 20 pg pRL-SV40 and, where indicated, 1 ng *VegT*, *Xbra* or *Eomes* RNA at the one-cell stage. Firefly luciferase activities were then normalised to renilla activities. Error bars indicate standard errors. Values significantly different ($p<0.05$) from background (+ lacZ) are indicated with an asterisk *.(B) Expression of *derrière* is induced in animal caps in response to *VegT*, *Xbra* and *Eomes*. Embryos were injected with the indicated RNAs at the one-cell stage. Animal pole regions were dissected at stage 8.5, cultured to stage 10.5, and then assayed by RNase protection for expression of *derrière*.

d851 is able to drive expression of a GFP transgene in an almost endogenous pattern

To address the question of the *in vivo* function of the *derrière* promoter fragment, a transgenic analysis was undertaken in parallel to the *in vitro* assays. The same 851 bp of upstream sequence was cloned into a vector containing a green-fluorescent protein (GFP) reporter gene (see ***Materials & Methods***). This construct was then used to produce transgenic embryos as described by Sparrow et al. (2000), a simplified version of the protocol of Kroll and Amaya (1996). These embryos were fixed at early gastrula (stage 10.5) and processed by *in situ* hybridisation to detect the GFP reporter RNA. *In situ* hybridisation was carried out instead of directly visualising the fluorescence of the GFP protein for two reasons. First, at gastrula stages the vegetal pole (prospective endoderm) of the *Xenopus* embryo contains a large supply of yolk granules, which auto-fluoresce at a very similar wavelength to that of GFP, making visualisation technically difficult. Second, I felt that *in situ* hybridisation would more accurately measure transcriptional output.

Analysis of these embryos showed that this 851 base-pair fragment is sufficient for correct initial induction of the GFP transgene in both the mesoderm and the endoderm of early gastrula embryos (Fig. 5.5A–B). It appears, however, that the transgene is not down-regulated in involuted mesoderm, as is the endogenous gene, because GFP RNA persists in these cells (Fig. 5.5C). One explanation of this observation is that there is a negative element, not present in the 851 base-pair 5' region, which leads to downregulation of transcription in involuted mesodermal cells. Another possibility is that the GFP RNA is more stable than the endogenous *derrière* RNA. Thus, although transcription of the GFP transgene has ceased, perdurance of the GFP RNA would give the impression of ectopic expression. At the moment, it is not possible to distinguish between these two possibilities.

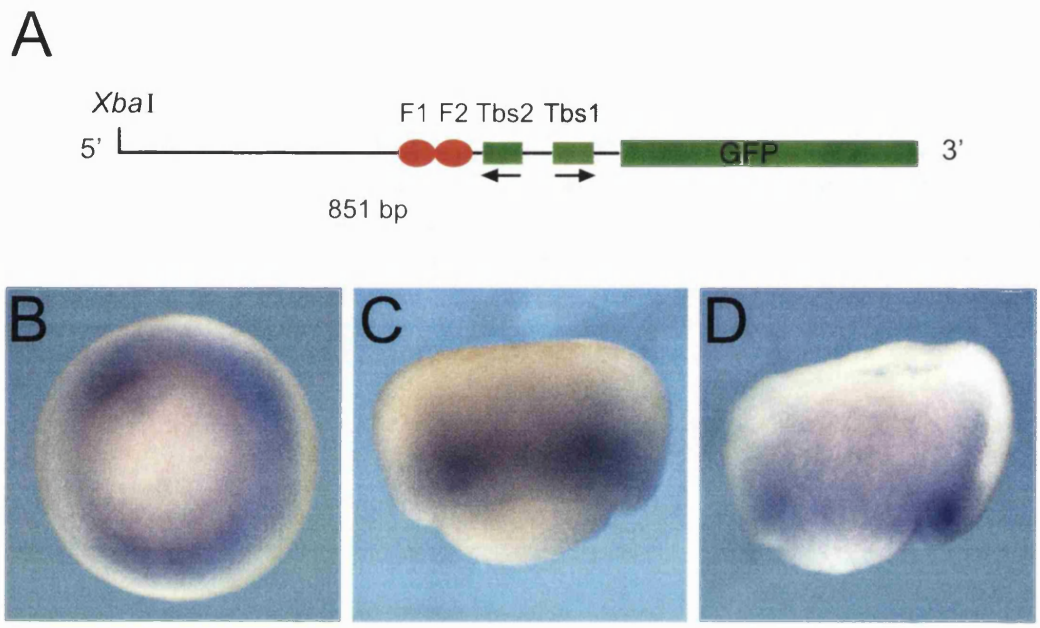


Fig. 5.4. d851 drives expression of a GFP reporter gene in transgenic embryos. Expression of wild-type transgene in transgenics. (A) Diagram of transgene. The entire 851 base-pairs of derrière upstream sequence was cloned into pGL3-GFP upstream of the GFP cDNA. (B–D) Transgenic embryos carrying the transgene. In situ hybridisation for GFP RNA. (B) Stage 10.5 Vegetal view. d851-GFP is able to drive expression in both the mesoderm and endoderm of gastrula stage embryos. (C) Dorsal view of embryo in B. (D) Lateral view of a stage 10.5 embryo with dorsal blastopore lip to the right. This embryo was bisected before the staining procedure. Expression of the transgene persists in the involuted mesoderm, which differs from the endogenous pattern.

DISCUSSION

Regulation of *derrière* expression

The preliminary analysis described in this chapter shows that both VegT and Xbra can induce expression from a *derrière* promoter fragment in the same manner as the endogenous gene. Interestingly, the relative effectiveness of the proteins varies between the *in vitro* assay and the endogenous situation. Why is it that *VegT* and *Xbra* appear equally effective in the *in vitro* assay and yet *VegT* is 3.5-fold more effective in the endogenous context than either *Xbra* or *Eomes*?

The simplest explanation is that there are differences in the amount of Xbra, VegT or eomesodermin protein produced from the same amount of RNA. Also, it may be that the promoter fragment being used here does not completely recapitulate the response of *derrière* to exogenous factors.

Another possibility is that a component of the regulation of the *derrière* promoter is at a structural level that again is not mimicked by the plasmid-based luciferase system. If the exogenous plasmid reporters are not packaged in the same chromatin configuration as the embryo's genomic DNA this could lead to differences in the effects of regulatory proteins. These assays, however, do show that VegT is capable of activating this promoter fragment and that the sequence contains at least one functional T-box site for direct regulation.

VegT is unable to bind Tbs2 *in vitro*

Tbs2 did not bind VegT in EMSAs. Tbs2 varies from the consensus at two positions and as such may not function as a T-box binding site *in vivo*. However, there is evidence from other systems that similarly divergent sequences can nevertheless be important for transcriptional responses.

In *Drosophila*, the gene *orthopedia* (*otp*) is activated by Brachyenteron, a homologue of Brachyury (Singer et al., 1996). Analysis of the *otp* promoter revealed three regions containing matches to the consensus T-box binding site (Kusch et al., 2002). Two of these regions were able to drive expression in transgenic embryos and luciferase assays, whereas the third could not drive any expression at all.

This third fragment contained seven 'low-affinity' binding sites, which show low similarity to the consensus site. However, although this region was unable to activate transcription on its own, when coupled to one of the other two fragments it acted in a synergistic manner – more than doubling the amount of activation (Kusch et al., 2002).

Kusch et al. (2002) also showed that in one of the two regions that does activate transcription, one of the putative T-box sites is required for activity of the promoter fragment and mutation of this site leads to an almost total loss of expression. Despite this, mutation of any of the other four more divergent sites caused a drop in expression, showing that all of the sites are required for full activation of the promoter (Kusch et al., 2002). This suggests that even sites that are relatively divergent from the consensus TCACACCT can act as T-box sites in the presence of a site matching the consensus and can contribute to the overall level of expression.

Transgene expression is not down-regulated in the involuted mesoderm

I used transgenic embryos expressing GFP under the control of the *derrière* promoter to show that, although this promoter fragment is able to recapitulate the initial expression of *derrière*, driving expression in both the marginal zone and vegetal pole, the expression is not identical to the endogenous pattern. In particular, the GFP transgene remains expressed in the involuted mesoderm where it is normally down-regulated.

There are two simple explanations for this. These are outlined below.

1) Transcriptional control

If there is an element, missing from the current reporter construct, that controls the downregulation of *derrière* in involuted mesoderm, it should be possible to search for such a sequence using larger pieces of genomic DNA in an attempt to see if the GFP reporter RNA is down-regulated at the same time as the endogenous transcript.

2) RNA stability

One approach to the question of RNA stability is to test the differential stability of GFP versus *derrière* RNA in *Xenopus* embryos. This particular experiment might be technically difficult as the difference may only be apparent in the involuted mesoderm and it would also be difficult to distinguish between injected and endogenous *derrière* RNA. However, a chimeric GFP construct might be more instructive. The stability of many RNAs is dependent on sequences in the 5' and 3' untranslated regions (UTRs). A chimeric construct containing the GFP ORF (used earlier in this chapter) surrounded by the *derrière* 5' and 3' UTRs might show a decreased stability compared with GFP alone. This construct expressed under the control of the d851 promoter in transgenic embryos may show a pattern more like the endogenous gene. It would then be possible to carry out a deletion analysis to identify the specific sequences in the 5' or 3' UTR involved in the destruction of the RNA in involuted mesoderm.

Finally, when discussing the results obtained using this promoter fragment, it is important to remember that I may not have isolated all the regions that contribute to the correct expression of *derrière*; rather, I have merely analysed sequences capable of driving the initial induction in the vegetal hemisphere and marginal zone. There will clearly be other inputs to the expression of this gene. For example, *derrière* is expressed again after gastrulation in the left lateral plate mesoderm (Hanafusa et al., 2000) in a similar manner to *Xnr1* (Lowe et al., 1996). This expression is driven by a conserved TGF-β-responsive element in intron 1 of *Xnr1* and the related mouse gene *nodal* (Osada et al., 2000). The same may be true for *derrière*. Currently, I have not tested whether the promoter or intron sequence that I have sub-cloned can drive any expression at that later stage. There may also be regulatory elements 3' to the coding region, which would be interesting to study.

In the next chapter, I discuss my investigation of the regions of the promoter required for expression in both luciferase assays and transgenic embryos.

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Chapter 6. Mutational analysis of the *derrière* promoter

INTRODUCTION

Several reports have been published since the cloning of *derrière*, which give insights into its regulation. Initially, Sun et al. (1999) showed that *derrière* was inducible by over-expression of RNA for both T-box (*VegT* and *Xbra*) and TGF-β (*activin* and *BVg1*) molecules and also by treatment with bFGF and derrière protein. Since then, induction of *derrière* by VegT has been shown by other groups (Clements et al., 1999; Yasuo and Lemaire, 1999) and Kofron et al. (1999) showed that expression of *derrière* depends on the presence of VegT.

Yasuo and Lemaire (1999) studied the profile of *derrière* expression in the early embryo. In intact embryos, *derrière* is expressed starting at mid-blastula (stage 9), rising rapidly up to the start of gastrulation (stage 10). This expression profile is not affected by dissociating the cells from the 2-cell stage until blastula stage 8. However, if the embryos are dissociated from the 2-cell stage until gastrula stage 10, *derrière* is still expressed at the same level at stage 9, but expression levels do not rise between stage 9 and stage 10 (Yasuo and Lemaire, 1999). Also, CHX treatment of embryos from pre-MBT stages (stage 7) onwards leads to a decrease in *derrière* expression, although there is residual expression presumably activated by maternal factors (Yasuo and Lemaire, 1999). This suggests there are two phases of *derrière* expression; an initial cell autonomous induction by a maternal factor(s) and a later zygotic phase requiring cell-cell communication.

The induction of *derrière* by *VegT* in animal caps is mostly cell autonomous (Clements et al., 1999), consistent with the idea that the maternal factor might be VegT. Experiments using various inhibitors of signalling, aimed at identifying the molecules involved in the cell non-autonomous phase of expression, have been carried out by several groups. Over-expression of a dominant-negative form of the activin type II receptor (DN-ActRII) does not cause a decrease of *derrière* expression in vegetal explants at stage 10 suggesting that TGF-β signalling is not required for expression of *derrière* (Yasuo and Lemaire, 1999). This experiment did not, however, investigate the marginal expression of *derrière* at this time.

Expression of the nodal-specific inhibitor Cerberus-short (Cer-S) was used to investigate the involvement of nodal signalling in the control of *derrière* expression (Agius et al., 2000). Over-expression of Cer-S has no effect on the expression of *derrière* between stages 8.5 and 9.5. Also, expression of the dominant-negative FGF and activin receptors (XFD and ΔXAR) has no effect on expression of *derrière* at stage 9. However, both completely block *derrière* expression at stage 11 (White et al. 2002). These results confirm those of Yasuo and Lemaire (1999) that show that cell-cell communication is only required for the expression of *derrière* at later stages and this suggests that both TGF-β and FGF signalling may be involved in the late maintenance phase of expression.

I have shown (see ***Chapters 4 and 5***) that VegT is able to induce *derrière* in a direct manner and that the *derrière* promoter contains T-box binding sites. In order to show whether these binding sites are essential for expression of the reporter, I have undertaken a mutagenesis strategy using both the luciferase and transgenesis assays. It is important to remember that the promoter fragment we are using does not necessarily represent the entire control region for *derrière*. However, the fragment appears to recapitulate quite well the initial induction and maintenance phases of expression so the analysis will be limited to the early stages of expression from mid-blastula to mid-gastrula (stage 9–11).

RESULTS

VegT-responsiveness of d851

To assess the requirements for the two T-box sites (Tbs1 & 2) in VegT-responsiveness, both *in vitro* and *in vivo*, mutations were introduced into the sites both singly and together in the context of both reporter systems (luciferase and GFP). The mutations used were the same three base-pair changes shown using *in vitro* binding assays (***Chapter 5***) to disrupt binding of VegT (see Fig. 6.1A). The mutagenesis was carried out by two-step PCR. Two overlapping PCR products were produced using primers containing the mutations and primers outside the insert region. These products were then pooled and a further round of PCR using just the most 5' and 3' primers was used to produce the final mutagenised insert. This was then cloned back into the *Mlu* I/*Hind* III sites of pGL3-basic and pGL3-GFP to give pGL3–GFP -d851/ΔTbs1/ΔTbs2/ΔTbs1,2 (see ***Chapter 2*** and Fig. 2.1).

Tbs1 and Tbs2 are not required for reporter gene expression

These constructs were then tested in both luciferase assays and transgenic embryos. The results are shown in Fig. 6.1B. When tested using the luciferase reporter in animal caps, mutation of either T-box site singly or both together had no effect on the level of induction of the reporter in response to VegT. All constructs responded equally well to VegT giving inductions of approximately 30-fold over the negative (+ β-gal) controls.

In agreement with these results, transgenic embryos carrying any of the mutated constructs still show correct expression of the GFP transgene in both the endoderm and mesoderm. This is shown in Fig. 6.1C–F. These embryos have rather variable expression patterns and expression levels due to the fact that every expressing embryo has a different number of independent random insertion events, which leads to mosaicism and position effects. However, embryos consistently show expression in the correct domains and ectopic expression is limited to small patches of highly expressing cells.

It is clear from these experiments that although *VegT* is able to activate the *derrière* promoter directly, the two potential T-box sites currently identified are not necessary for this induction in animal caps. The two most obvious possible reasons for this are first, that this promoter fragment contains a T-box binding site as yet unidentified by sequence criteria that is sufficient for the wild-type level of induction of the reporter and second, that *VegT* is able to activate the promoter in an indirect manner. This is consistent with the results from the DEX/CHX experiment (Fig. 4.3) which showed that the induction of endogenous *derrière* is made up of both an early direct component and a later indirect one.

Table 6.1. Expression patterns of reporter gene constructs

Reporter Construct	Number of Experiments	Expression in Endoderm & Mesoderm	No expression	Embryos examined
pGFP-d851	6	121 (57.3%)	90	211
pGFP-d851 ΔTbs1	3	63 (53.4%)	55	118
pGFP-d851 ΔTbs2	3	54 (55.7%)	43	97
pGFP-d851 ΔTbs1,2	3	87 (56.9%)	66	153
pGFP-d851 ΔFast	1	8 (53.3%)	7	15
pGFP-d851 ΔTbs1,2 ΔFast	1	6 (50%)	6	12

Tbs1 and Tbs2 are required for the initial phase of *derrière* induction

To investigate the possibility of an early direct phase followed by a later indirect phase of induction by VegT, I performed a simple variant of the original luciferase assay. In this experiment, the animal cap explants are split into two groups. One group is cultured for the same length of time as previously (approximately 3–3.5 hours). The other is collected approximately one hour earlier. As can be seen in Fig. 6.2, this leads to a dramatic difference in the outcome of the experiment. Now, although induction of either the wild-type construct or ΔTbs1,2 again look identical after 3 hours (with an approximately 30-fold induction above negative controls), the induction of ΔTbs1,2 after only 2 hours is significantly reduced compared to wild-type and only slightly larger than the negative control (+ β-gal).

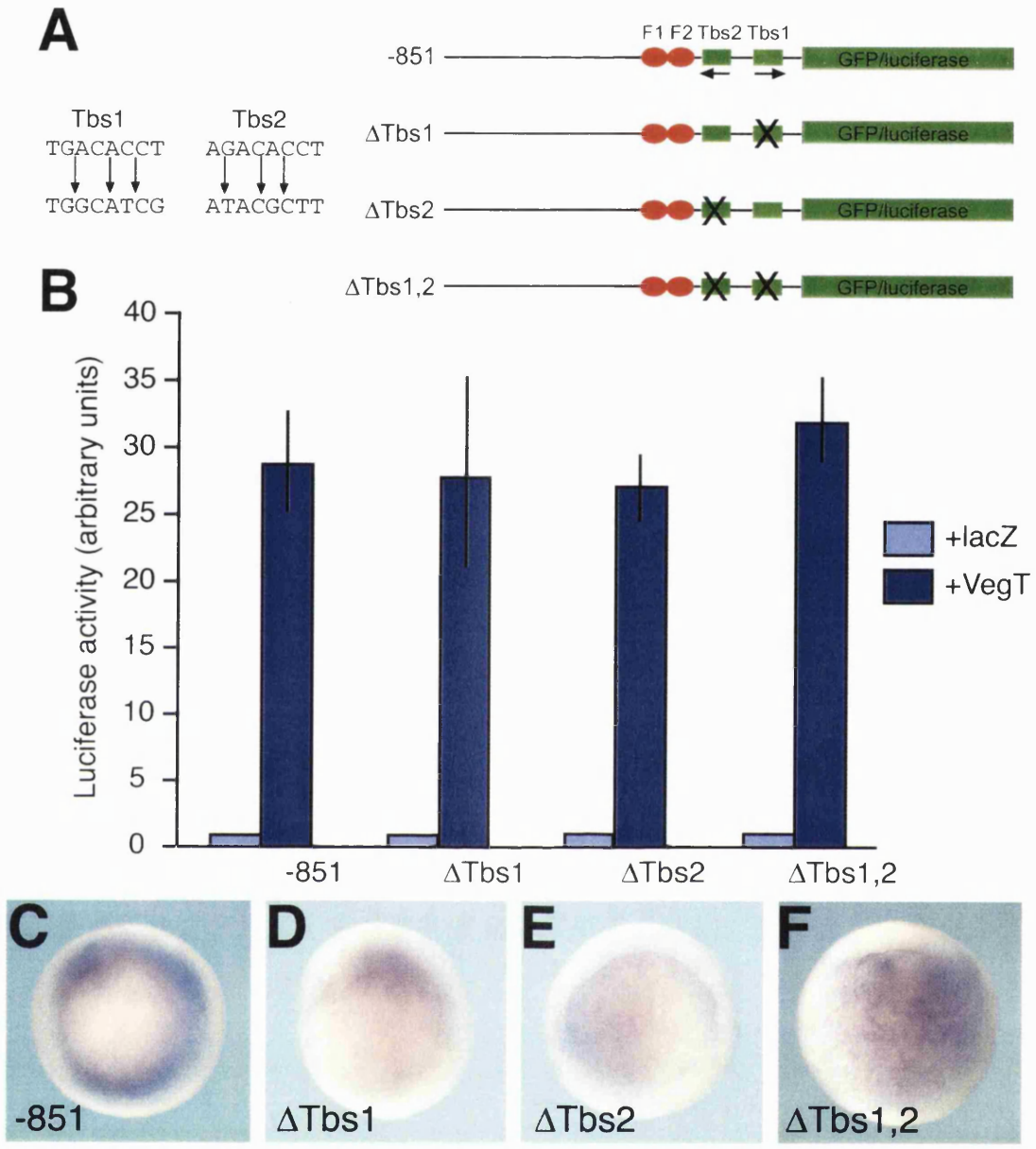

Fig. 6.1. Tbs1 & 2 are not required for induction by VegT. (A) Mutations and constructs. Tbs1 and Tbs2 were mutated singly and in combination in the context of the d851 promoter fragment. Three base-pair changes were made in each site, which have previously been shown to disrupt binding (Tada et al., 1998). (B) VegT is able to activate mutated constructs in luciferase assays in animal caps. Embryos were injected at the one-cell stage with 20 pg pRL-SV40, 1 ng lacZ or VegT RNA and, where indicated, 20 pg d851, ΔTbs1, ΔTbs 2 or ΔTbs 1,2. Animal caps were dissected at stage 8.5 and groups of five were assayed in triplicate for firefly and renilla luciferase activities 3 hours later. Firefly luciferase activities were then normalised to renilla activities. Error bars indicate standard errors. The constructs used are shown above the graph. (C–F) Constructs carrying mutations in Tbs1 and Tbs2 are still expressed in transgenic embryos. Stage 10.5 vegetal view.

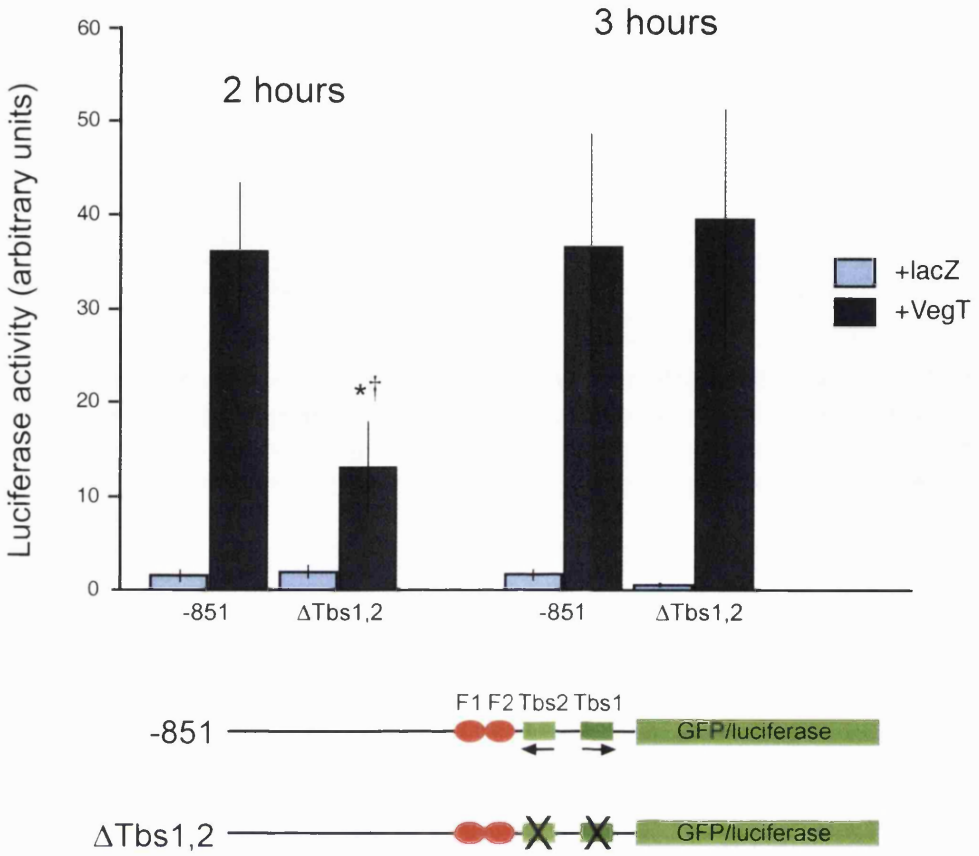

Fig. 6.2. Tbs1 and Tbs2 are required for initial induction of d851 by VegT. Embryos were injected at the one-cell stage with 20 pg pRL-TK , 1 ng lacZ or VegT RNA and, where indicated, 20 pg d851 or ΔTbs 1,2. Animal caps were dissected at stage 8.5 and groups of five were assayed in triplicate for firefly and renilla luciferase activities either 2 or 3 hours later. Firefly luciferase activities were then normalised to renilla activities. Error bars indicate standard errors. * = value significantly different ($p<0.05$) from pGL3–d851 + VegT (collected at same time). † = values not significantly different ($p>0.05$) from background control (+ lacZ). The constructs used are shown below the graph.

This result suggests that the second explanation given above is more likely to be correct. Thus, in the previous experiments, the initial direct induction of the promoter by *VegT* is probably reduced or absent, but there is sufficient time before stage 10.5, when the caps are collected, for an indirect mechanism of activation to recover the induction to wild-type levels. These results are consistent with the finding in ***Chapter 4*** that the induction of *derrière* by VegT is reduced by CHX treatment (Fig. 4.3).

This scenario would require *VegT* to activate the transcription of an intermediate gene which is then able to activate the d851 promoter fragment. This hypothetical factor would probably be another direct target of *VegT* and is most likely to be either a transcription factor or a signalling molecule. The results from Yasuo et al. (1999) suggest that cell-cell signalling is involved. Thus, the best candidates for this intermediate signal are *derrière* itself or any one of the nodal-related genes *Xnr1,2,4,5 & 6*).

Direct regulation of d851 by VegT

In order to investigate the direct effects of *VegT* on d851 it was necessary to find some method of negating the indirect effects of VegT. A variety of methods were considered and these are discussed below:

1) DN-ALK4

Chang et al. (1997) constructed a dominant-negative form of the type I activin receptor, which lacks the intracellular domain. It does not possess the kinase domain, which is necessary for signalling. This construct should be able to dimerise with the wild-type type II receptor, but not initiate signalling and so should act in a dominant-negative fashion, inhibiting all activin-like signalling. There are two concerns with this approach. First, the specificity of this construct is currently unclear, as it is unknown to what extent it inhibits signalling by other activin-like TGF-β molecules such as the *nodal-related* genes and *derrière*. Second, even if completely effective, this construct would only inhibit the indirect effects of activin-like TGF-β signalling and, although it is clear that this can occur (see ***Feedback by TGF-β signalling***), we have not ruled out indirect effects by another mechanism.

2) Inhibition of protein synthesis

One way to inhibit any indirect effects by intermediate genes would be to inhibit protein synthesis using CHX, as in the original *VegT–GR* experiments. The only drawback to this is that the luciferase assay relies on the production of the luciferase protein and CHX would inhibit this as well as all other protein synthesis. This experiment would require using a method of detection based on RNA rather than protein. For example, it would be feasible to use RT-PCR to assay the level of induction of firefly luciferase RNA relative to renilla luciferase RNA. I considered using this approach, but the two methods described below would not require changing the assay, but would still stop indirect effects.

3) Luciferase assays in tissue culture cells

In an attempt to avoid anything other than the direct effects of VegT a series of experiments was carried out in COS cells. This heterologous system should allow only direct effects of VegT protein. This involved transfecting the cells with DNA for both reporters (Renilla and Luciferase) and a plasmid to drive expression of the test inducer (β-gal or VegT) and assaying the cell lysate for luciferase activity. However, these experiments were abandoned as it was never possible to get an appropriately high and consistent induction from the wild-type construct, leaving nothing with which to compare the mutated versions.

4) Luciferase assays in oocytes

Mature (stage VI) *Xenopus* oocytes are transcriptionally silent, because their genomes are in an inactive chromatin state (Davidson, 1986). However, the transcription and translation machinery are present, so that gene activation can occur subsequently at MBT. Thus, the apparatus necessary to transcribe and translate the luciferase reporter genes is present. However, the oocyte's endogenous genome will not respond to the over-expressed VegT protein, and so only the direct effects of VegT protein on the promoter fragment will be measured.

Tbs1 is required for expression in oocytes

In light of the problems encountered using COS cells, it was decided to carry out these experiments in oocytes. Oocytes were obtained and defolliculated by collagenase treatment. Mature stage VI oocytes were selected for these assays (as they have an inactive arrangement of chromatin) and were left in fresh medium overnight. Surviving oocytes were then injected in the vegetal pole (in order to avoid the germinal vesicle) with 4 ng β-gal or VegT RNA. The oocytes were cultured for 24 hours to allow translation of the injected RNA, at which point DNA was injected into their germinal vesicles. The DNA mixtures contained pRL-TK (renilla) and either pGL3-d851, ΔTbs1, ΔTbs2 or ΔTbs1,2.

After another 24 hours, oocytes were collected and assayed. Each sample was measured in triplicate with approximately 10 oocytes per replicate. The results are shown in Fig. 6.3. The wild-type construct (d851) shows a consistent large induction on addition of VegT RNA. This induction is almost completely abolished when Tbs1 is mutated and the induction is lowered further when both Tbs1 and Tbs2 are mutated. Loss of Tbs2 on its own appears to have no effect on the behaviour of the reporter. However, in the absence of Tbs1 it can still drive a low level of expression and this is abolished on mutation of Tbs2. These results, together with those shown above, imply that the early direct effects of VegT on transcription from this promoter are mediated by Tbs1 and to some extent Tbs2 and that they are required for this phase of expression. However they are not required to produce the appropriate level of expression at early gastrula, suggesting that the indirect effects of VegT, acting towards the beginning of gastrulation, are sufficiently strong and rapid to produce wild-type levels of expression by early gastrula.

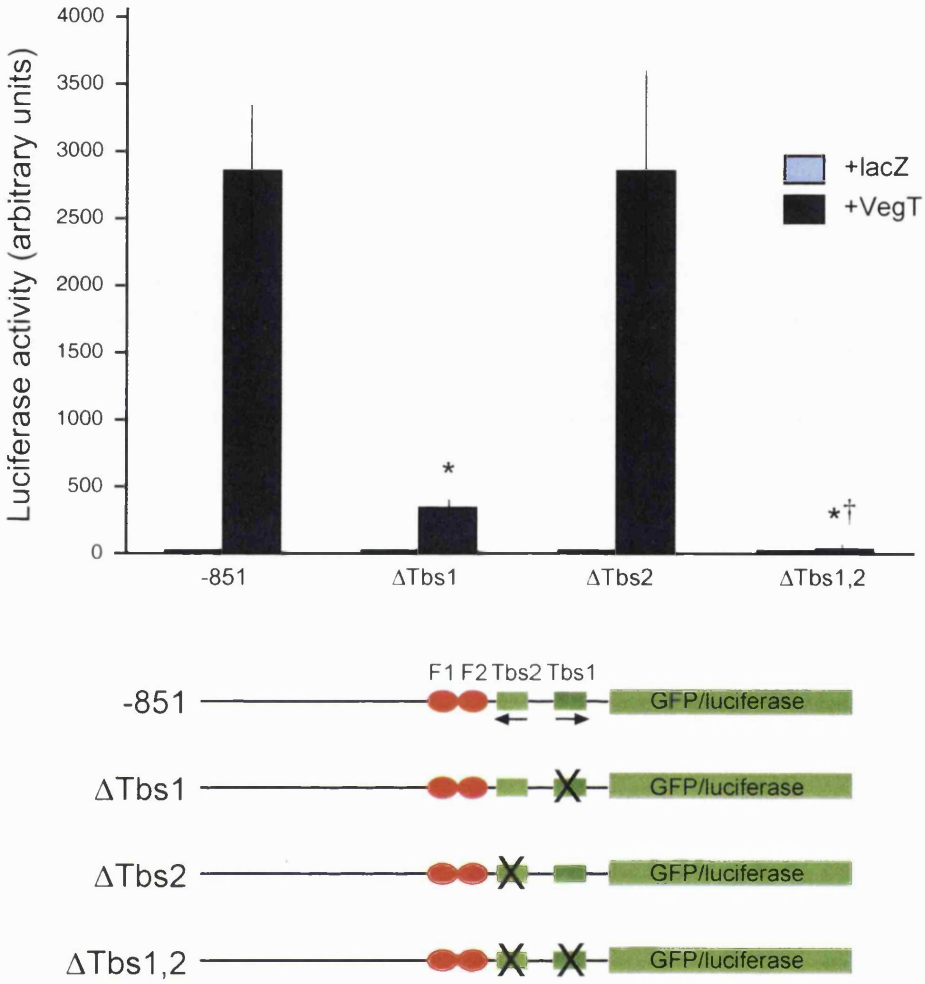

Fig. 6.3. Tbs1 and Tbs2 are required for induction of d851 in oocytes. Oocytes were injected with 4 ng lacZ or VegT RNA. After 24 hours they were injected with 1 ng d851 or ΔTbs1,2 and 1 ng pRL-TK. Groups of 10 oocytes were collected after another 24 hours and assayed in triplicate. Firefly luciferase activities were then normalised to renilla activities. Error bars indicate standard errors. * = values significantly different ($p<0.05$) from pGL3–d851 + VegT. † = values not significantly different ($p>0.05$) from background control (+ lacZ). The constructs used are shown below the graph.

Feedback by TGF-β signalling

It is already known that TGF-β molecules are able to regulate their own transcription. For example, *Xnr1* responds to TGF-β signalling via a Fast-responsive module in its first intron (Osada et al., 2000). Therefore, in the case of the *derrière* promoter, positive feedback may account for the fact that *VegT* is able to activate this promoter fragment indirectly. The diagram in Fig. 6.4 shows how this would produce the results seen.

According to this model, embryos over-expressing *VegT* would activate the wild-type reporter gene. When both Tbs1 and Tbs2 are mutated, even though the over-expressed *VegT* is unable to bind directly to the T-box sites present in the d851 promoter, it can still activate endogenous TGF-β genes including *derrière* itself and *Xnr1*, *2*, *4*, *5* and *6*. These could then activate the promoter indirectly by signalling through ALK4 and stimulating the formation of ARF1/2, a transcriptional complex containing SMAD2, SMAD4 and XFast1/3 (Howell et al., 1999).

The d851 fragment contains three consensus Fast binding sites (positioned at 194, 209 and 690 nucleotides 5' of the transcription start site). It is therefore possible that these sites comprise a TGF-β-response element, which could account for the indirect induction by *VegT*. To investigate this possibility, this promoter fragment was tested for TGF-β-responsiveness.

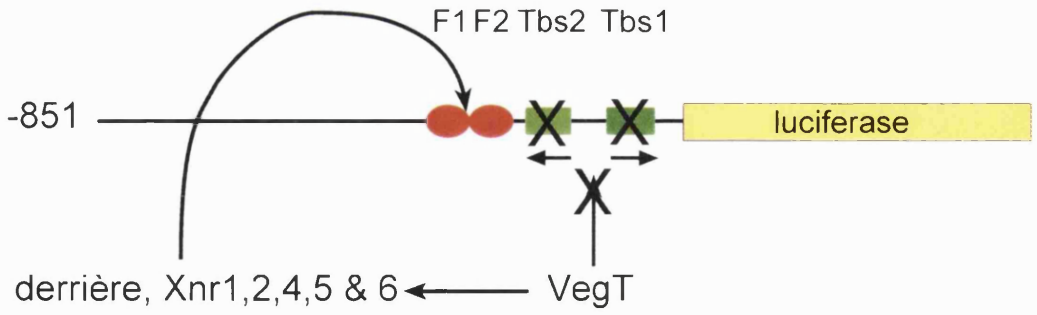

Fig. 6.4. Indirect induction of ΔTbs1,2 by VegT-induced TGF-β signalling. With the T-box sites mutated, VegT is unable to induce expression by directly binding to Tbs1 and Tbs 2 (shown in green), but it induces expression of the TGF-β family members *derrière*, *Xnr1*, *Xnr2*, *Xnr4*, *Xnr5* and *Xnr6*. Signalling by TGF-β then activates the promoter through the Fast sites (shown in red).

Activin-responsiveness of d851

To test the TGF-β-responsiveness of d851, luciferase assays were performed in animal cap explants as before, except that the inducing molecule was changed to *activin* RNA. Here, *activin* was used as a generic test TGF-β as there are so many endogenous candidates that it may not be helpful to try to single molecules out before testing the initial premise. A small deletion series of the reporter constructs was prepared by PCR and used in this assay to isolate a TGF-β responsive region. The results are shown in Fig. 6.5.

The full-length wild-type construct responds to activin stimulation, showing an 11-fold induction above the background expression level seen with co-injection of β-gal RNA. The deletion series shows that *activin*-responsiveness is confined to a 127-bp region (ΔTBS), which coincides with two of the Fast sites (at 194 and 209 nucleotides 5' of the transcription start site) and both T-box sites. To assess the relative importance of these sites, constructs were prepared using PCR containing mutations in both Fast sites either with or without the ΔTbs1,2 mutations. The mutations introduced into the Fast sites were single base-pair changes in each site in the 7th base-pair (AATNNACA → AATNNAGA). These have been shown previously to completely disrupt binding of Fast1 (Yeo et al., 1999; Zhou et al., 1998).

Two Fast sites and Tbs1 & 2 are required for activin-responsiveness

When these mutations are introduced into the reporter construct *activin*-responsiveness is reduced from 25-fold in these particular experiments to 10-fold and in combination with ΔTbs1,2 induction is completely abolished (See Fig. 6.6). Thus, both Fast sites and T-box sites are required for full induction by *activin*. It is probable that *activin* expression initiates a positive feedback loop as *activin* is also able to activate the expression of T-box genes such as *VegT* and *Xbra*. The interpretation of this experiment is that the Fast sites are the transducers of the direct activin-induced stimulation and the T-box sites provide an indirect mechanism of induction through the activation of T-box genes.

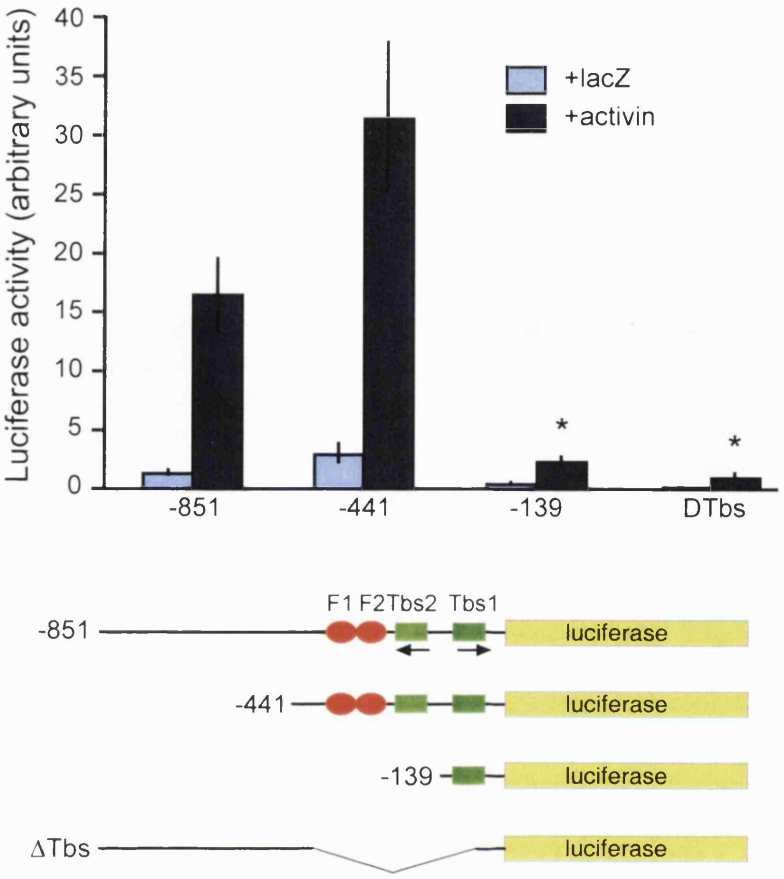

Fig. 6.5. Activin responsiveness of d851. Deletion analysis of the *derrière* promoter. Embryos were injected at the one-cell stage with 20 pg pRL-TK, 1 ng lacZ or activin RNA and, where indicated, 20 pg d851, d441, d139 or ΔTBS. Animal caps were dissected at stage 8.5 and groups of five were assayed in triplicate for firefly and renilla luciferase activities 3 hours later. Firefly luciferase activities were then normalised to renilla activities. Error bars indicate standard errors. * = value significantly different ($p<0.05$) from pGL3–d851 + activin. The constructs used are shown below the graph.

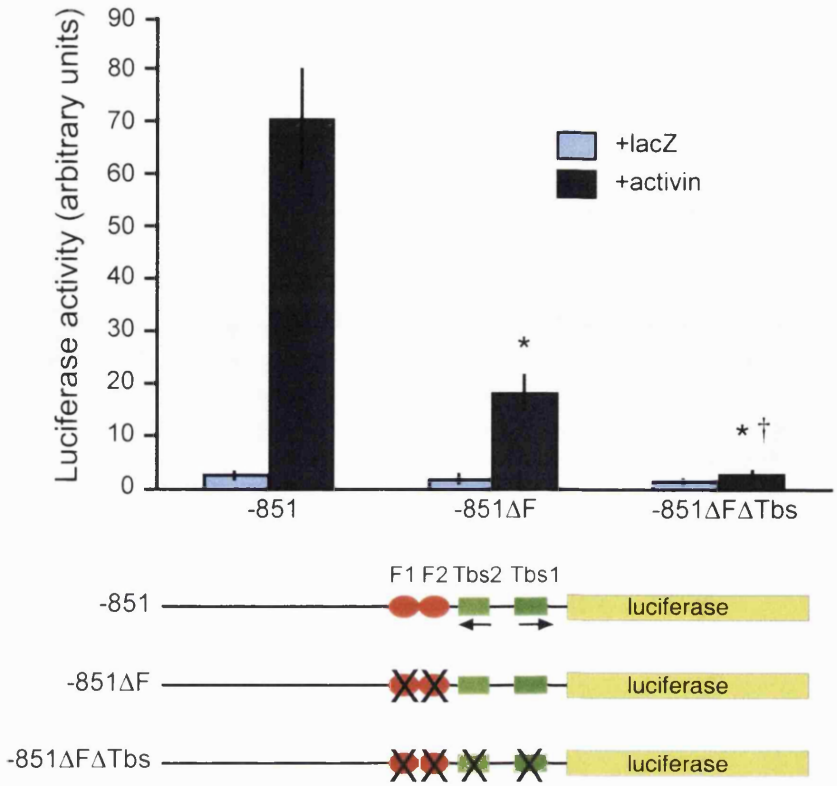

Fig. 6.6. F1 and F2 are required for activin responsiveness of d851 . Embryos were injected at the one-cell stage with 20 pg pRL-TK, 1 ng lacZ or activin RNA and, where indicated, 20 pg d851 , ΔFast, ΔFast or ΔTbs1,2. Animal caps were dissected at stage 8.5 and groups of five were assayed in triplicate for firefly and renilla luciferase activities 3 hours later. Firefly luciferase activities were then normalised to renilla activities. Error bars indicate standard errors. * = value significantly different ($p<0.05$) from pGL3–d851 + activin. † = values not significantly different ($p>0.05$) from background control (+ lacZ). The constructs used are shown below the graph.

Fast1 is able to bind a 35-bp region containing two Fast sites.

Electrophoretic mobility shift assays were carried out to test if the identified sites are functional Fast sites. Flag-tagged XFast1 protein was produced both *in vitro* using rabbit reticulocyte lysate and *in vivo* by producing an embryo extract from embryos injected with both activin (200 pg) and XFast1 (1.5 ng) RNA. The probe used in each case was a 35 base-pair region of the *derrière* promoter containing both Fast sites. As shown in Fig. 6.7, both sources of protein give specific mobility shifts consistent with XFast1 binding to these sites. Sequence specificity is shown using a 100-fold excess of unlabelled competitor (lanes 4 & 9). This binding is not competed by a mutated probe showing that the mutation is sufficient to disrupt any binding by XFast1 (lanes 5 & 10). Supershift assays using antibodies specific for XFast1 and the Flag-tag produced a low mobility complex showing that the original mobility shifts were produced by the tagged Fast1 protein (lanes 6 & 11). This indicates that these are bona-fide Fast sites capable of specifically binding Fast proteins.

Mutations in both Fast sites cause an increase in expression

The mutated ΔFast reporter constructs described above were tested in luciferase assays, in response to over-expression of *VegT*. In this case, they produced an unexpected increase in overall expression levels by early gastrula (stage 10.5). Currently, we do not know the reason for this. Mutation of the Fast sites appears to produce some sort of derepression (although basal transcription from the promoter is unchanged). This effect occurs in response to *VegT* but not in response to *activin* (compare Fig. 6.6 and 6.8). A similar phenomenon was also observed in the response of the endogenous gene to *VegT* by Clements et al. (1999). They looked at the effects of disaggregating cells from animal caps over-expressing *VegT*. Induction by *VegT* of *derrière*, *activin* B and *Xnr4* were markedly increased in disaggregated cells, suggesting the existence of a diffusible inhibitor.

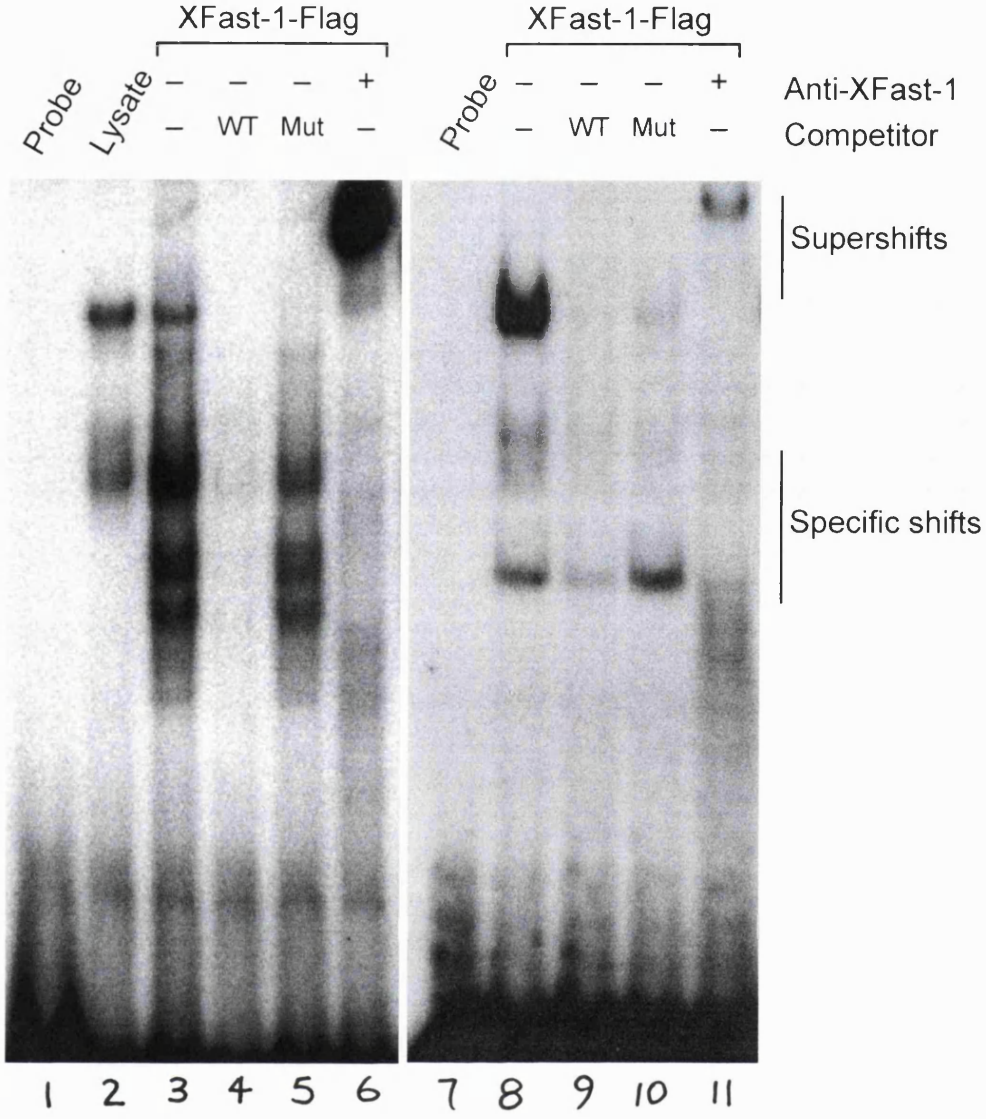

Fig. 6.7. Fast1 is able to bind a 35-bp region containing two Fast sites. Electrophoretic mobility shift assay demonstrating that Fast1 protein binds to the two Fast sites F1 and F2. A [^{32}P]-labelled 37 base-pair probe containing F1&2 was incubated with either in vitro translated XlFast1 or an embryo extract from embryos over-expressing 1.5 ng XlFast1 and 200 pg activin to promote the formation of ARF1. (Lanes 1,2 and 7) Probe alone and probe incubated with unconditioned reticulocyte lysate to test for non-specific binding. (Lanes 3–5) XlFast1 + probe. Competition experiments were carried out by incubating protein with a 100-fold molar excess of unlabelled probe, including a mutated version of the probe (Mut), prior to addition of probe. (Lanes 8–10) Embryo extract + probe. Competition experiments were carried out as above (Lanes 6 and 11) XlFast1 + probe + α–Fast1 antibody. Super shift assays were carried out by incubating DNA–protein complexes with a polyclonal Fast1 antibody (Howell et al., 2002) for 10 minutes on ice.

Interestingly, although mutation of both Fast sites leads to an increase of expression at both stages tested (late blastula and early gastrula), additional mutation of Tbs1 and Tbs2 decreases the derepression at late blastula (Fig. 6.8). This suggests that in some way, mutation of just the Fast sites leads to an increased direct response to VegT as well as some other factor. By early gastrula stages both mutant constructs behave in the same way showing approximately 3-fold more induction than wild-type or ΔTbs1,2 constructs. These results suggest two things; First, the Fast sites within this promoter can mediate negative as well as positive effects and second there is at least one other factor downstream of VegT that is able to activate this promoter.

Transgenic embryos show no ectopic expression

One transgenesis experiment, involving a very limited number of embryos, was performed, using constructs both carrying the Fast sites mutated and both the Fast and T-box sites mutated. Due to the small number of cases the results are not conclusive, however, expression is still seen in the embryos carrying constructs containing mutations in both the T-box and Fast sites. It is possible that expression levels are elevated in the vegetal region, although it is very difficult to say as expression levels tend to be very variable anyway as each embryo carrys a different number of transgene insertions at different integration sites.

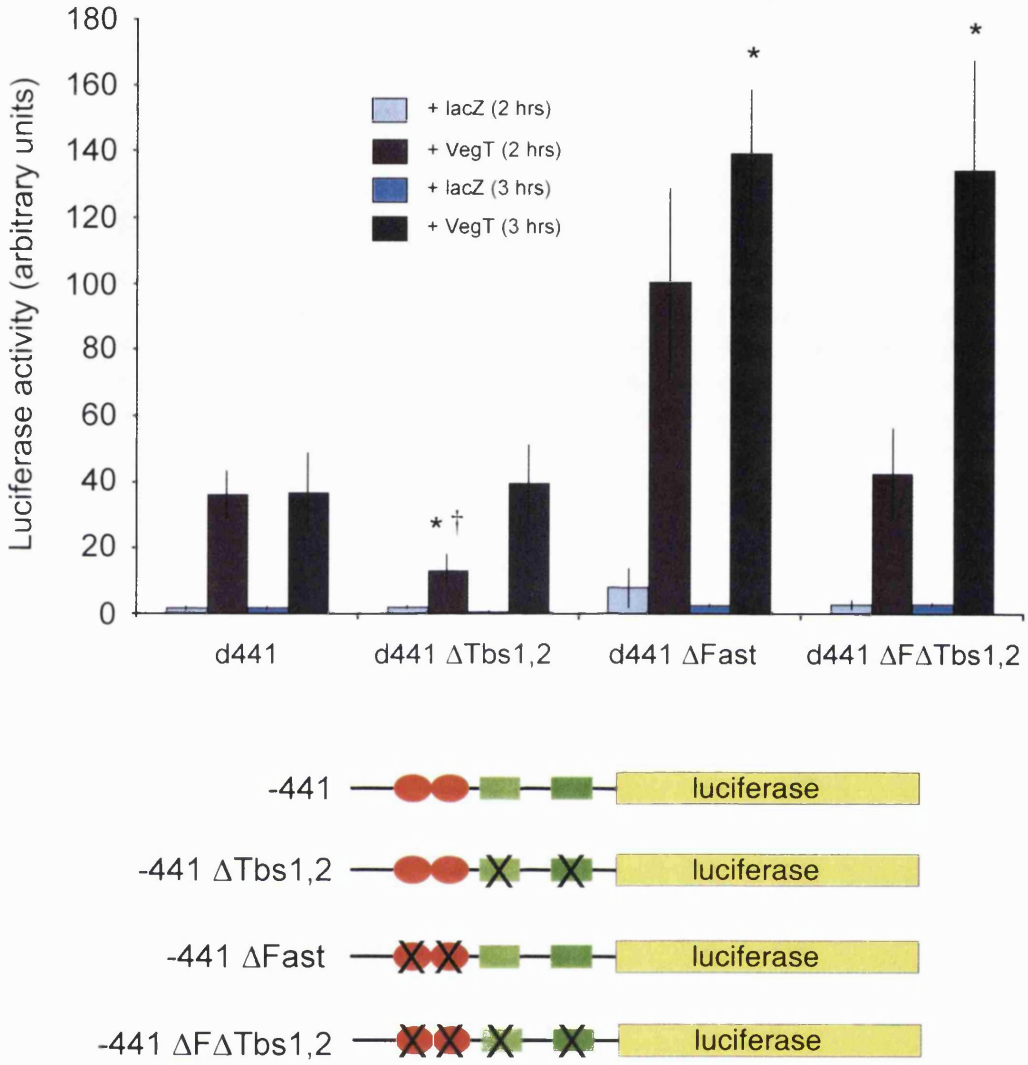

Fig. 6.8. Mutations in both Fast sites cause an increase in expression. ΔTbs1,2 ΔFast in luciferase assays. Embryos were injected at the one-cell stage with 20 pg pRL-TK, 1 ng lacZ or VegT RNA and, where indicated, 20 pg d441, d441ΔTbs 1,2, d441 ΔFast or d441 ΔF ΔTbs1,2. Animal caps were dissected at stage 8.5 and groups of five were assayed in triplicate for firefly and renilla luciferase activities either 2 or 3 hours later. Firefly luciferase activities were then normalised to renilla activities. Error bars indicate standard errors. * = value significantly different ($p<0.05$) from pGL3–d441 + VegT (collected at same time). † = values not significantly different ($p>0.05$) from background control (+ lacZ). The constructs used are shown below the graph.

DISCUSSION

The direct effects of VegT on *derrière* expression

Experiments in oocytes show that both Tbs1 and Tbs2 contribute to the direct effects of VegT on the d851 *derrière* promoter fragment. In fact, these two sites are solely responsible for the direct effects of VegT on the *derrière* promoter. This result is consistent with previous knowledge and shows that the sites defined by binding site selection do represent real T-box sites. These sites do not appear to be required for expression of reporter genes *in vivo* though as mutation of these sites does not affect the level of expression from reporter constructs in luciferase assays.

Why are Tbs1 and Tbs2 not required for expression of reporter genes?

VegT is able to drive expression of the pGL3–d851 luciferase reporter construct in animal caps and when samples are collected 3 hours after dissection, mutation of Tbs1 and Tbs2 has no effect on reporter gene activty. However, when the samples are collected 2 hours after dissection, mutation of the T-box sites abolishes luciferase activity. Tbs1 and Tbs2 are also required when the reporter gene assays are performed in oocytes. These results suggest that Tbs1 and Tbs2 are required for an initial phase of *derrière* expression, which has been shown to be directly under the control of a maternal factor (Clements et al., 1999; Yasuo and Lemaire, 1999). The second maintenance phase, which requires protein synthesis after MBT and cell-cell signalling (Yasuo and Lemaire, 1999), does not rely on the T-box sites.

These results complement those obtained with transgenic embryos carrying transgenes with both T-box sites mutated. Tbs1 and Tbs2 are not required for expression of a GFP reporter gene in both vegetal and marginal cells. However, the embryos were only assayed at gastrula stage (stage 10.5) and so it is possible that the expression of the GFP reporter shows a delay of expression as in the luciferase assays (Fig. 6.2). This might be harder to detect in the transgenic embryos as the assay technique is different. In situ hybridisation is less sensitive than the luciferase assay and it is not quantitative. Also, the inherent variability in expression levels due to differences in the number and position of integration sites might make a conclusive difference difficult to detect.

Tbs1 and Tbs2 are only required for very early stages of reporter expression, because indirect effects of genes also activated by VegT are able to compensate for the lack of early direct induction. At least some of this indirect activation could be provided by TGF-β signalling.

Feedback by TGF-β signalling

The results shown in Fig. 6.5 and 6.6 suggest that signalling by TGF-β contributes to the control of *derrière* expression. However, its exact role has been hard to define. Mutation of the two Fast sites within the promoter fragment leads to a loss of activin-responsiveness. This suggests that the effects of TGF-β signalling on this promoter are mediated by Fast proteins, probably acting as part of a complex such as the activin response factor (ARF) identified by Howell et al. (1999). Indeed, there are several Smad4 binding sites located near to the Fast sites.

However, the same mutations actually lead to an increase of reporter expression in response to VegT. This suggests that Fast proteins also mediate negative effects on this promoter fragment. It is possible that in the absence of signalling Fast proteins are bound to DNA without Smad 2 and 4, and this acts negatively on the promoter.

I have shown in this chapter that a TGF-β positive feedback loop could be operating in these assays increasing expression of the *derrière* promoter, but it would be important to demonstrate that such feedback is occurring in the embryo, as mutation of the Fast sites does not lead to the expected outcome. One way to show this would be to repeat the reporter assays in the presence of various inhibitors of TGF-β signalling. For example the dominant-negative ALK4 receptor could be used to inhibit all TGF-β signalling and reagents such as cm-derrière and Cer-S could ask if a specific TGF-β molecule is involved.

If positive feedback is occurring in the embryo what purpose would it serve in induction/patterning? I have outlined three potential reasons below.

1) Maintenance

TGF-β signalling could function simply to reinforce and maintain expression of *derrière* after the initial induction by VegT, allowing the embryo to rapidly accumulate *derrière* transcripts.

2) Signalling relay

Signalling might serve to induce expression of *derrière* in cells joining the marginal zone later on during gastrulation (see Clements et al., 1999; Yasuo and Lemaire, 1999). As gastrulation proceeds, cells that were initially located animally with respect to the equator, enter the marginal zone. These cells did not inherit any maternal VegT protein to induce *derrière* expression. Signalling from cells already expressing *derrière* would serve to induce expression. This possibility is consistent with the fact that TGF-β signalling is only required at later stages of gastrulation (White et al., 2002). Given this possibility, it would be interesting to test transgenic embryos carrying constructs with both Fast sites mutated. One might expect to lose expression of the GFP reporter gene at later stages when TGF-β signalling is required.

3) To regulate negative feedback

If expression of *derrière* is regulated by negative feedback by lefty-like molecules then some positive input would be required to stop the complete loss of expression in the marginal zone. A balanced system of both positive and negative TGF-β signalling could function to maintain expression in the marginal zone while ensuring a lack of expression outside the equatorial zone, especially in involuted mesoderm which down-regulates *derrière* expression.

The real situation may be a combination of all three suggestions, with positive feedback by TGF-β signalling acting to maintain expression in marginal zone cells already expressing *derrière*, to induce new expression in cells entering the marginal zone during gastrulation and to balance the negative effects of TGF-β feedback inhibitors.

Another factor(s)?

My experiments suggest that there is another factor downstream of VegT which is also able to activate the d851 promoter. There are two obvious strategies to search for this additional factor.

1) Deletion analysis

A deletion analysis similar to that used to test activin-responsiveness could be used in luciferase assays to identify the regions required for induction by *VegT*. Also, a linker scanning approach, mutating small regions of the required sequence, could help to identify the actual binding sites.

2) Candidate approach

One candidate for another factor capable of activating the d851 promoter is *eFGF*. It has already been shown that *FGF* is able to activate endogenous *derrière* (Sun et al., 1999) and it has recently been shown that FGF signalling is required for the expression of *derrière* at gastrula stages (White et al., 2002). It would be possible to test this easily by carrying out the luciferase assays in the presence of the dominant-negative FGF receptor, XFD.

Summary

In this chapter, I have shown that *derrière* is subject to a regulatory network with the maternal determinant VegT at the top of the hierarchy. There are two main phases of *derrière* expression; an early phase involving the direct action of VegT on the two T-box sites Tbs1 and Tbs2 and a later maintenance phase requiring both TGF-β and FGF signalling, which no longer relies on the direct effects of VegT.

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Chapter 7. General Discussion

Control of expression of *derrière*

In this thesis, I have investigated the regulation of *derrière* by *VegT*. I have attempted to understand the spatial and temporal expression pattern of *derrière* and the requirements for that expression. In this discussion I should like to review the current data both from this thesis and from other work on the regulation of *derrière*.

Derrière expression pattern

Derrière is expressed at the start of zygotic transcription and its pattern of expression closely matches that of *VegT* and then *Apod* (the zygotic isoform of *VegT*).

- **Stage 9.5** – *Derrière* is expressed in both the vegetal pole and the marginal zone.
- **Stage 10.5** – *Derrière* is still expressed throughout the vegetal pole cells. Expression in the marginal zone is stronger on the dorsal side.
- **Stage 12.5** – *derrière* is no longer expressed in the vegetal pole region. It is still expressed in the marginal zone around the closing blastopore, with the exception of the most dorsal region.

As discussed previously, there appears to be two phases to the regulation of *derrière*: a cell-autonomous early phase and a later cell-cell signalling-dependent phase.

Early cell-autonomous phase

Experiments, both in this thesis and by others, have shown that the early phase of *derrière* expression (stage 9–10) is dependent mostly on cell-autonomous factors (Clements et al., 1999; Yasuo and Lemaire, 1999). Cells dissociated from the two-cell stage onward still express *derrière*, although this residual expression does not increase from stage 9 to stage 10 as the endogenous transcript does (Clements et al., 1999; Yasuo and Lemaire, 1999), suggesting some cell non-autonomous input at this stage. This input is probably TGF-β signalling and possibly FGF signalling as well, although, at this stage, expression of *derrière* in the marginal zone has been shown to be not dependent on TGF-β or FGF signalling (Agius et al., 2000; White et al., 2002; Yasuo and Lemaire, 1999).

Expression of reporter constructs at this time is dependent at least to some extent on the two T-box binding sites Tbs1 and Tbs2 (Fig. 6.2). To test how important this direct action of VegT is in terms of the spatial arrangement of *derrière* expression one could treat embryos with cycloheximide (CHX) from stage 8 onwards and assay expression of *derrière* in the vegetal pole region compared with that in the marginal zone. Is expression generally lower everywhere or is it specifically lost in the marginal zone? Also, it would be interesting to repeat the experiment shown in Fig. 6.2 with embryos over-expressing a truncated activin receptor (tALK4) to test whether removing the T-box sites and inhibiting TGF-β signalling leads to a more permanent loss of expression.

Interestingly, in the experiments of Yasuo and Lemaire (1999), the effect of dissociating cells appears to be different from the effects of CHX treatment. CHX treatment appears to cause a more severe loss of expression, although the two situations were never directly compared in the same experiment. If this is the case, it suggests a role for another cell-autonomous factor downstream of *VegT* or some other maternal factor. This could be a known gene like *Xsox17α/β* or one of the *Mix/Bix* genes or something as yet unidentified.

I have shown that while the direct action of VegT is required for the initial expression of *derrière*, it very quickly becomes unnecessary. This is due to the activation by VegT of other molecules which can then substitute for the direct effect of VegT on the promoter. At least some part of this indirect activation is supplied by TGF-β and FGF signalling, which are both required for *derrière* expression from mid-gastrula stages onwards (stage 11).

Late cell-cell signalling phase

At this stage, cells that did not inherit any VegT protein are entering the marginal zone from originally more animal positions. Therefore, they may rely on cell-cell signalling to induce and maintain *derrière* expression. Thus, as cells enter the marginal zone, they are induced to express *derrière* and other marginal zone markers by combined TGF-β and FGF signalling.

The loss of *derrière* expression in the vegetal pole may be due to this dependence on FGF signalling. There is no FGF signalling in the vegetal region at this time and therefore, vegetal cells which no longer contain VegT protein (Stennard et al., 1999) fail to maintain *derrière* expression. This suggestion could be tested be over-expressing *eFGF* in vegetal cells to see if it results in ectopic *derrière* expression.

The role of *Apod* in regulating *derrière* expression

Apod begins to be expressed at stage 9.5 in the dorsal marginal zone. Its expression spreads laterally and ventrally until it is expressed throughout the marginal zone. It is unclear what role *Apod* plays in the regulation of *derrière*. Because the T-box sites within the *derrière* promoter are not required for correct expression from reporter constructs it is unlikely that Apod is required to activate the promoter directly, but it may participate in a auto-regulatory loop with *derrière* in a similar manner to that which occurs between *Xbra* and *eFGF* (Isaacs et al., 1994; LaBonne et al., 1995; Schulte-Merker and Smith, 1995; Umbhauer et al., 1995). Expression levels of *derrière* appear to mirror levels of *Apod* expression, such that *derrière* is expressed more strongly on the dorsal side.

Expression of *derrière* is down-regulated in the most dorsal region of the marginal zone, coincident with the loss of *Apod* from the same area. It may be that the system responsible for down-regulating *Apod* also down-regulates *derrière*. Or it could be that the loss of *Apod* leads to lack of maintenance of *derrière* expression. It would be interesting to see whether the *derrière* promoter region so far isolated recapitulates this part of the expression pattern when tested in transgenic embryos. If so, it should be possible to isolate the region responsible.

Models of VegT action

When it was discovered that VegT was required for both mesoderm and endoderm specification, three models were proposed by Zhang et al. (1998) to explain how VegT might act to specify both germ layers (Fig. 7.1).

Model 1 – A signalling model. VegT specifies endoderm in the vegetal pole region and then signalling molecules, induced by VegT and released by the vegetal cells, induce mesoderm in the overlying equatorial cells.

Model 2 – A combinatorial model, in which VegT acts directly to specify both germ layers, but some other factor(s) expressed in the marginal zone acts to distinguish the two areas as mesoderm and endoderm.

Model 3 – A Gradient model. An inherited gradient of VegT protein which determines both endoderm and mesoderm; high levels of VegT in the vegetal pole region leads to endoderm formation and lower levels in the marginal zone produce mesoderm. In this, instance VegT acts as a morphogen.

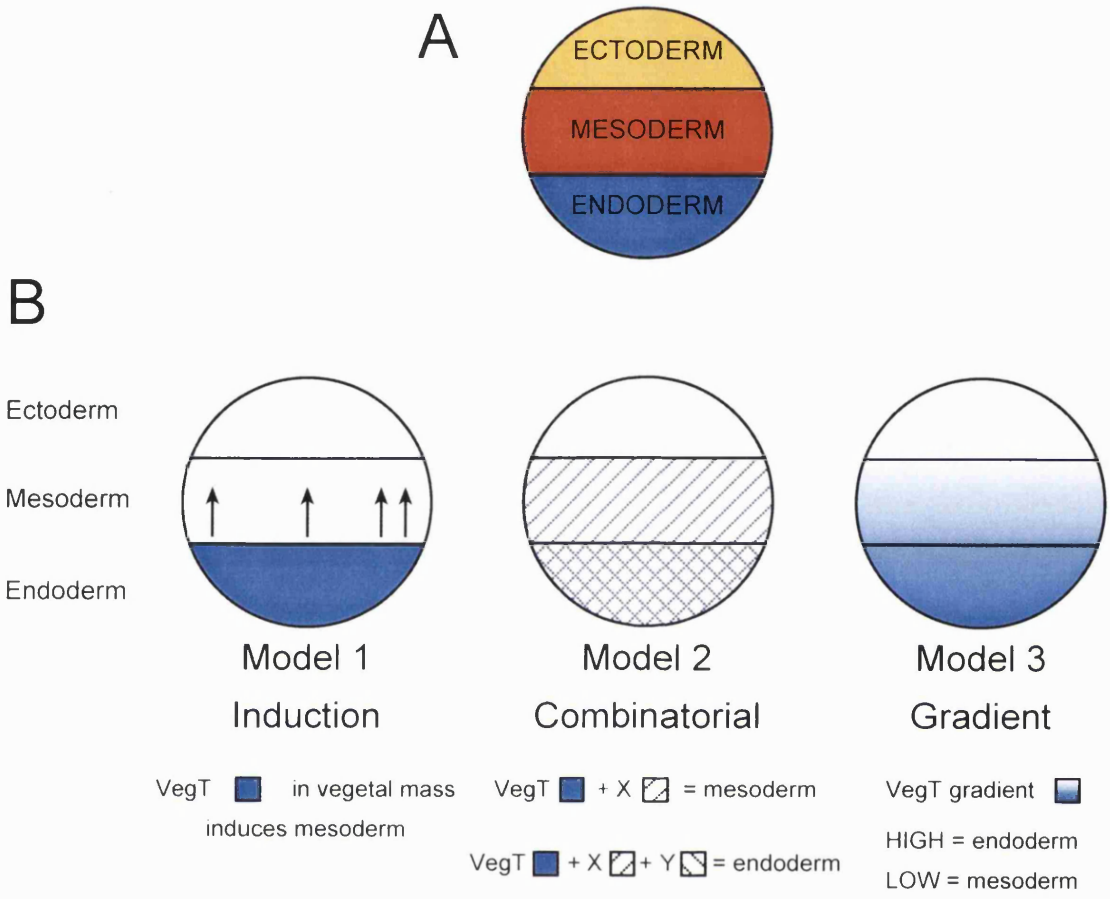

Fig. 7.1. Proposed models of action of VegT. (A) The arrangement of germ layers in the *Xenopus* blastula. Endoderm forms from the vegetal mass (shown in blue), whereas ectoderm forms from the animal cap (shown in yellow) and mesoderm forms in the equatorial region (shown in red). (B) Models of how VegT acts to generate both endoderm and mesoderm. Model 1 – Induction. VegT specifies endoderm cell-autonomously and activates the expression of signalling molecules to induce mesoderm. Model 2 – Combinatorial. Co-factors help to discriminate between germ layers. In this example, two other factors (X and Y) are required with VegT to generate endoderm. Only VegT and X are expressed in the equatorial region, together giving mesoderm. Model 3 – Gradient. A vegetal to animal gradient of protein is present in the egg and specifies endoderm at high concentrations and mesoderm at low concentrations. Adapted from Zhang et al. (1998)

With respect to *derrière*, VegT functions as a combination of models 1 and 2. Initial induction can occur in the absence of signalling throughout the entire vegetal hemisphere. TGF-β signalling is sufficient but not necessary for activation of the promoter. This very early stage of promoter activation, requires intact T-box binding sites, although this requirement is soon bypassed, possibly by TGF-β and FGF signalling. From stage 11 onwards, TGF-β signalling and FGF signalling is essential for expression of *derrière* in the marginal zone. This may explain why expression is lost from vegetal region where there is no longer any VegT protein and FGF signalling is not active. The later stages of expression of VegT are independent of the T-box sites in the promoter, but are indirectly downstream of VegT.

In this thesis, I have shown that derrière is a good candidate for one of the endogenous mesoderm-inducing signals and that it is regulated both directly and indirectly by *VegT*. *Derrière* is able to activate the expression of *Xbra* at several cell diameters away from its site of production and an antisense morpholino oligonucleotide causes a defect in the trunk and tail of the embryo. The regulation of *derrière* consists of two phases downstream of the maternal determinant VegT: an early phase involving the direct action of VegT on the two T-box sites Tbs1 and Tbs2 and a later maintenance phase requiring both TGF-β and FGF signalling, which no longer relies on the direct effects of VegT.

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