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Making mesoderm–upstream and downstream of *Xbra*

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ABSTRACT The mesoderm of the amphibian embryo is formed through an inductive interaction in which cells of the vegetal hemisphere of the embryo act on overlying equatorial cells. My laboratory is studying the *Brachyury* gene, which plays a key role in this interaction, being both necessary and sufficient for normal mesoderm formation. In this article I describe our attempts to understand how *Xenopus Brachyury* (*Xbra*) is activated in the right cells at the right time, and then to understand how *Xbra* exerts its effects.

KEY WORDS: *Xenopus, Brachyury, mesoderm, induction, transcription.*

Introduction

It is now well known, thanks to the pioneering work of Nieuwkoop, that the mesoderm of the amphibian embryo is formed through an inductive interaction in which cells of the vegetal hemisphere act on overlying equatorial cells (reviewed by Harland and Gerhart, 1997). It is the aim of my laboratory to understand this process in as detailed a fashion as possible. In short, I wish to identify the mesoderm-inducing signal(s), to understand how their production is regulated, and to ask whether they can exert long-range effects. I next want to identify the cell-surface receptors which bind the inducing factors, to investigate the signal transduction pathways that they employ, and isolate their target genes. And finally I should like to understand how those target genes are regulated, to identify genes that are regulated by those targets, and to continue this process until I can explain what makes a mesodermal cell mesodermal: what makes such a cell undergo gastrulation movements, for example, and what makes it differentiate as notochord, muscle or blood. This task is clearly a tall order, but I believe it is possible to approach the problem by analysis of the *Xenopus* homologue of *Brachyury*, *Xbra*.

Xenopus Brachyury

The *Brachyury*, or *T*, mutation was first described in 1927; heterozygous mutant individuals had a short tail (thus giving rise to the names of the mutation), while homozygous mutant embryos lacked mesoderm posterior to somite 7 and notochord differentiation was severely impaired (Dobrovolskaïa-Zavadskaïa, 1927; Gluecksohn-Schoenheimer, 1938). The *Brachyury* gene was cloned in 1990 (Herrmann *et al.*, 1990), and expression analysis showed that transcripts were present in those structures that were affected in mutant animals: at early stages the gene is activated throughout the primitive streak, while later it is expressed in the notochord and tail-bud (Wilkinson *et al.*, 1990).

Brachyury proved to encode a protein with sequence-specific DNA-binding activity which functions as a transcriptional activator (Conlon *et al.*, 1996; Kispert and Herrmann, 1993; Kispert *et al.*, 1995), and work in *Xenopus* demonstrated that the ability of Brachyury to activate transcription is essential for its biological function (Conlon *et al.*, 1996). A PCR-based binding-site selection experiment identified a preferred Brachyury binding site as the palindrome T(G/C)ACACCTAGGTGTGAAATT (Kispert and Herrmann, 1993), although the more recent identification of *bona fide* Brachyury targets suggests that a 'half-site' may be sufficient *in vivo* (see below). Subsequent work revealed that Brachyury is the founder member of a family of genes known as the T-box family, members of which contain a DNA-binding domain homologous to that of Brachyury (reviewed by Papaioannou and Silver, 1998; Smith, 1999; Smith, 1997). One such T-box gene, *VegT*, is discussed below.

Xenopus Brachyury proved to be expressed in a pattern analogous to its mouse homologue. Transcripts are first detected throughout the marginal zone (prospective mesoderm) of the embryo, and expression then refines to include the involuting circumblastoporal mesoderm and the notochord (Smith *et al.*, 1991). To the student of mesoderm formation, the expression pattern of *Xbra* provides the first reason for working on the gene. However, several other features mark out *Xbra* as a key gene in the process of mesoderm formation. First, as would be predicted from the mouse mutant phenotype, *Xbra* function is essential for normal mesoderm formation in *Xenopus*. This was demonstrated by expressing a construct in which the transcription activation domain

Abbreviations used in this paper: xbra, Xenopus brachyury.

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of *Xbra* was replaced by the repressor domain of *Drosophila* engrailed (Xbra-En^R). Such a construct interferes with the activity of the wild-type protein, and when expressed in *Xenopus* it caused loss of posterior structures and, frequently, impairment of notochord differentiation (Conlon *et al*., 1996). These defects were presaged by an inhibition of gastrulation movements, suggesting that *Xbra* function is also required for morphogenetic movements (Conlon and Smith, 1999). This work indicated that *Xbra* is necessary for normal mesoderm formation. Additional experiments indicate that *Xbra* is also sufficient for normal mesodermal differentiation; when the gene is mis-expressed in prospective ectodermal tissue those cells activate mesoderm-specific genes and go on to form mesodermal cell types including mesenchyme and muscle (Cunliffe and Smith, 1992, 1994; O'Reilly *et al.*, 1995).

The final reason for studying *Xbra* in the analysis of mesoderm formation is based on its response to mesoderm-inducing factors such as activin. Expression of *Xbra* can be induced in prospective ectodermal tissue by cells of the vegetal hemisphere and by candidate mesoderm-inducing factors such as the TGFβ family member activin (Smith *et al*., 1991). Intriguingly, the response of *Xbra* to activin is strictly concentration-dependent: low concentrations of activin do not induce expression, intermediate concentrations do, and high concentrations do not (Green *et al*., 1992; Green *et al*., 1994; Gurdon *et al*., 1994; Gurdon *et al*., 1995). This dose-dependent response may underlie the expression pattern of the endogenous gene: concentrations of the mesoderm-inducing factor may be too high in the vegetal hemisphere of the embryo for expression of *Xbra* to occur, and too low in the animal hemisphere, but just right in the marginal zone.

Overall, this summary of the properties of *Xbra* suggests that if we can understand how the gene is activated in the right cells at the right time, and then how it exerts its effects, then we could claim to have quite a good understanding of mesoderm induction. And, for good measure, we might also gain insights into the control of gastrulation and the interpretation of morphogen gradients. I now discuss the progress we have made towards solving these problems.

Regulation of *Xbra*

Our attempts to understand the regulation of *Xbra* have involved an 'outside-in' approach, in which we attempt to follow the sequence of events from membrane to gene, and an 'inside-out' regimen in which we have investigated the *Xbra* promoter and attempted to work 'backwards' towards the membrane. Here I shall describe data obtained with the latter approach; for results obtained with the former see Umbhauer *et al.* (1995) and Armes *et al.* (1997; 1999).

Our first attempts to understand the regulation of *Xbra* involved injecting reporter constructs into *Xenopus* embryos, dissecting animal pole regions, and asking if reporter gene expression could be induced by treatment with the mesoderm-inducing factors FGF and activin. These experiments gave rather variable results, due, we think, to the fact that reporter DNA was not incorporated into the chromosomes of the injected embryo (Latinkic *et al*., 1997). Nevertheless, we were able to conclude that 381 base pairs 5' of the *Xbra* transcription start site are sufficient to confer responsiveness to mesoderm-inducing factors and, importantly, that they can also reproduce the dose-dependent response to activin, such that intermediate, but not high, concentrations of activin will induce reporter gene expression (Latinkic *et al*., 1997).

Attempts to understand the concentration-dependent response of the –381 base pair reporter constructs have met with limited success. One promising approach was inspired by the observations that the homeobox-containing gene *goosecoid* is activated by the high concentrations of activin that suppress *Xbra* (Green *et al*., 1992; Green *et al*., 1994; Gurdon *et al*., 1994; Gurdon *et al*., 1995) and that *goosecoid* can suppress expression both of endogenous *Xbra* and of *Xbra* reporter constructs (Artinger *et al*., 1997; Latinkic and Smith, 1999; Latinkic *et al*., 1997). More recent work, however, indicates that inhibition of *goosecoid* function in the *Xenopus* embryo does not prevent the down-regulation of *Xbra* at high concentrations of activin (Papin and Smith, 2000). Goosecoid cannot, therefore, be the only gene product responsible for the repression of *Xbra.*

In the future, we plan to study the dose-dependent response of *Xbra* to activin using transgenic *Xenopus* embryos in which reporter constructs become integrated into host DNA (Amaya and Kroll, 1999; Kroll and Amaya, 1996). We have recently demonstrated that the 381 base pairs which confer concentration-dependent responsiveness to activin are also sufficient to drive expression of a reporter gene throughout the marginal zone of the *Xenopus* embryo at the early gastrula stage, although expression is rapidly down-regulated in the prospective notochord thereafter (Fig. 1 and Lerchner *et al*., 2000).

The most significant points to emerge from our transgenic study have been that the correct spatial expression of *Xbra* occurs more through the relief of repression than through specific activation. For example, mutation of the homeodomain sites thought to be involved in the suppression of *Xbra*-381 activity at high levels of activin (Latinkic *et al*., 1997) causes ectopic activation in dorsal mesoderm and ectoderm at mid-gastrula stages, with additional weak expression in endoderm and the rest of the ectoderm (Lerchner *et al*., 2000).

Perhaps of even greater note, however, is the observation that disruption of a bipartite δEF-1 binding site also causes widespread reporter gene activation, with loss of the mesoderm-ectoderm and mesoderm-endoderm boundaries early in gastrulation and particularly strong ectopic expression in dorsal tissues (Fig. 1 and Lerchner *et al*., 2000; Remacle *et al*., 1999). The δEF-1 binding site identified in the *Xbra* promoter consists of an upstream CACCT sequence and a downstream CAGGTG. These motifs interact with the two zinc fingers of SIP1, a novel Smad interacting protein of the δEF-1 family (Verschueren *et al*., 1999) which, when mis-expressed in the *Xenopus* embryo, eliminates endogenous *Xbra* expression (Verschueren *et al*., 1999). SIP1, like other members of the δEF-1 family (Sekido *et al*., 1997) functions as a transcriptional repressor (Verschueren *et al*., 1999), and we speculate that SIP1 is bound to its binding site in the absence of Smad signalling, but changes its conformation and dissociates from DNA when associated with an activated Smad molecule (Lerchner *et al*., 2000). Recent experiments show that *Xenopus SIP1* is indeed expressed during early gastrula stages, although highest expression occurs subsequently, in the neural plate (Eisaki *et al*., 2000; van Grunsven *et al*., 2000).

Downstream of Xbra

eFGF

To understand how *Xbra* does what it does, one has to identify its target genes, and we have adopted two approaches to this end. The first is the simplest, and is frequently called a 'candidate gene

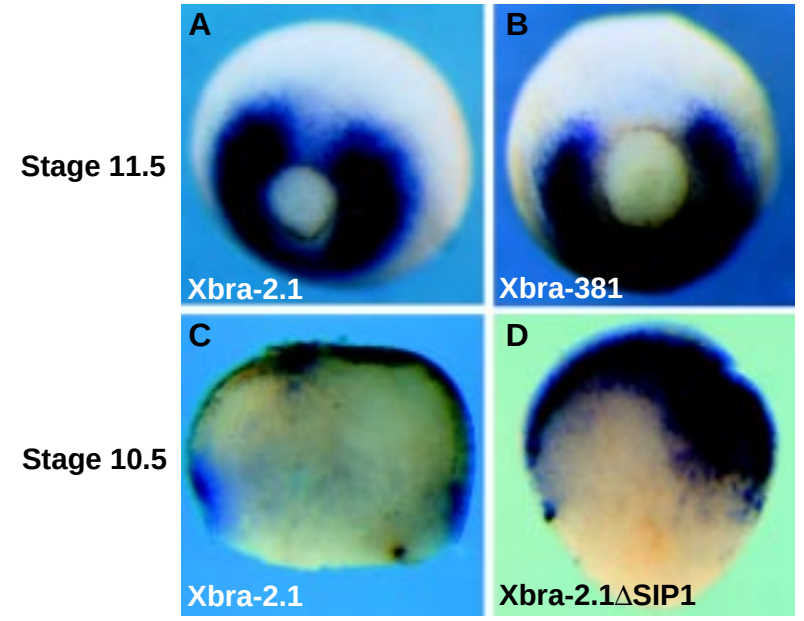

Fig. 1. Identification of *Xbra* 5′ regulatory sequences. *Expression patterns of the indicated Xbra promoter constructs at the indicated stages.* **(A,B)** *2.1 kb and 381 base pairs of 5′ Xbra sequence are both sufficient to drive expression of a reporter gene throughout the involuting mesoderm at stage 11.5, but expression does not occur in the presumptive notochord.* **(C,D)** *Bisected Xenopus embryos at stage 10.5 showing expression of a reporter gene driven by 2.1 kb of wild-type 5′ Xbra sequence (C) and expression driven by 2.1 kb of 5′ sequence in which the proximal zinc finger target sequence CAGGTG is mutated to CAGATG (D). Note widespread activation of the reporter gene in (D).*

approach', although 'guesswork' might be more accurate. The first guess we made was *eFGF*. This member of the fibroblast growth factor family is expressed in a very similar pattern to that of *Xbra* (Isaacs *et al.*, 1995), and it is involved in an autoregulatory loop in which *Xbra* activates *eFGF* and *eFGF* maintains expression of *Xbra* (Isaacs *et al.*, 1994; Schulte-Merker and Smith, 1995). In a series of preliminary experiments, we showed that interference with *Xbra* function caused a down-regulation of *eFGF* expression, and we used a hormone-inducible version of Xbra, termed Xbra-GR (Tada *et al.*, 1997), to show that activation of *eFGF* could be induced by *Xbra* in isolated animal pole regions in the presence of cycloheximide (Casey *et al.*, 1998). The latter observation indicated that Xbra can induce *eFGF* in the absence of intervening protein synthesis, making it likely that *eFGF* is a direct, rather than indirect, target of Xbra.

Sequencing of the *eFGF* 5' regulatory region revealed a single 10 base pair element TTTCACACCT located 936 nucleotides upstream of the transcription start site, with a related sequence AACCACACCT positioned 123 nucleotides downstream of the transcription start site (Casey *et al.*, 1998). The first of these sequences corresponds precisely to half of the previously-identified Brachyury binding site (Kispert and Herrmann, 1993), and its significance is emphasised by the fact that the 5' regulatory regions of human and mouse *FGF-4*, to which *eFGF* is closely related (Isaacs *et al.*, 1992), also contain a Brachyury half-site within 1 kb of their transcription start sites (Casey *et al.*, 1998). Electrophoretic mobility shift assays demonstrated that the Brachyury T box can recognise the half-site as a monomer, and a single copy of the half-palindrome, when placed upstream of a minimal promoter was able, in a specific manner, to drive reporter gene expression in response to Xbra (Casey *et al.*, 1998). Finally, both half sites proved to be necessary for full induction of a 2.5 kb *eFGF* reporter construct when assayed in *Xenopus* oocytes, while the half-palindrome TTTCACACCT, when placed upstream of a minimal promoter, was able to drive reporter gene expression following injection into tier C (prospective mesoderm) of the 32-cell *Xenopus* embryo but not following injection into tier A (prospective ectoderm) (Casey *et al.*, 1998).

Together, these experiments identified the first putative target of *Xbra*, and revealed that a half-palindrome can function as a Brachyury binding site. They also provided evidence that the *Xbra*/*eFGF* autoregulatory loop is direct in the sense that Xbra acts directly on the *eFGF* promoter while previous work had shown that FGF activates *Xbra* expression directly through the MAP kinase pathway (Gotoh *et al.*, 1995; LaBonne *et al.*, 1995; Umbhauer *et al.*, 1995).

The Bix *genes*

A complete understanding of the function of Xbra will require the identification of all its target genes, and it is unlikely that one will be able to guess each and every one. To address this issue, we have made use of hormone-inducible Xbra to construct cDNA libraries enriched for targets of *Xbra* alone (Tada *et al.*, 1998) and for targets of *Xbra* plus *Pintallavis* (Saka *et al.*, 2000). The latter library was made because *Xbra* alone is unable to induce notochord from *Xenopus* animal pole regions, whilst a combination of *Xbra* and *Pintallavis* induces this tissue with high frequency (O'Reilly *et al.*, 1995; Saka *et al.*, 2000); the first library is therefore unlikely to include targets of *Xbra* that are expressed exclusively in the notochord. Screening of the two libraries identified five gene products, including the *Bix* family, *Xwnt11*, *1A11*, *Xegr-1* and *Xbtg1* (Saka *et al.*, 2000; Tada *et al.*, 1998). I shall discuss the first two of these.

One of the first genes identified in our differential screen, *BIG4* (Brachyury-induced gene 4), had no homology with known sequences in GenBank. The cDNA was, however, incomplete, and screening of a gastrula cDNA library using this as a probe yielded four novel homeobox-containing genes which we designated *Bix1–4* (Brachyury-induced homeobox) (Tada *et al.*, 1998). The homeodomain of Bix1 was 68%, 68% and 65% identical to those of *Xenopus* Mix.1 (Rosa, 1989), Mix.2 (Vize, 1996) and Mixer (Henry and Melton, 1998), respectively, and the gene subse-

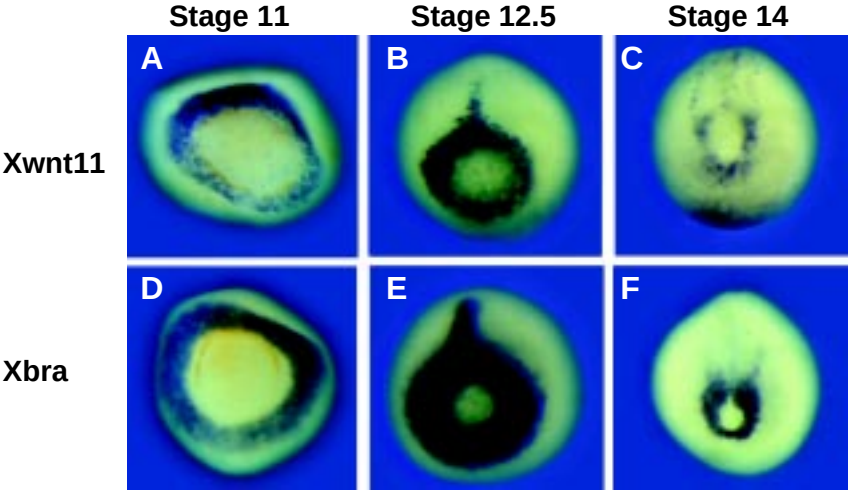

Fig. 2 Expression patterns of *Xbra* and *Xwnt11*. *Comparison of the expression patterns of Xwnt11* **(A-C)** *and Xbra* **(D-F)** *at stages 11 (A,D), 12.5 (B,E) and 14 (C,F).*

quently proved to be identical to *Mix.4* (Mead *et al.*, 1998). *Bix2* is identical to *Milk* (Ecochard *et al.*, 1998).

As might be predicted, *Bix1* is expressed in the mesoderm of the *Xenopus* early gastrula, and expression of the gene can be induced in animal pole tissue by hormone-inducible Xbra in the presence of cycloheximide, suggesting that it is a direct target of Xbra (Tada *et al.*, 1998). More surprisingly, however, *Bix1* is also expressed in the vegetal hemisphere of the embryo, and its expression actually precedes that of *Xbra*. These results suggested that *Bix1* is regulated by factors in addition to *Xbra*, and one strong candidate was the vegetally-localised maternal T-box gene *VegT* (also known as *Antipodean*, *Xombi* and *Brat*) (Horb and Thomsen, 1997; Lustig *et al.*, 1996; Stennard *et al.*, 1996; Zhang and King, 1996). Consistent with this suggestion, VegT, like Xbra, can induce expression of *Bix1* in animal pole regions (Tada *et al.*, 1998).

Study of the regulation and role of the *Bix* family of homeobox-containing genes continued with the closely-related *Bix4*. Expression of *Bix4*, like that of *Bix1*, is induced by *VegT* and, significantly, we found that depletion of maternal *VegT* transcripts by means of antisense oligonucleotides prevents subsequent expression of *Bix4*, indicating that *VegT* function is essential for *Bix4* expression (Casey *et al.*, 1999). Sequencing of the *Bix4* 5' regulatory region revealed three motifs resembling the Brachyury half site. The most distal sequence CCTGACACCT (T_d) is positioned 85 base pairs 5' of the transcription start site, a middle sequence CTTCACACCT (T_m) is positioned 15 nucleotides downstream of T_d, and a proximal sequence ATTCACACGT (T_p) is located a further 9 nucleotides downstream (Casey *et al.*, 1999). In electrophoretic mobility shift assays, the Xbra DNA-binding domain proved to interact with both T_m and T_p (albeit only weakly with the latter), while VegT interacted only with T_m (Tada *et al.*, 1998).

The importance of these sites in the normal expression of *Bix4* was demonstrated using transgenic *Xenopus* embryos in which 1.6 kb of *Bix4* upstream regulatory sequence was used to drive expression of reporter genes. Transgenic embryos carrying the wild-type promoter region expressed reporter genes in mesoderm and endoderm in a pattern resembling the endogenous gene, but simultaneous mutation of T_m and T_p caused a complete loss of reporter gene expression (Casey *et al.*, 1999). Mutation of single sites produced more complicated phenotypes, suggesting, for example, that T_d might be involved in repressing *Bix4* expression. Nevertheless, the data show clearly that Brachyury half-sites in the *Bix4* promoter are essential for normal expression of the gene.

What of the role of *Bix4*? Embryos lacking maternal *VegT* transcripts fail to form endoderm and vegetal blastomeres lose the ability to induce mesoderm (Zhang *et al.*, 1998). Injection of *Bix4* RNA into embryos depleted of maternal *VegT* causes partial rescue of endoderm formation, but cannot restore mesoderm-inducing activity to vegetal tissue (Casey *et al.*, 1999). This suggests that additional VegT targets may be involved in both processes, and particularly in mesoderm induction, where the *nodal*-related genes (Jones *et al.*, 1995; Joseph and Melton, 1997; Smith *et al.*, 1995) and *derrière* (Sun *et al.*, 1999) are strong candidates (Kofron *et al.*, 1999). Little is known about the molecular function of Bix4, although the related molecules Mixer and Bix2 (Milk) have recently been shown to interact with Smad2 and thereby mediate activin/TGF-beta-induced transcription (Germain *et al.*, 2000).

Xwnt11

Another gene isolated in the screen for Xbra targets encoded *Xenopus* Wnt11 (*Xwnt11*) (Ku and Melton, 1993). The zygotic expression pattern of *Xwnt11* proved to resemble that of *Xbra* very closely (Fig. 2; Saka *et al.*, 2000; Tada and Smith, 2000), and had we been aware of this sooner, *Xwnt11* would undoubtedly have been a strong candidate in the 'guesswork' screen. Like the *Bix* genes, *Xwnt11* can be activated by hormone-inducible Xbra in the presence of cycloheximide, and inhibition of Xbra function by means of the engrailed repressor construct (Conlon *et al.*, 1996) causes the down-regulation of *Xwnt11* expression (Tada and Smith, 2000).

The role of *Xwnt11* was investigated by means of a dominant-negative Xwnt11 construct based on a previous dominant-negative version of Xwnt8 designed by Moon and colleagues (Hoppler *et al.*, 1996). When expressed in *Xenopus* embryos, dominant-negative *Xwnt11* proved to inhibit gastrulation movements, but not to interfere with mesoderm-specific gene expression, raising the possibility that the inhibition of morphogenesis caused by Xbra-En^R (Conlon and Smith, 1999) is due to the down-regulation of *Xwnt11*.

How might *Xwnt11* regulate gastrulation? One possibility is that it affects cell adhesion, perhaps through a mechanism involving the translocation of β-catenin from membrane to nucleus. However, our results show that over-expression of wild-type or dominant-negative *Xwnt11* does not affect blastomere adhesion to fibronectin (Tada and Smith, 2000), and indeed several lines of evidence suggest that Xwnt11 does not signal through the canonical Wnt signalling pathway involving GSK-3, β-catenin and Tcf-3 (Cadigan and Nusse, 1997). For example, although over-expression of a dominant-negative *Tcf-3* blocks the canonical Wnt signalling pathway as judged by inhibition of axis formation in *Xenopus* (Molenaar *et al.*, 1996), it cannot, in contrast to dominant-negative *Xwnt11*, inhibit the elongation of *Xenopus* animal pole regions in response to activin (Tada and Smith, 2000). Consistent with this observation, the Dishevelled construct *Dsh-DEP+* has no effect on the canonical Wnt pathway but is a potent inhibitor of gastrulation. And finally, over-expression of another Dishevelled construct, *Dsh-ΔN*, can rescue the inhibitory effects of dn-wnt11 on gastrulation but cannot activate the Wnt pathway involving β-catenin (Tada and Smith, 2000).

Together, this work suggests that although the effects of Xwnt11 in regulating gastrulation are mediated through Dishevelled, subsequent signalling events occur through a β-catenin-independent pathway. Such a pathway has been recently implicated in 'planar polarity' signalling in *Drosophila* (Boutros and Mlodzik, 1999), where certain mutations in *Dishevelled* cause defects in the orientation of cells within epithelia of the wing, thorax and eye. For example, hairs in the wing usually point distally; the *dsh*^{1} allele causes these hairs to become orientated in a highly abnormal fashion (Adler, 1992). Genetic and biochemical studies show that the signalling pathway required to establish correct cellular orientation does not involve GSK-3, β-catenin and Tcf-3. Rather, it consists of small GTPases such as RhoA and Rac followed by the activation of JNK/SAPK-like kinases (Boutros *et al*., 1998; Strutt *et al*., 1997).

These observations suggest that Xwnt11 might act to control cell polarity during gastrulation, and this conclusion is supported by elegant work in which time-lapse confocal microscopy was used to observe cell polarity during *Xenopus* gastrulation in a direct fashion (Wallingford *et al*., 2000). In these experiments, cells lacking Dishevelled function suffered from defects in polarity, as judged by the orientation of membrane protrusions from individual cells, and failed to undergo proper convergent extension. In the future we plan to investigate this aspect of gastrulation by taking advantage of the fact that the zebrafish *silberblick* locus (Heisenberg *et al*., 1996) encodes Wnt11 and that, as in *Xenopus*, Slb/Wnt11 activity is required for zebrafish cells to undergo normal gastrulation movements (Heisenberg *et al*., 2000).

Conclusions

The work described in this paper describe the attempts of my laboratory to understand mesoderm formation. The approach focuses on the mesoderm-specific transcription factor *Xbra*, which I regard as a significant gene: as I say in the Introduction, if we can understand how *Xbra* is activated in the correct spatial and temporal manner, and if we can understand how it exerts its effects, then we should gain quite a reasonable understanding of mesoderm induction, of gastrulation and of the interpretation of morphogen gradients. Some progress has been made, but there is still some way to go.

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