Mesoderm formation in response to *Brachyury* requires FGF signalling

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Background: The *Brachyury* (*T*) gene is required for the formation of posterior mesoderm and for axial development in both mouse and zebrafish embryos. In these species, and in *Xenopus*, the gene is expressed transiently throughout the presumptive mesoderm, and transcripts then persist in notochord and posterior tissues. In *Xenopus* embryos, expression of the *Xenopus* homologue of *Brachyury*, *Xbra*, can be induced in presumptive ectoderm by basic fibroblast growth factor (FGF) and activin; in the absence of functional FGF or activin signalling pathways, expression of the gene is severely reduced. Ectopic expression of *Xbra* in presumptive ectoderm causes mesoderm to be formed. As *Brachyury* and its homologues encode sequence-specific DNA-binding proteins, it is likely that each functions by directly activating downstream mesoderm-specific genes.

Results: We show that expression in *Xenopus* embryos of RNA encoding a dominant-negative FGF receptor inhibits the mesoderm-inducing activity of *Xbra*. We demonstrate that ectopic expression of *Xbra* activates transcription of the embryonic FGF gene, and that embryonic FGF can induce expression of *Xbra*. This suggests that the two genes are components of a regulatory loop. Consistent with this idea, dissociation of *Xbra*-expressing cells causes a dramatic and rapid reduction in levels of *Xbra*, but the reduction can be inhibited by addition of FGF.

Conclusion: Formation of mesoderm tissue requires an intact FGF signalling pathway downstream of *Brachyury*. This requirement is due to a regulatory loop, in which *Brachyury* activates expression of a member of the FGF family, and FGF maintains expression of *Brachyury*. The existence of this loop explains why embryos lacking an FGF signalling pathway appear similar to those mutant for *Brachyury*, and why over-expression of truncated FGF receptors seems to inhibit induction of *Brachyury* expression by activin.

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Background

The *Brachyury* (*T*) gene is required for the formation of posterior mesoderm and for axial development in both mouse and zebrafish embryos [1–4]. In all vertebrates, the gene is initially expressed throughout the presumptive mesoderm, and transcripts then persist in notochord and in posterior tissue [5,6]. In *Xenopus* embryos, expression of *Xbra*, the *Xenopus* homologue of *Brachyury*, can be induced in presumptive ectoderm by the mesoderm-inducing factors basic (b) fibroblast growth factor (FGF) and activin [7]. If signalling in response to FGF or activin is inhibited by over-expression of truncated versions of the FGF or activin receptors, expression of *Xbra* in the intact embryo is severely reduced [8–10]. Expression of *Xbra* is also inhibited if cells of the *Xenopus* embryo are dispersed during cleavage and blastula stages, thereby disrupting cell–cell communication [11].

Over-expression of *Xbra* RNA in the presumptive ectoderm of *Xenopus* embryos causes ventral mesoderm formation [12], and co-expression of *Xbra* with the *noggin* gene, which encodes a secreted dorsalizing factor [13,14], leads to the formation of dorsal tissues, such as notochord and skeletal muscle [15]. *Brachyury* and its homologues encode sequence-specific DNA-binding proteins [16], suggesting that each exerts its mesoderm-inducing effects by directly activating downstream mesoderm-specific genes. In this study, however, we show that formation of both dorsal and ventral mesoderm requires an intact FGF signalling pathway downstream of *Brachyury*. This requirement results from a regulatory loop, in which *Brachyury* activates expression of a member of the FGF family and FGF maintains expression of *Brachyury*. The existence of this regulatory loop may explain why embryos lacking a functional FGF signalling pathway [8] appear so similar to those mutant for *Brachyury* [1,3]. It also explains why over-expression of truncated FGF receptors in the animal caps of *Xenopus* embryos seems to inhibit the induction of *Xbra* expression by activin [17–19]. Our results show that *Xbra* expression is induced by activin under these circumstances, but that in the absence of a functional FGF signalling pathway, *Xbra* expression is not maintained. Finally, bearing this observation in mind, we discuss whether the apparent inhibition of *Xbra* expression in intact embryos that results from over-expression of truncated FGF receptors [9] similarly arises as a result of failure to maintain *Xbra* transcription, rather than failure to induce transcription.

Results

Xenopus Brachyury function requires an intact FGF signalling pathway

Over-expression of *Xbra* RNA in animal caps of *Xenopus* embryos leads to formation of ventral mesoderm [12]. In preliminary experiments, *Xbra* RNA was injected into the animal pole regions of fertilized *Xenopus* eggs, either alone or in the presence of RNA encoding a truncated

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FGF receptor (*XFD*) [8] or a truncated activin receptor (*Δ1XAR1*) [10]. Animal pole regions were dissected from these embryos at the mid-blastula stage, cultured, and assayed for extension movements. Explants derived from embryos injected with *Xbra* RNA alone, or with *Xbra* and *Δ1XAR1* RNAs, elongated slightly and differentiated into vesicles containing mesenchyme, mesothelium, and occasionally muscle. However, explants derived from embryos injected with *Xbra* and *XFD* RNAs showed no signs of mesoderm formation and differentiated into atypical epidermis. This suggested that the effects of *Xbra* require an intact FGF signalling pathway.

To investigate whether the same is true for *Xbra* function in dorsal mesoderm, the experiment was repeated in the presence of *noggin* RNA [13,14], which dorsalizes the response to *Xbra* [15]. Over-expression of RNAs encoding noggin, Xbra and HAVØ, a non-functional form of the FGF receptor, leads to elongation and the differentiation of muscle, notochord and neural tissue (Fig. 1). The same is true for explants derived from embryos injected with *noggin*, *Xbra* and *Δ1XAR1* RNAs (Fig. 1). In the presence of *XFD*, encoding the truncated, dominant-negative FGF receptor, however, explants do not elongate or form mesoderm, but differentiate into neural tissue and occasionally cement glands (Fig. 1). These tissue types are observed after over-expression of *noggin* RNA alone [15,20], and are also formed if *noggin* and *XFD* RNAs are co-injected with *ΔXbra* RNA, encoding a non-functional form of Xbra (Fig. 1).

Consistent with the findings of these morphological and histological analyses, RNAase protection assays reveal that cardiac actin RNA is expressed in explants derived from embryos injected with *Xbra*, *noggin* and *HAVØ*, or *Xbra*, *noggin* and *Δ1XAR1* RNAs, but not in explants over-expressing *Xbra*, *noggin* and *XFD*, the truncated FGF receptor (Fig. 2). All explants injected with *noggin* RNA express the neural marker N-CAM (Fig. 2), indicating that *XFD* does not block *noggin*'s function. Thus, in both ventral and dorsal presumptive mesoderm, a functional FGF receptor pathway, but not a functional activin receptor pathway, is required downstream of *Xbra* for normal *Xbra* function.

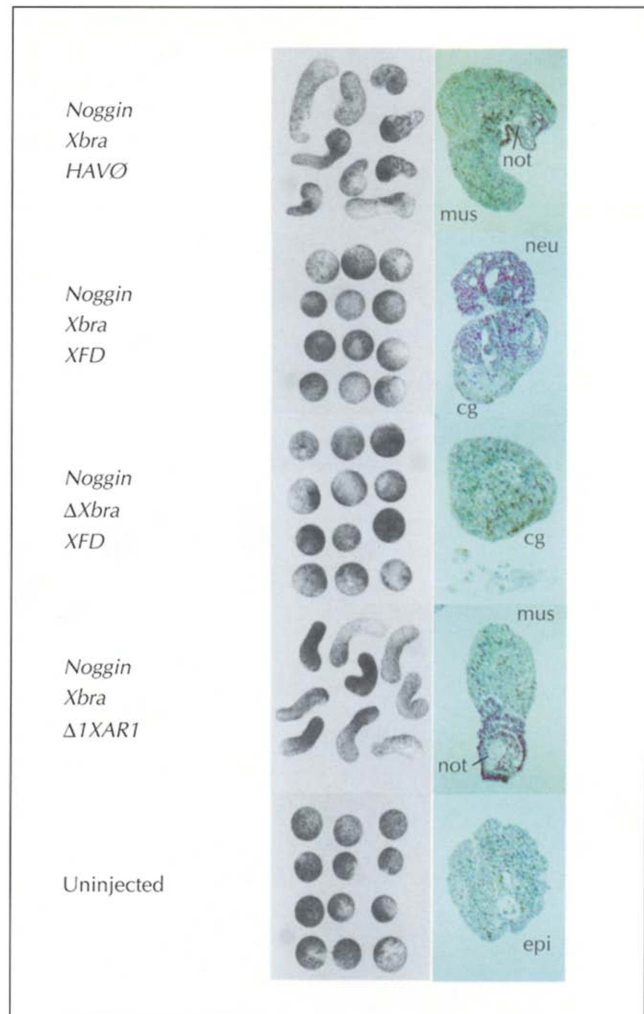

Fig. 1. *Xbra* function is blocked by co-expression of RNA encoding a truncated FGF receptor (*XFD*), but not a non-functional FGF receptor (*HAVØ*) or a truncated activin receptor (*Δ1XAR1*). Animal caps derived from embryos injected with the indicated combinations of RNA were assayed for elongation (left-hand column) and for differentiation of mesodermal cell types (right-hand column). Mesoderm formation in response to full-length *Xbra* RNA is inhibited by *XFD* but not by *HAVØ* or *Δ1XAR1*. The neural-inducing effects of *noggin* are not inhibited by *XFD*. Abbreviations: not, notochord; mus, muscle; neu, neural tissue; cg, cement gland; epi, atypical epidermis.

Xbra and embryonic FGF induce each other's expression

The simplest interpretation of these results is that *Xbra* induces the expression of a member of the FGF family, and that this molecule is necessary for *Xbra* function. One potential target of *Xbra* is embryonic (e) FGF, zygotic expression of which begins shortly before gastrulation [21], with transcripts then restricted to posterior tissue in a pattern resembling the posterior expression of *Xbra*. To investigate whether *Brachyury* induces the expression of eFGF, RNA encoding *Xbra* or *no tail* (the zebrafish *Brachyury* homologue) was injected into the animal pole regions of *Xenopus* embryos at the one-cell stage. Animal caps were isolated at the mid-blastula stage, cultured until the early gastrula stage (stage 11) and assayed for expression of eFGF RNA by RNAase protection. Both *Xbra* (not shown) and *no tail* induced expression of eFGF (Fig. 3a).

The experiment described above indicates that *Brachyury* activates expression of eFGF, a member of the FGF family. A reciprocal influence of FGF family members on *Xbra* expression might occur at either of two stages of development. The first of these is before gastrulation. The FGFs might act as mesoderm-inducing factors; treatment of animal caps with bFGF is already known to induce expression of *Xbra* [7] and we show here that eFGF has the same effect (Fig. 3b). We also demonstrate that *no tail* can induce expression of *Xbra*; animal caps derived from embryos injected with *no tail* RNA go on to express *Xbra* (Fig. 3b). This observation confirms previous results:

Brachyury can induce its own expression [22]. The autoinduction is likely to occur indirectly, through induction of eFGF expression, rather than by direct interaction of the *no tail* product with the *Xbra* promoter, because animal caps derived from embryos over-expressing *no tail* in the presence of *XFD* RNA do not activate transcription of *Xbra* (our unpublished observations).

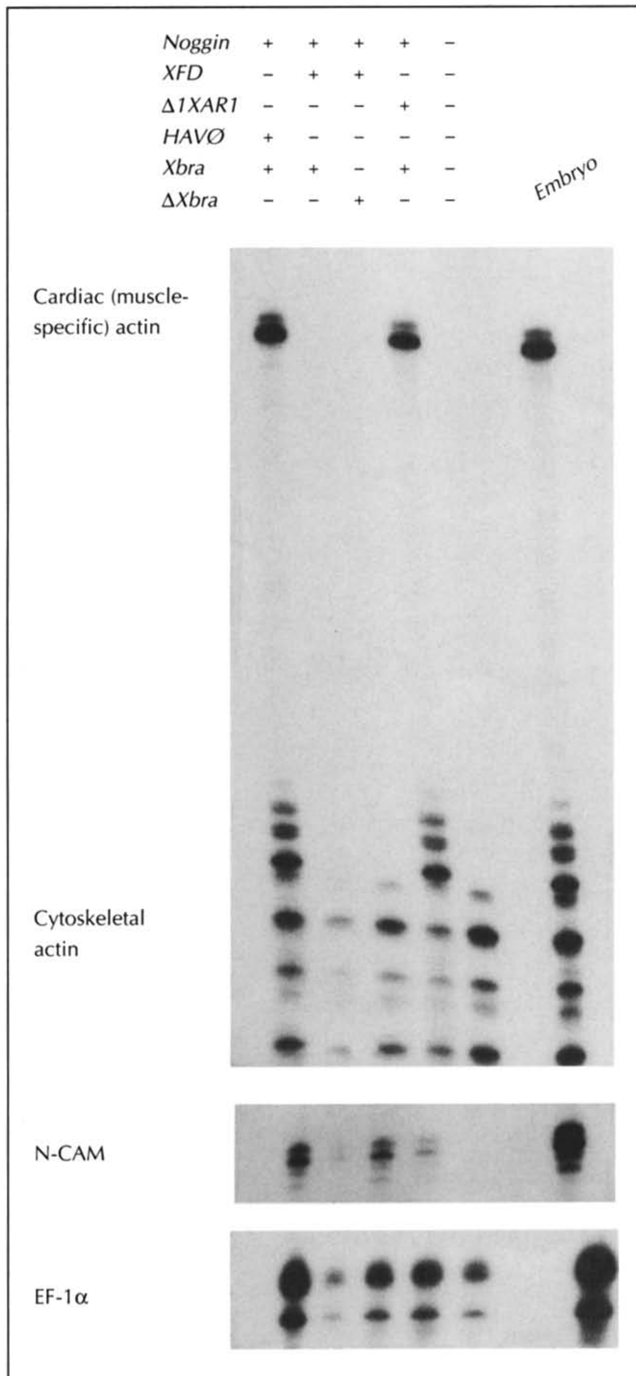

Fig. 2. Animal caps derived from embryos injected with the indicated combinations of RNA were assayed for expression of muscle-specific cardiac actin RNA by RNAase protection. Cardiac actin gene expression in response to full-length *Xbra* RNA injection is inhibited by *XFD*, but not by *HAVØ* or *Δ1XAR1*. The same RNA samples were also probed for expression of N-CAM. All explants derived from embryos injected with *noggin* RNA express N-CAM, confirming that *XFD* does not block the function of *noggin*.

Maintenance of *Xbra* transcription requires FGF signalling

The second time during development at which FGF family members might influence expression of *Brachyury* is during gastrulation. Animal pole tissue loses the ability to respond to FGF at the onset of gastrulation [23,24]. This loss of ability is correlated with the loss of functional FGF receptors from animal pole cells at this time [25]. FGF receptors persist in presumptive mesoderm, however, and particularly in posterior tissue, where *Xbra* is expressed [26]. This observation, together with the data presented above, suggests that FGF signalling may be involved in *Xbra* function at these stages. To investigate this possibility, ventral and dorsal marginal zone tissue was dissected from *Xenopus* embryos at the early gastrula stage (stage 10.5), after the onset of *Xbra* expression [7].

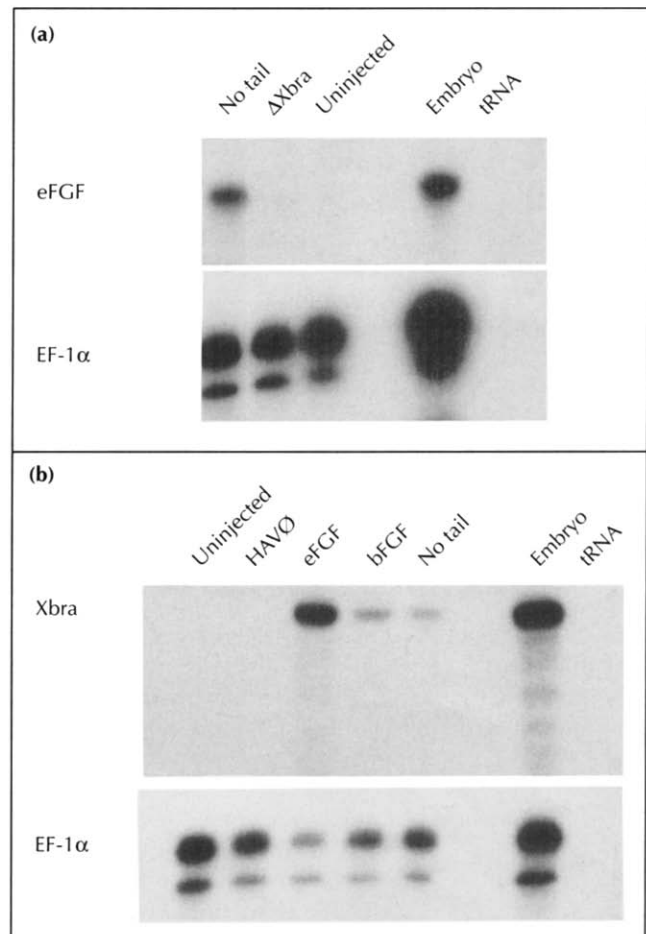

Fig. 3. Induction of eFGF expression by *Brachyury* and induction of *Xbra* expression by FGF family members and *no tail*. **(a)** Animal caps derived from embryos injected with *no tail* RNA express eFGF, whereas caps derived from uninjected embryos or from embryos injected with RNA encoding a truncated form of *Xbra* (*ΔXbra*) show no signal. **(b)** Animal caps derived from embryos injected with RNA encoding eFGF or with *no tail* RNA express *Xbra*. Exposure of animal cap explants to 20 U ml^{-1} soluble bFGF served as a positive control (see [29] for definition of a unit of mesoderm-inducing activity), whereas animal caps from control-injected and uninjected embryos were used as negative controls.

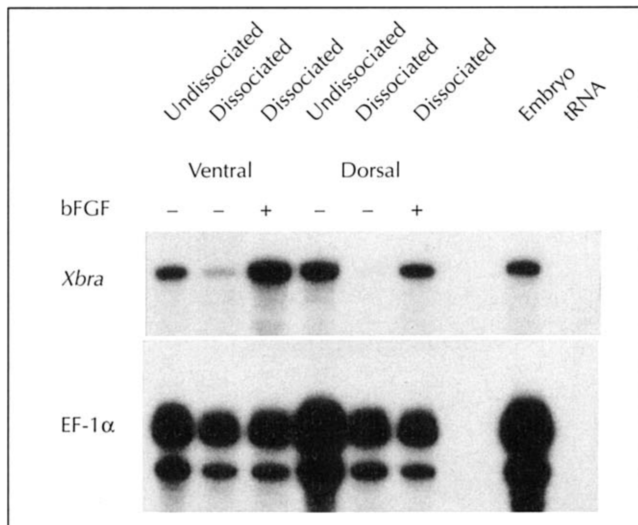

Fig. 4. FGF is required for the maintenance of *Xbra* expression. Intact ventral and dorsal marginal zone tissue maintains *Xbra* expression after culture to gastrula stages, whereas dissociated marginal zone cells maintain expression only in the presence of 20 U ml^{-1} bFGF. Marginal zone regions were dissected at stage 10.5 and cultured to stage 12, either intact or in a dissociated state.

The tissue was then cultured intact or, in an effort to dilute the effects of endogenous FGF, as dissociated cells. Samples were collected 90 minutes after complete dissociation of the explants (stage 12). Expression of *Xbra* persisted in the intact marginal tissue, but had fallen almost to undetectable levels in dissociated cells (Fig. 4). Addition of bFGF to the dissociated cells, however, maintained expression of *Xbra* (Fig. 4).

Activin-mediated induction of *Xbra* does not require FGF signalling

The observation that the FGF signalling pathway is required for maintenance of *Xbra* expression sheds light on experiments in which animal pole explants derived from *XFD*-injected embryos are exposed to activin [17–19] or the mature region of the Vg1 signalling protein [19]. Such explants do not exhibit extension movements, and the induction of some mesodermal markers, such as *Xbra*, seems to be inhibited. One interpretation of this result is that the activin and Vg1 signal transduction pathways require components of the FGF signalling pathway. An alternative explanation, however, is that induction of *Xbra* expression occurs in response to activin, but that, in the absence of FGF signalling, this expression of *Xbra* is only transient.

We examined this possibility by applying activin to animal pole explants derived from *XFD*-injected embryos (expressing the truncated FGF receptor) and assaying for expression of *Xbra* at intervals thereafter (Fig. 5). Expression of *Xbra* was consistently observed 200 minutes after exposing caps to activin, demonstrating that activin can induce expression of *Xbra* in the absence of a functional FGF signalling pathway. Transcripts became undetectable within 60 minutes, however, indicating that, under these circumstances, *Xbra* RNA undergoes very rapid turnover. This rapid turnover may account for the low initial level of expression of *Xbra* observed in these experiments (Fig. 5).

Discussion

Our data indicate that *Xbra* and eFGF are components of a regulatory loop, in which each can induce expression of the other, and in which eFGF (or another member of the FGF family) maintains expression of *Xbra*. This loop is required for the mesoderm-inducing effects of *Xbra*, because if it is disrupted, by interfering with FGF signalling, mesoderm formation is blocked. These results are consistent with data recently reported by Isaacs and colleagues [27], who also demonstrated that *Xbra* induces eFGF expression and that dissociation of marginal zone tissue causes a decline in levels of *Xbra*. Our data add to theirs, however, by demonstrating that expression of

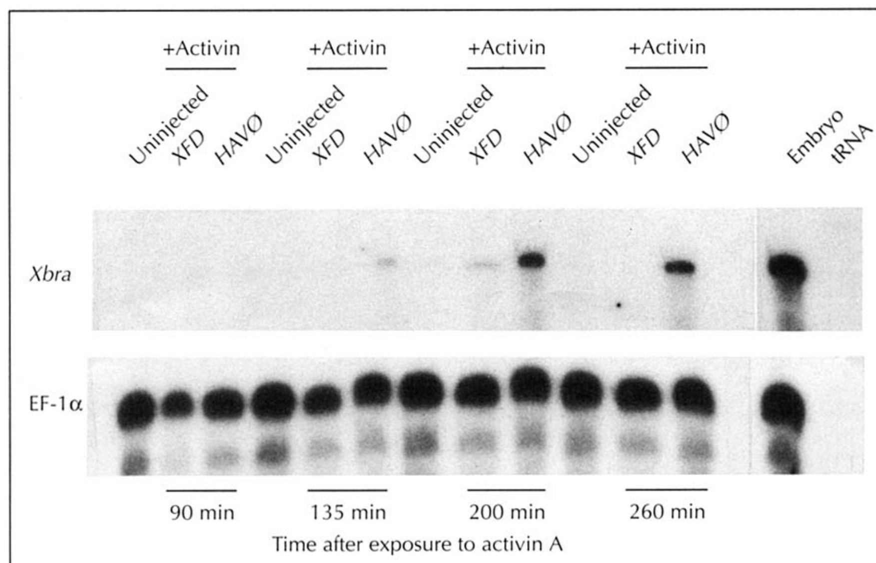

Fig. 5. The FGF receptor pathway is required for maintenance, but not for initiation, of activin-induced expression of *Xbra*. Animal caps derived from embryos injected with *XFD* or *HAVØ* at the one-cell stage were incubated in 12 U ml^{-1} activin from stage 8 and harvested at the indicated times (see [29] for the definition of a unit of mesoderm-inducing activity). As a negative control, animal caps were dissected from uninjected embryos and cultured in the absence of activin. Activin causes persistent expression of *Xbra* in animal caps derived from embryos injected with *HAVØ* RNA, but only transient expression in caps derived from embryos injected with *XFD* RNA. This experiment has been performed three times, with identical results.

eFGF is a very rapid response to *Xbra*, and that levels of *Xbra* decline precipitously if FGF signalling is inhibited. Furthermore, co-expression of *Xbra* mRNAs with *XFD*, encoding the truncated dominant-negative FGF receptor, enables us to demonstrate unequivocally that FGF signalling is a downstream requirement for *Xbra* function. These results raise several issues.

FGF and the *Brachyury* mutant phenotype

The existence of the regulatory loop described in this paper may explain, at least in part, why the phenotypes of embryos lacking components of the FGF signalling pathway are similar to those mutant for *Brachyury* or its homologues [1,3,8]. In both cases, embryos lack a differentiated notochord and posterior structures, although head structures are unaffected. But *Xenopus* embryos expressing truncated FGF receptors usually have no somites [8], whereas mutant mouse embryos have seven somites [1] and mutant fish embryos have 17–19 somites [3]. Whether the complete absence of somites in *Xenopus* embryos represents a species difference, or whether FGF signalling is also required for the formation of the most anterior somites, remains to be seen.

The proposed regulatory loop involving *Brachyury* and a member of the FGF family would also be disrupted by the absence of functional *Brachyury* protein, and we note that in both mouse and zebrafish embryos mutant for *Brachyury*, levels of *Brachyury* RNA are reduced [4,5]. It will be important to examine whether a homologue of *Xenopus* eFGF exhibits reduced levels of expression in *Brachyury* mutant mouse and zebrafish embryos.

Implications for other experiments involving truncated FGF receptors or dissociation procedures

Our conclusion that FGF signalling is required for the maintenance of activin-induced expression of *Xbra*, but not for the initial activation of the gene, has several implications. First, as discussed above, it suggests that the activin signal transduction pathway does not require components of the FGF signalling pathway. In addition, the finding affects the interpretation of several recent experiments involving the dispersal of embryonic cells. Lemaire and Gurdon [11], for example, have shown that *Xbra* transcripts cannot be detected in cells derived from embryos that are kept dissociated from the morula stage to the early gastrula stage. By contrast, expression of *goosecoid* and *Xwnt-8* does occur. This may mean that expression of *goosecoid* and *Xwnt-8* is controlled differently from the expression of *Xbra*, but an alternative explanation is that activation of *Xbra* occurs in these experiments, but in the absence of cell-cell contact, and therefore of FGF signalling, this expression is weak and transient.

It is also possible that the apparent lack of *Xbra* expression in embryos over-expressing truncated FGF receptors [9] reflects a lack of maintenance of expression, rather than inhibition of the initial activation of the gene. If this were the case, there might be a brief period of expression of *Xbra* at early gastrula stages, followed by a rapid decline in transcript levels. We have investigated this question using the sensitive technique of RNAase protection, but at no stage do we observe complete extinction of *Xbra* expression. We do not know whether this is due to incomplete blockage of FGF signalling, or whether other mesoderm-inducing factors, such as activin, Vg1 or BMP-4, are inducing *Xbra* expression.

Conclusions

The data in this paper show that the FGF receptor signalling pathway is required for maintenance of the expression of *Xbra*, and therefore for *Xbra* function. We provide evidence for a regulatory loop in which *Xbra* activates expression of a member of the FGF family and FGF maintains expression of *Xbra*. These findings add significantly to our understanding of the roles of *Brachyury* and its homologues, and of the FGF family of signalling molecules, in vertebrate early development, and will help in the interpretation of other experiments involving over-expression of dominant-negative FGF receptors, or experiments involving dissociation procedures.

Materials and methods

RNA injections and processing of explants

Various combinations of synthetic RNAs (1 ng *Xbra* ; 0.6 ng Δ*Xbra*; 1 ng *no tail*; 100 pg *noggin*; 1 ng Δ*1XAR1*; 1 ng *XFD*; 1 ng *HAV∅*) were injected into the animal pole regions of *Xenopus* embryos at the one-cell stage. Animal caps were dissected at stage 8 [28] and cultured to stage 15 for morphological analysis, stage 40 for histological analysis, or to the stages indicated in the text for RNAase protection analyses. Synthesis of RNA, microinjection, histological analysis and RNAase protection assays were carried out as described [19].

Dissociation of marginal zones

Marginal zone regions were dissected at stage 10.5 and dissociated. Dissociation was carried out in CMFME (44 mM NaCl, 2.5 mM sodium phosphate, pH 7.5, 0.1 mM KCl, 0.5 mM EDTA), and was complete by stage 11. Where appropriate, 20 U ml^{-1} bFGF was added to the CMFME. Samples were collected after 90 min, when controls had reached stage 12. Intact marginal zones were cultured in 75 % normal amphibian medium (NAM [24]).

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