Activin has direct long-range signalling activity and can form a concentration gradient by diffusion
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Background: Activin has strong mesoderm-inducing properties in the early *Xenopus* embryo, and has a long-range signalling activity that activates genes in cells distant from a source in a concentration-dependent way. It has not yet been established what mechanism of signal transmission accounts for this and other examples of long-range signalling in vertebrates. Nor is it known whether activin itself acts on distant cells or whether other kinds of molecules are used for long-range signalling. Here we have used a well characterised model system, involving animal caps of *Xenopus* blastulae treated with activin or transforming growth factor β, to analyze some fundamental properties of long-range signalling and of the formation of a morphogen gradient.

Results: We find that cells distant from the source of activin require functional activin receptors to activate *Xbrachyury*, a result suggesting that activin itself acts directly on distant cells and that other secondary signalling molecules are not required. We also find that the signals can be transmitted across a tissue that cannot respond to it; this argues against a relay process. We provide direct evidence that labelled activin forms a concentration gradient emanating from its source and extending to the distant cells that express *Xbrachyury*. Lastly, we show that there is no inherent polarity in the responding tissue that influences either the direction or rate of signalling.

Conclusions: The long-range signalling mechanism by which activin initiates the transcription of genes in a concentration-dependent manner depends on a process of rapid diffusion and the establishment of an activin gradient across the tissue. It cannot be explained by a relay or wave propagation mechanism. Activin itself is the signalling molecule to which distant cells respond.

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Background

It is becoming clear that signalling between cells is an important mechanism by which the diversity and spatial arrangement of cells is generated in vertebrate development. In Amphibia, where these processes have been most fully investigated in the early stages of development, a series of cell interactions takes place at intervals of a few hours. These result in the induction and patterning of the mesoderm, neural tissue, and many other tissues and organs.

Signalling processes fall into two classes. Short-range inductions involve direct contact between inducing and responding cells, and are prevalent in embryos and organs consisting of small cell numbers — for example, in the 4-cell-stage nematode embryo [1]. In contrast to these cases, the second class of signalling processes is long range and includes examples where cells respond to signals originating in other cells with which they have no contact. The signalling process must therefore be able to spread over more than one cell diameter, and may do so over several diameters in a few hours. Special interest attaches to these examples of long-range effects where the signalling molecule acts as a morphogen and is able to induce different cell fates at different concentrations. Thus a single signalling event — the release of a morphogen — can generate a diversity of cell-types in a defined spatial relationship to each other. The amphibian inductions described above seem to fall into this category, about which there has been a long history of theoretical discussion and experimental contribution. These ideas are summarised in a number of important reviews [2–7].

The experiments reported here concern activin as an example of a morphogen. Activin, a member of the transforming growth factor β (TGFβ) family of growth factors, has potent mesoderm inducing activity in *Xenopus* [8], and is present in *Xenopus* eggs [9] and early embryos [10,11]. A new design of dominant-negative activin receptor has recently been described [12]. This specifically inhibits activin, but does not affect Vg1 or any other known mesoderm inducer effective in the *Xenopus* embryo; since it disrupts mesoderm formation, it seems that activin does indeed have an essential role in early

Xenopus development. This result extends earlier work in *Xenopus* [13] and in *Medaka* fish [14].

Activin signalling in *Xenopus* has the properties expected of a morphogen. Using dissociated blastula cells, Green, Smith and colleagues [15,16] demonstrated decisively that different concentrations of activin elicit different gene activations. We have described an experimental system using *activin*-mRNA-containing cells, or activin protein attached to beads, and intact blastula tissue, in which activin appears to elicit different gene activations at different concentrations [17]. We have used directional conjugates of this kind to show that an increasing dose of activin causes cells distant from the activin source to express different genes [18,19]. There are two possible mechanisms that can account for the long-range signalling of a morphogen like activin. One possibility is that the signal is transmitted across the tissue to distant cells through a series of short-range interactions, in which one cell signals to its immediate neighbour, causing it to emit either the same or different molecules to its neighbours. In this way, the signal is propagated from cell-to-cell by a relay mechanism. Alternatively, long-range signalling could occur by diffusion into the surrounding tissue of either the initial signalling molecule itself, or *via* the activation and diffusion of a second long-range signal. Until these two mechanisms are distinguished, we do not know the identity of the molecule to which cells respond in long-range signalling.

Evidence against a relay concept and in favour of a diffusion model has been provided in *Xenopus* [20] and in *Drosophila* [21,22]. It was found that a constitutively active receptor, in the absence of ligand, cannot generate long-range signalling. However, this approach does not rule out the involvement of a second long-range signalling molecule that is activated by the ligand-induced receptor but not by the constitutive receptor. In contrast to the conclusions from the constitutive receptor experiments, Reilly and Melton [23] argue that, in *Xenopus*, patterning at a distance by TGFβ family members takes place by a process of relay rather than by diffusion, with secondary signalling molecules participating in the process.

Here, we use tissue and bead recombination experiments to provide positive evidence that the morphogen behaviour of activin, as well as that of its related family member TGFβ, is attributable to the diffusion of the molecules themselves and does not depend on a relay process. Furthermore, secondary signalling molecules appear not to be involved, since cells distant from the morphogen source respond to the original signalling molecules, activin or TGFβ. This conclusion therefore identifies activin as a molecule that forms a diffusion gradient and whose concentration is directly assessed by the responding cells.

Results
Activin activates distal *Xbra* directly

In our directional conjugate assay [17], in which two blastula stage animal caps are sandwiched around activin-coated beads, we showed by *in situ* hybridisation that activin is able to induce, at a distance from the beads, a band of *Xbrachyury* (*Xbra*) mRNA, an immediate early response to mesoderm induction [24]. However, it is possible that activin is required only for the initiation, but not for the continuation, of signalling in our system. For example, it may be that activin travels only as far as the first layer of responding cells, where it activates a second signal and it is this which acts at long range to induce *Xbra*. Such a process takes place in *Drosophila* wing and leg discs, where Hedgehog acts at short range to induce a long-range morphogen signal, Decapentaplegic (Dpp) [25]. Likewise, in the limb bud, Sonic hedgehog appears to act at short range to induce the long-range proliferative agent bone morphogenetic protein 2 (BMP2) [25].

To distinguish a direct from indirect mechanism of signalling, we have asked whether activin is required in distal cells as well as in cells near the source. The experimental design is a modification of the directional conjugate assay [17]. A three-layer conjugate was constructed in which an intervening sheet of cells was sandwiched between an animal cap and the activin beads (Figure 1a). This sheet is an animal cap from which the outer impermeable cell layer, which surrounds all *Xenopus* embryos, had been previously removed. We labelled this permeable 'decoated' sheet with the lineage label rhodamine lysinated dextran (RLDx) to distinguish it from the upper animal cap, which contains 2 ng of mRNA for the type 2 dominant-negative activin receptor (ActRIIBdn) [13]. Hence, a band of *Xbra* seen in the cells containing ActRIIBdn would suggest that a second, non-TGFβ signal had replaced the original activin signal in the decoated sheet of cells. Alternatively, the absence of an *Xbra* band in the ActRIIBdn cells would suggest that activin induces *Xbra* directly. The bottom animal cap acts as a control to verify that the beads are of sufficient strength to induce *Xbra* in distant cells.

The result can be seen in Figure 1b,c. Whereas an *Xbra* band was induced in the bottom (control) cap and scattered staining could also be seen in the decoated wild-type cells, no *Xbra* band was present in the upper ActRIIBdn cells. Similar results were obtained with beads of higher concentration (data not shown). These experiments show that functional activin receptors are required in the distal range of signalling and that this process does not depend on a second signalling molecule unrelated to activin.

The dominant-negative receptor used above does not distinguish signalling by activin from that by other TGFβ molecules. It is possible, therefore, that signalling by a type of TGFβ other than activin is involved. A new type

Figure 1

Activin does not induce *Xbra via* secondary signalling. Two types of directional conjugate were constructed (see text). **(a)** A decoated animal cap of RLDx-labelled, wild-type cells separates the ActRIIBdn cap (above) from the activin beads. The lower wild-type cap supporting the conjugate acts as a positive control. **(b)** No *Xbra* band is seen in the upper ActRIIBdn cells although scattered staining can be seen at the distal edge of the decoated cells and in the bottom control cap. **(c)** RLDx labelling distinguishes the wild-type decoated cells from the dominant-negative cells. In none of the samples was an *Xbra* band seen in the body of the ActRIIBdn animal cap (total 47). **(d)** Experimental design for ActRIIBexd: the activin signal passes from the beads into the RLDx-labelled animal caps. Following bead removal, one of the activin-influenced caps is then placed next to either an ActRIIBexd or a wild-type cap. **(e,g)** An *Xbra* band has been induced in the cells of the wild-type cap (12 out of 17 conjugates with 5 beads of either 40 or 16 nM). **(f,h)** No *Xbra* band, however, is present in the ActRIIBexd cells (0 out of 18 conjugates). The black lines demarcate the boundaries.
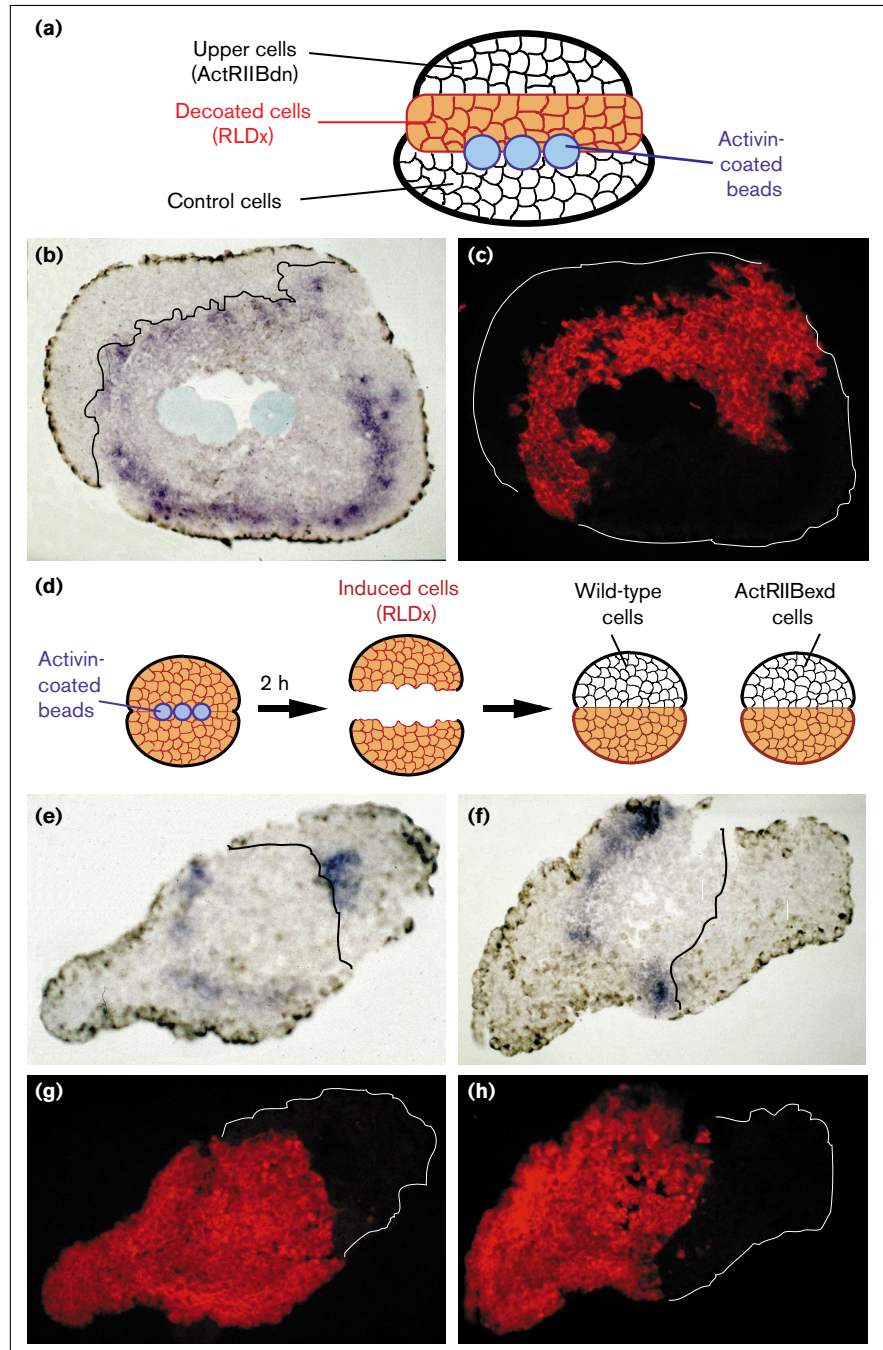
of dominant-negative receptor (ActRIIBexd) specifically inhibits activin signalling when expressed at concentrations of up to 1 ng of mRNA per animal cap [12]. In the previous design of experiment, the concentration of activin necessary to send the signal across the intervening cell layer is too high to be completely blocked by only 1 ng of *ActRIIBexd*. That is, at such high concentrations of activin, the cells expressing ActRIIBexd are still able to partly respond (data not shown). We have therefore used

the following experimental design (Figure 1d). Beads with a high concentration of activin were sandwiched between two rhodamine-labelled animal caps and the conjugates cultured for 2 hours; this is sufficient time for the signal to spread into the caps. The conjugate was then dismantled and the beads removed; one of the caps was placed next to a wild-type, non-activin-treated blastula cap, while the other was placed next to a cap injected with the *ActRIIBexd*. Conjugates were then cultured for a further

2 hours until the early gastrula (stage 10.5) when they were fixed and stained for *Xbra* induction by *in situ* hybridisation. The results are shown in Figure 1e–h. While the activin-treated, rhodamine-labelled cap induced an *Xbra* band in the non-activin-treated, wild-type cap, it did not induce *Xbra* in the ActRIIBexd cells. We thus conclude that activin itself is acting as the long-range signal to induce *Xbra* in cells distant from its source.

TGFβ2 protein can act directly as a long-range morphogen by a mechanism of diffusion

The above result led us to ask whether the activin reaches the distant cells by a mechanism of diffusion or relay. To do this, we modified the design of the three-layer conjugate shown in Figure 1a so that, in this case, the middle layer of decoated tissue cannot respond to the signal, while the upper animal cap cells can respond. Initially, we used the receptor ActRIIBdn to block response in the decoated cells. However, we found that the type 2 dominant-negative receptor sequestered the activin when over-expressed in the decoated cells. This is because the truncation does not eliminate the ligand-binding domain of the type 2 monomer. Therefore, to obtain cells which cannot respond and which will not sequester ligand, we used the non-endogenous signalling molecule TGFβ2. Although closely related to activin, neither TGFβ1 nor TGFβ2 are present in *Xenopus* embryos and the cells do not contain receptors for them. However, provided the embryo has been previously injected with mRNA encoding the type 2 TGFβ receptor (TGFβIIR), these ligands can activate a wide range of mesodermal genes in an induction similar to activin or Vg1 ([23,26] and our unpublished data). The ability of either TGFβ1 or TGFβ2 to have long-range signalling effects like activin, across cells able to respond, has not however been reported.

To test this, we sandwiched TGFβ2-containing beads between two animal caps. One cap had been injected with mRNA for TGFβIIR and the other, unable to respond, served as a control (Figure 2a). Conjugates were assayed by *in situ* hybridisation for the induction of *Xbra*. Beads loaded with a low concentration of TGFβ2 induced *Xbra* in their immediate vicinity (Figure 2b), whereas high concentration beads induced an *Xbra* band at a distance in a manner analogous to activin (Figure 2c). We conclude that TGFβ2 protein can act as a morphogen and has long-range signalling properties that are similar to activin.

To test whether this long-range action is due to the TGFβ itself or to the production of a secondary signal, we repeated an assay similar to that described in Figure 1a for activin. Wild-type cells, which cannot respond to TGFβ2, were used in the upper layer in place of cells containing dominant-negative receptor, while *TGFβIIR*-injected cells provided the responding middle cell layer. Following *in situ* hybridisation, an *Xbra* band was absent from the upper

non-responsive cell layer in all conjugates analysed (six conjugates in two separate experiments; data not shown). We therefore conclude that TGFβ2 signalling, like activin signalling, is required over the whole of its range and that secondary signalling molecules are not involved.

TGFβ2 therefore has the properties necessary of a long-range signal and so can be used to distinguish diffusion from relay. The experimental design, as explained earlier, involved a three-layered conjugate in which the middle layer cannot respond to the signal. In this case, as illustrated in Figure 2d, the middle decoated layer is wild-type (rhodamine-labelled) and therefore is unable to respond to the TGFβ ligand, while the upper layer contains TGFβIIR and so can respond. Hence, if an *Xbra* response is induced in these TGFβIIR cells, the TGFβ must have passed across the intermediate, non-responsive cells by diffusion through or around them. Alternatively, absence of an *Xbra* band would suggest that a cell must receive and respond to a signal in order to transmit it; this would support a process of relay.

The results are shown in Figure 2e,f. A band of *Xbra* was observed in the upper cells containing TGFβIIR in all 26 conjugates analysed, while *Xbra* expression was never seen in the intervening wild-type cells nor in the lower control cells. Another immediate early gene, *Xgoosecoid* (*gsc*) [27], was also assayed by *in situ* hybridisation and found to be expressed in these upper cells (data not shown). Since the cells of the middle layer are entirely non-responsive to TGFβ2, signal passage across these cells cannot have been by a relay mechanism. We therefore conclude that passage of the signalling molecule across this middle layer took place by diffusion and not by a relay process.

Our results appear to differ from those of Reilly and Melton [23] who used *TGFβ* mRNA, injected into the 32-cell-stage embryo, rather than protein bound to beads. Since they did not observe an *Xbra* response in a clone of distant TGFβRII cells, they concluded that TGFβ1 cannot diffuse across non-responding cells and that signalling must occur by a mechanism of relay. To investigate this difference in the long-range signalling properties of cells expressing *TGFβ1* mRNA compared to mature protein on beads, we asked whether an animal cap containing *TGFβ1* mRNA can induce *Xbra* at a distance in an opposed cap containing TGFβII receptor and RLDx. We found no induction of *Xbra* when either 500 pg or 1 ng of *TGFβ1* mRNA was injected. With 2 ng and 4 ng of *TGFβ1* mRNA, *Xbra* induction was rarely seen, and even then only in responding cells close to the signalling cells (6 conjugates out of 75; data not shown). Furthermore, the 4 ng concentration also caused autoinduction in the signalling cells. This result would therefore suggest that, unlike beads coated with a mature form of the protein, cells

Figure 2

TGFβ2 can act as a morphogen and pass across tissue composed of cells unable to respond to it. **(a–c)** TGFβ2 can act as a morphogen. (a) Two TGFβ2 beads were sandwiched between an animal cap supplied with TGFβIIR and a non-responsive (wild-type), rhodamine-labelled animal cap. (b) With weak beads (1 nM), the band in the receptor injected cells is in the vicinity of the beads (5 out of 5 conjugates), while stronger beads (20 nM) induce a band further away (5 out of 5 conjugates) (c). The black lines demarcate the boundary between the receptor injected and RLDx cells (not shown). **(d)** A modified version of the three-layer conjugate, described previously in Figure 1a. The middle decoated layer (RLDx) is wild-type and hence cannot respond to the TGFβ2 signal from the beads, while cells of the upper animal cap can respond since they contain TGFβIIR. **(e)** A strong band of *Xbra* expression is induced in the upper responding cap (26 conjugates out of 26). **(f)** Rhodamine labelling demonstrates that the wild-type decoated tissue forms a continuous layer separating the receptor injected cells from the beads.
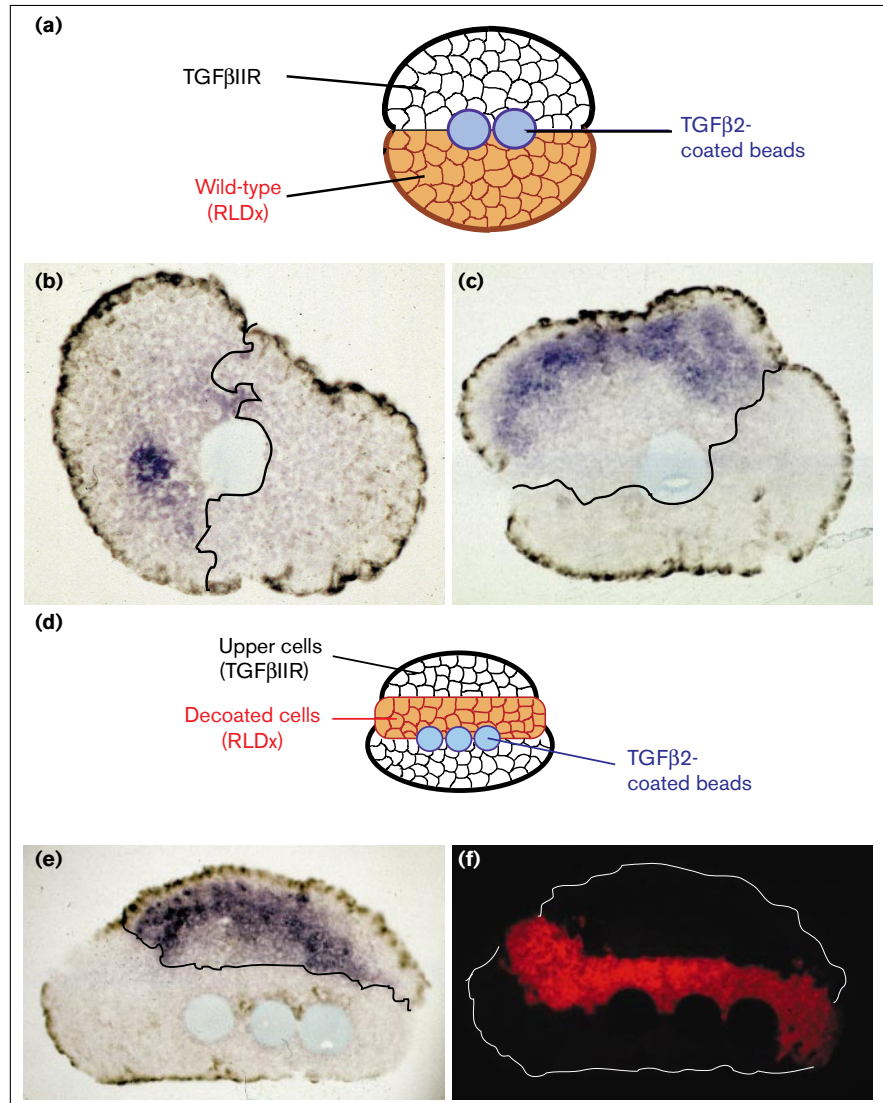

expressing *TGFβ1* mRNA do not have long-range signalling properties (see Discussion).

Labelled activin enters animal cap tissue

Our experiments provide evidence that activin and TGFβ exert their long-range effects *via* diffusion rather than by a relay mechanism and we expect that these molecules must diffuse from the beads into the responding tissue. We therefore asked if activin can be found in animal caps after activin-loaded beads have been removed. To this end, we generated ^{35}S-labelled activin protein from the medium of *Xenopus* oocytes microinjected with synthetic *activin* mRNA.

Each oocyte was injected with 50 ng of *activin* mRNA and cultured in the presence of ^{35}S-methionine for 3 days. Naturally occurring activin is secreted from cells as a complex between a mature processed dimer, which is responsible for signalling, and a biologically inactive pro-region [28–30]. Figure 3a shows reducing SDS-polyacrylamide gel electrophoresis (PAGE) analysis indicating that the medium from *activin*-mRNA-injected oocytes was highly enriched in activin protein. Mature processed activin (14.5 kDa) and the larger processed pro-region (approximately 40 kDa) together account for 75–85% of all the labelled proteins secreted by *activin*-mRNA-injected oocytes. In order to demonstrate that the activin in the oocyte medium was biologically active and to estimate its concentration, we compared our activin medium to purified activin in its ability to induce mesoderm in animal cap tissue. Animal caps were treated with a dilution series of either pure activin or oocyte medium and were assessed by morphology and by RNase protection for the expression of the mesodermal genes *Xbra* and *gsc* (data not

Figure 3
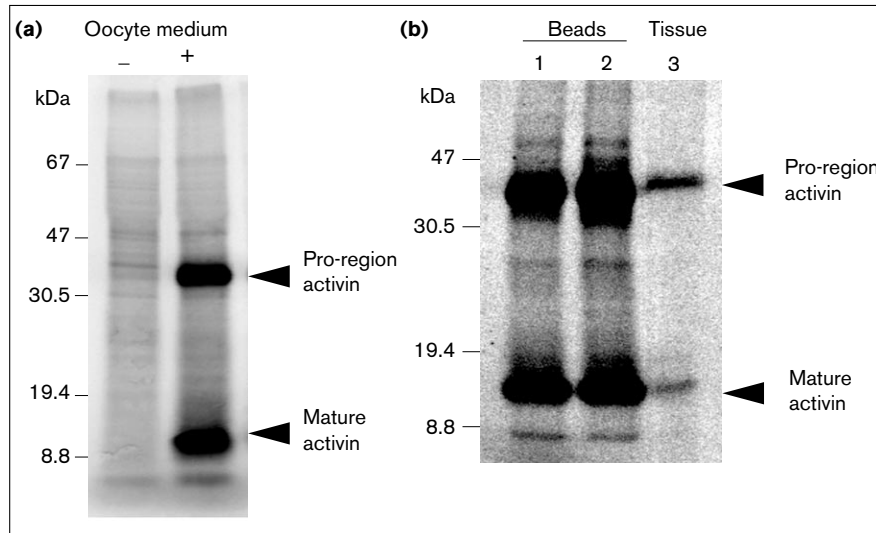
³⁵S-labelled activin protein can spread from beads into tissue. **(a)** Synthetic mRNA encoding activin βB was micro-injected into *Xenopus* oocytes that were cultured for 3 days in the presence of ³⁵S-methionine and ³⁵S-cysteine. The labelled proteins in the conditioned oocyte medium were resolved by 15% SDS-PAGE in reducing conditions and visualised by fluorography. Oocytes injected with *activin* mRNA (+) contain both the mature processed activin (~14 kDa) as well as a larger (~40 kDa) secreted form with a size consistent with that of unprocessed activin. **(b)** Affigel beads were loaded with labelled activin oocyte medium and implanted into animal cap conjugates. After 4 h of culturing, the beads were recovered from the tissue. The labelled proteins present in the tissue or still bound to the beads were resolved by 15% SDS-PAGE in reducing conditions and visualised by fluorography. Lane 1 shows protein bound to beads before implantation. Lane 2 shows the proteins remaining on the beads after removal from tissue and lane 3 shows the activin protein present in the tissue

shown). Typical activin concentrations in our oocyte medium preparations were 100–400 ng/ml (5–20 nM) with a specific activity of about $1-2 \times 10^8$ cpm/µg.

We used the labelled ³⁵S-activin to look for radioactivity diffusing from the beads into the tissue of the animal cap conjugates. Incubation in 20 nM ³⁵S-activin resulted in 1000–4000 cpm of ³⁵S-activin (equivalent to 10–40 pg of mature activin) per bead. Such beads were placed between two animal caps and after approximately 4 hours, a time during which we know that mesodermal genes are normally activated in distant cells, the beads were removed from the tissue. The material from 20 such conjugates was pooled and the labelled protein in the tissue and that remaining on the beads was resolved on reducing SDS-PAGE and visualised by fluorography. Figure 3b shows that some ³⁵S-activin had been released from beads and was present in the animal cap tissue, as would be expected if the long-range activin effect depends on diffusion. About 5% of the activin loaded on to the beads enters the tissue. This would be equivalent to an average concentration of about 10–80 pM if the amount released from the beads is spread evenly throughout the animal cap tissue.

We conclude from these experiments that activin leaves the beads and enters the tissue. However, we have not ruled out the possibility that the activin we detected in the tissue was entirely bound to the cells immediately adjacent to the beads, rather than having spread into the tissue.

Direct visualisation of an activin concentration gradient

We have used the ³⁵S-labelled activin in an attempt to visualise directly a concentration gradient of activin in the tissue of our animal cap conjugates. Beads loaded with ³⁵S-activin from oocyte medium were placed between two animal caps and cultured for approximately 4 hours, during which time *Xbra* was activated in distant cells. After fixation, conjugates were sectioned and the location of the labelled activin protein was determined by autoradiography. Figure 4a,c show dark field views of typical autoradiographs resulting from these experiments. Over 12 conjugates from three separate experiments were analysed. A white halo of autoradiographic silver grains can be seen in the tissue around the bead. The halo of silver grains appears to form a gradient, with the highest density of silver grains in the tissue near the bead and with a progressive dilution of grains in the tissue further away from the bead. Figure 4b,d show bright field and fluorescence views of the same conjugate. The conjugates containing activin beads exhibit a characteristic morphology indicating the induction of mesodermal tissue by the activin when compared to control conjugates (vertical elongation in Figure 4a compared to the flat conjugate in Figure 4g).

We have considered two ways in which our interpretation of the results could be incorrect. One is that our ³⁵S-activin preparation might contain residual amounts of free ³⁵S-methionine and ³⁵S-cysteine (typically 0–5% in our preparation), which could form a gradient of incorporation in the

Figure 4

Visualisation of an activin morphogen gradient in tissue. Between 10 and 40 pg (1000–4000 cpm per bead; see Materials and methods) of ^{35}S-labelled activin βB from oocyte medium was loaded on to Affigel beads, the beads were implanted in animal cap conjugates and after 4 h of culturing conjugates were fixed, sectioned and subjected to autoradiography. **(a,b)** Low magnification of an activin bead in a conjugate; (a) dark field view; (b) bright field view; the white area in (a) is a dark field view of autoradiographic grains. **(c)** A dark field view of (a) at higher magnification. **(d)** Hoechst nuclear stain; the dotted circle indicates the position of the bead. **(e)** Bright field view of a section through a conjugate in which the bead was dislodged before autoradiography. **(f)** Bright field view of an activin-bead containing conjugate, in which the section shown has passed ~25 μm above the level of the edge of the bead. **(g)** Dark field view of a conjugate with two beads loaded with ^{35}S-methionine and ^{35}S-cysteine only (no activin); the flat morphology shows no response to activin. **(h)** High magnification of a bright field autoradiograph shows a concentration gradient. **(i)** NIH image software was used to quantitate the average silver grain density over sections of tissue. The results were plotted relative to distance from the edge of the bead position (referred to as tissue edge). The graph depicts the concentration gradient of activin across the tissue.
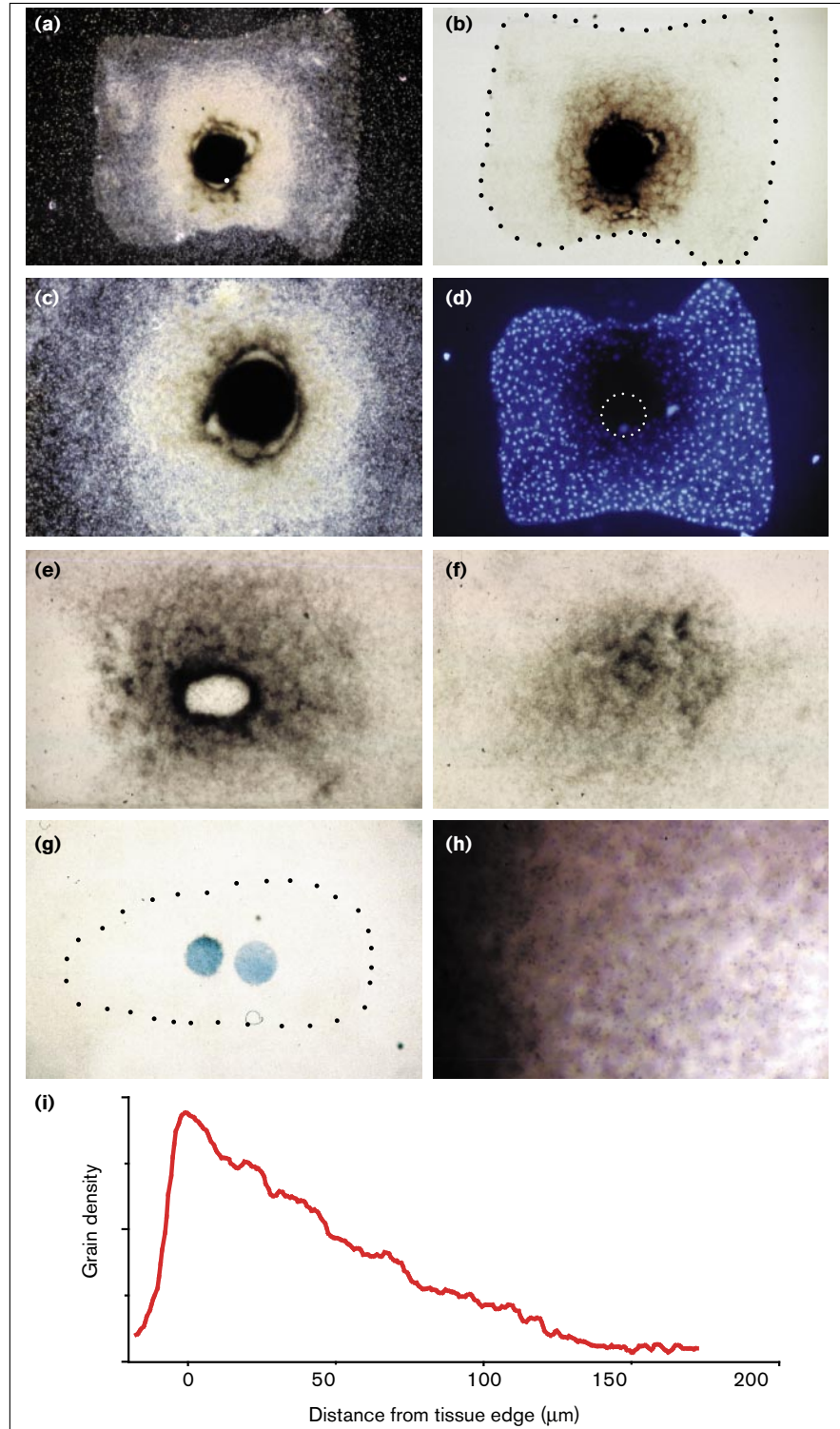
tissue if it were released progressively from the beads. In order to control for any possible autoradiographic signal due to this remaining free label, we prepared beads to which was absorbed a greater amount of ^{35}S-methionine and ^{35}S-cysteine than would have been present in the ^{35}S-activin beads. When free ^{35}S-methionine and ^{35}S-cysteine containing beads were implanted into conjugates and cultured for 4 hours, equivalent autoradiographs showed that

Figure 5
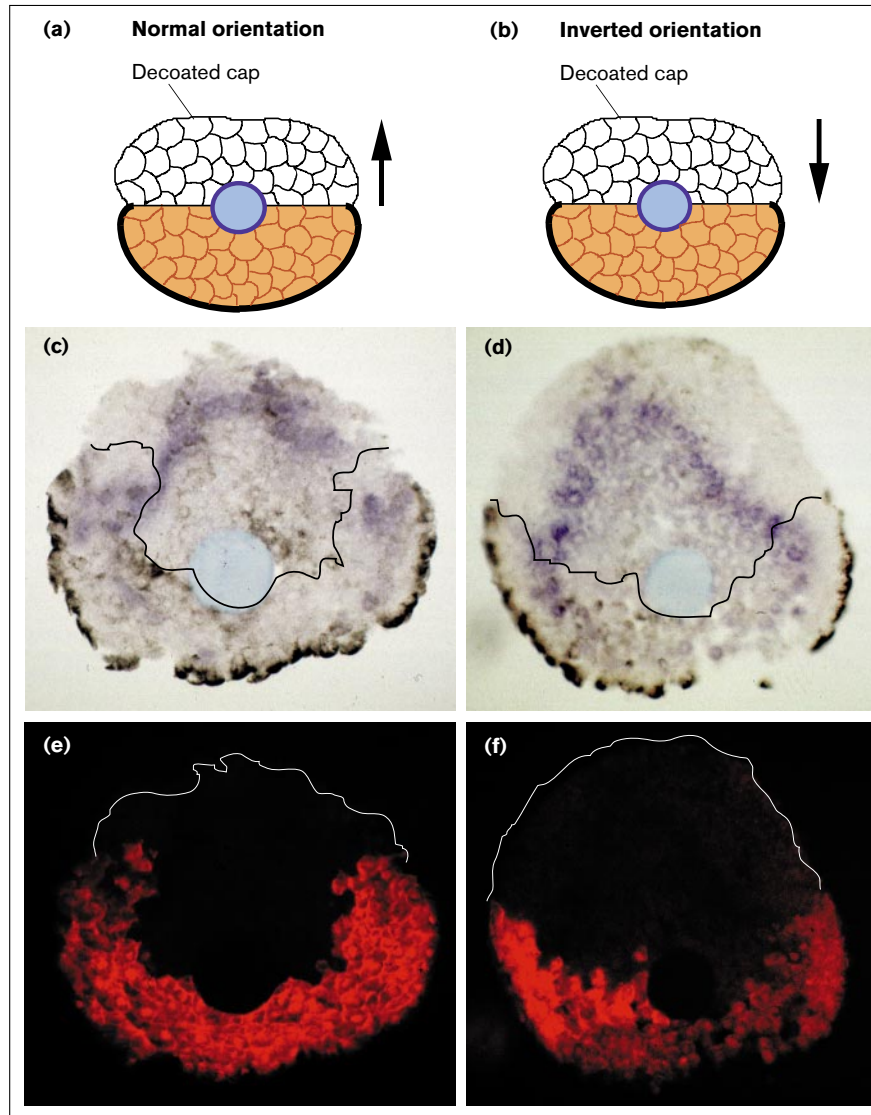
The polarity of responding tissue does not affect direction or rate of signalling. **(a,b)** The design of the experiment: the outer impermeable surface coat was removed from an animal cap and the tissue placed in either an inverted or normal orientation. Cells expressing *Xbra* RNA are the same distance from an activin bead, whether the upper decoated cap is in a normal orientation **(c)** or 180 degrees inverted **(d)**. The lower animal cap has a normal surface coat (pigmented) and has been injected with rhodamine lysinated dextran (RLDx). The black line (c,d) demarcates the boundary between decoated and rhodamine labelled cells **(e, f)**.

minimal grain density was not arranged in a gradient (Figure 4g). This is probably because the free amino acids diffuse away from the tissues during fixation and washing.

A second way in which our results could be misinterpreted relates to the path length of ^{35}S radioactivity. Could the gradient of autoradiographic grains centred on a bead represent emissions from the molecules on the bead rather than from molecules located under the grains in the tissue? We exclude this interpretation for two reasons. First, gaps between beads and nearby tissue (Figure 4a,c) leave grain-free areas and therefore, even with a strong source of radioactivity on the bead, the effective path length of ^{35}S emissions is less that 10 μm. Second, under conditions where a bead has been removed before sectioning (Figure 4e) or when the sections are at least 25 μm

above or below the bead (Figure 4f), we still see a clear gradient of autoradiographic grains in the section. In these cases, the bead is completely absent from the sections during autoradiography and therefore it is impossible for any silver grains in the tissue to have come from the radioactivity of molecules on the beads.

In the activin gradient that we see, autoradiographic grains can be readily detected above background for at least 120 μm away from a bead, a distance of about 7 diameters of cells in end to end contact (Figure 4d). With the strength of beads used in these experiments, we typically see activation of the high-response mesodermal gene *gsc* within this 120 μm area where a gradient is clearly observed. However, since the strength of beads used in these experiments causes *Xbra* expression in cells at the outer periphery of the

conjugate, and since low concentrations of the activin gradient would not be seen by autoradiography, the actual activin gradient is likely to extend to the edges of the conjugates. To obtain quantitation of the activin gradient, we have examined the autoradiographs of conjugates of the kind shown in Figure 4h using NIH imaging software. The graph shown in Figure 4i is derived from preparations where the bead is absent (as in Figure 4e) and illustrates diagramatically an activin concentration gradient across the tissue. These results provide positive evidence that activin can diffuse through solid tissue and is able to reach cells distant from an activin source.

Direction and rate of signal diffusion are not affected by polarity of the responding tissue

An interesting possibility is that signal diffusion is influenced by a polarisation of the responding tissue. Such a polarisation, if due to a differential distribution of extracellular matrix molecules, could aid the diffusion of activin in one direction and thus help to establish the form of the concentration gradient. We have therefore asked whether the direction and rate of signal passage is affected by the polarity of the animal cap along the vegetal–animal axis — from the inside (blastocoel roof) surface towards the outer surface.

The experimental design involved sandwiching two animal caps around activin-coated beads. One of these caps had previously been decoated. This enabled this decoated cap to be inverted by 180° compared to its normal orientation (Figure 5a,b). The distance of the *Xbra* band from the beads could therefore be compared in the inverted cap and in a decoated cap which had not been inverted.

The results are shown in Figure 5c–f. In all cases where an *Xbra* band was observed in the normally orientated cap, a similar band was observed in the inverted cap (Figure 5c,d). Furthermore, the rate of signalling, measured by distance of the centre of the band from the signalling source, was similar in the two types of conjugate. This was true when beads of different activin doses were used and also when the activin source was a cap injected with *activin* mRNA (Table 1). Altogether, 23 conjugates with inverted caps and 22 conjugates with normally orientated caps were tested and gave the results described. We therefore conclude that the blastula animal tissue does not have a polarisation which influences either the direction or rate of signal diffusion.

Discussion
Long-range signalling by activin is direct
A major conclusion from the work presented here is that the long-range signalling process initiated by activin is also mediated in the most distant responding cells by activin itself. This therefore eliminates the involvement of a non-activin secondary signalling molecule and identifies activin as the molecule to which cells respond in a

Table 1

Rate of signalling is not influenced by the polarity of the responding tissue.

Activin dose	5 nM beads		10 nM beads	
	Normal	Inverted	Normal	Inverted
Number of samples	7	7	9	10
Minimum distance (µm)	100	100	140	160
Maximum distance (µm)	180	190	260	240

Table comparing the minimum and maximum distances (µm) between the activin source (beads) and the centre of the *Xbra* band, for either a 'normal' or 'inverted' conjugate. Over the range of activin concentrations used, the distances observed were similar for both types of conjugate. Similar results were obtained when using *activin*-mRNA-injected caps as the signalling source and placed next to either normal or inverted animal caps and stained for *Xbra* expression.

concentration-dependent manner. The system we have used for analysis is experimental and, although the role of endogenous activin in mesoderm formation has yet to be fully understood, it is clear that activin can behave as a morphogen in *Xenopus* animal caps. The mechanism by which it does so is likely to be helpful in understanding other long-range signalling events in early development.

The experimental system used here evades a number of uncertainties that limit the interpretation of other examples of long-range signalling. Most importantly, there is very little cell division or cell movement [17], and the long-range signalling effects cannot be explained by the division and movement of cells after a short-range interaction (see [31]).

Diffusion not relay
All of our results support a diffusion rather than a relay mechanism of signalling. The fact that activin is required for response by the distant cells does not itself preclude the possibility that a relay process is involved, activin being released, at an attenuated level, by each step in the relay series. However, we have two kinds of evidence in favour of diffusion. First, we show that activin-related TGFβ2 can spread through tissue unable to respond to or synthesise it. Hence the spread cannot be by relay. Second, we show that radioactive activin, labelled by synthesis and not by attachment of a tag, leaves the signalling source and enters the responding tissue reaching cells not in contact with the signal source. We conclude that, in the model system used here, activin signalling takes place by diffusion, not relay. It is therefore reasonable to suppose that, in normal development, long-range signalling by activin also occurs by a mechanism of diffusion. It is possible that, in normal development, a relay system may be operative in addition to diffusion.

Our evidence that both TGFβ and activin can diffuse is consistent with our earlier experiments showing that

activin can spread through re-aggregated endoderm (non-responsive) tissue, as well as through re-aggregated cyclo-heximide-treated cells, which cannot therefore synthesize activin [17]. Furthermore, we believe that our results are consistent with those of Reilly and Melton [23] who also used non-endogenous TGFβ, but conclude that it signals by a relay mechanism. It is likely that the apparent difference in results may be due to their use of cells expressing *TGFβ1* mRNA rather than beads loaded with TGFβ1 protein as the source of ligand. In our hands, while the mature form of the protein has efficient long-range signalling properties across responsive (receptor injected) cells, *TGFβ* mRNA injection does not induce *Xbra* in distant responding cells. This may be contrasted with our previous work [17] which showed that activin expressed from mRNA-containing blastula cells does exhibit long-range signalling properties, unlike *TGFβ1* mRNA. The lack of long-range signalling following mRNA injection of TGFβ1 could be due to inefficient processing of a protein that is a non-endogenous molecule. The ability of endogenous TGFβs to exhibit long-range signalling may therefore be determined by how efficiently they are processed and secreted from the cells in which they are synthesised. Clear evidence for this was demonstrated by Jones *et al.* [20]. They showed that a more efficiently processed form of Xnr2 diffused from cells and exhibited a distance effect, while the poorly processed wild-type form did not.

Properties of the activin gradient

There are many examples of long range signalling in development that suggest the operation of a morphogen gradient. However, there has so far been no direct demonstration of an extracellular gradient of signalling molecules in the responding tissue. Thaller and Eichele [32] obtained four decreasing values for retinoic acid in a chick wing bud which had been exposed to retinoic acid beads. It is not, however, clear that retinoic acid functions as a morphogen in this system, at the nanomolar concentrations observed. In many other cases, molecules with morphogenetic effects — such as basic fibroblast growth factor, TGFβ, BMP4, Dpp, and so on — have not been detected outside the cells in which they are synthesized. This is probably because they act as morphogens at extraordinarily low concentrations (in the picomolar range) and cannot be detected in a gradient by even the most sensitive antibody procedures (our unpublished results) or tagging methods [23]. The reason why we have been able to see an activin gradient in our experiments is probably due to the very high specific activity of relatively pure activin synthesised and secreted by mRNA-injected oocytes and loaded onto beads.

We estimate the average concentration of activin in responding animal caps to be in the range of 10–80 pM, based on our estimate of the specific activity of our oocyte-synthesised ^{35}S-labelled activin. If the average concentration of activin in our animal cap experiments is 50 pM, the *Xbra*-inducing concentration, which is in the most distant cells in these experiments, is likely to be several fold lower, in reasonable agreement with the 20 pM concentration of activin found by Green *et al.* [16] to induce *Xbra* in dissociated animal cap cells.

It is usual to discuss morphogen gradients in terms of a source and a sink [33] and to assume that cells respond to an equilibrium gradient. We suggest that response to a changing or 'dynamic' gradient may be more normal in early development when new patterns of gene activation take place at intervals over a period of a few hours or less. We have shown that *Xenopus* blastula cells respond to increasing activin concentrations by a ratchet-like process [18], that is to the highest concentration that they experience within their period of competence. It would therefore be unnecessary for an equilibrium gradient to be reached before cells should respond to it. If an equilibrium gradient had to be achieved within a few hours, it would be difficult to ensure that cells would not respond to it prematurely. We therefore think that cell response to a progressively increasing gradient, by a ratchet-like mechanism, would place less demands on the precision of early developmental processes.

Materials and methods

Embryo injections
For the directional and multilayer conjugate experiments, embryos were microinjected at the 2-cell stage in 1× MBS [34], 4% Ficoll, with either 2 ng *ActRIIBdn* mRNA, 1 ng *ActRIIBexd* mRNA, 4 ng TGFβII receptor (*TGFβIIR*) mRNA [26], 500 pg, 1 ng, 2 ng or 4 ng *TGFβI* mRNA, or 30 ng rhodamine lysinated dextran (RLDx, Mr 10^4, Molecular Probes).

Embryological manipulations and bead preparation
Animal caps were cut at stage 8 and conjugates made as described in text. Recombinant bovine activin βA (Genentech) and TGFβ2 (R and D Systems) beads (Affi-gel blue, 100-200 mesh, Bio-Rad) at the concentrations cited in text and figure legends, were prepared according to [17–19]. Animal cap constructs were cultured at 23°C in 1× MBS. Where decoated caps were used, the outer impermeable cell layer was removed from stage 8.5 embryos, in 1× MBS, by gently peeling it away using fine forceps.

RNA expression constructs
Synthetic, capped mRNA was synthesized as described previously [35,36] using a Mega-Script *in vitro* transcription kit (Ambion) and cap analogue (New England Biolabs). *Xenopus ActRIIBdn* in pSP64T (a gift from A. Hemmati-Brivanlou and D. Melton) was linearised with *Sfi*I and transcribed using T3 RNA polymerase. *ActRIIBexd* in pBluescrip-tRN3P (a gift from S. Dyson) was linearised with *Sfi*I and transcribed with T3 RNA polymerase. The TGFβ1 expression construct was derived from pRKβ1E.C2S2 (a gift from R. Derynck) by cloning into pBluescriptRN3P [36]. For capped sense mRNA it was linearized at the *Sfi*I site and transcribed using T3 RNA polymerase. TGFβIIR in pSP64T (a gift from A. Bhushan) was linearized with *Eco*RI and transcribed using SP6 RNA polymerase.

In situ *hybridisation*
In situ hybridizations, using either *Xbra* or *Xgsc* antisense probes, were performed on sections as described by [36].

Autoradiography
Ilford K5 emulsion film was pipetted onto slides containing sectioned material. After exposure for 3–6 weeks, slides were developed by standard procedures.

Acknowledgements

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