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The role of fibroblast growth factor in early *Xenopus* development

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Summary

In early amphibian development, the mesoderm is formed around the equator of the blastula in response to an inductive signal from the endoderm. A screen of candidate substances showed that a small group of heparin-binding growth factors (HBGFs) were active as mesoderm-inducing agents *in vitro*. The factors aFGF, bFGF, kFGF and ECDGF all show similar potency and can produce inductions at concentrations above about 100 pM. The product of the murine *int-2* gene is also active, but with a lower specific activity. Above the induction threshold there is a progressive increase of muscle formation with dose. Single blastula ectoderm cells can be induced and will differentiate in a defined medium to form mesodermal tissues. All inner blastula cells are competent to respond to the factors but outer cells, bearing oocyte-derived membrane, are not.

Inducing activity can be extracted from *Xenopus* blastulae and binds to heparin like the previously described HBGFs. Antibody neutralization and Western blotting experiments identify this activity as bFGF. The amounts present are small but would be sufficient to evoke inductions *in vivo*. It is not yet known whether the bFGF is localized to the endoderm, although it is known that inducing activity secreted by endodermal cells can be neutralized by heparin.

The competence of ectoderm to respond to HBGFs rises from about the 128-cell stage and falls again by the onset of gastrulation. This change is paralleled by a rise and fall of binding of ^{125}I-aFGF. Chemical cross-linking reveals that this binding is attributable to a receptor of relative molecular mass about 130×10^3. The receptor is present both in the marginal zone, which responds to the signal *in vivo*, and in the animal pole region, which is not induced *in vivo* but which will respond to HBGFs *in vitro*.

In the embryo, the induction in the vicinity of the dorsal meridian is much more potent than that around the remainder of the marginal zone circumference. Dorsal inductions contain notochord and will dorsalize ventral mesoderm with which they are later placed in contact. This effect might be due to a local high bFGF concentration or, more likely, to the secretion in the dorsal region of an additional, synergistic factor. It is known that TGF-β1 and -2 can greatly increase the effect of low doses of bFGF, although it has not yet been demonstrated that they are present in the embryo. Lithium salts have a dorsalizing effect on whole embryos or on explants from the ventral marginal zone, and also show potent synergism when applied together with HBGFs.

Key words: *Xenopus laevis*, mesoderm induction, mesoderm-inducing factors, fibroblast growth factor, fibroblast growth factor receptor, transforming growth factor beta, competence, morphogens.

Introduction

Work in experimental embryology has given us a fairly detailed picture of the processes of regional specification occurring in the *Xenopus* embryo prior to gastrulation. These processes are collectively called 'mesoderm induction' because they lead to the formation of a ring of mesodermal tissue around the equator of the blastula (Nieuwkoop, 1969; Dale *et al.* 1985; Gurdon *et al.* 1985; Jones and Woodland, 1987). This knowledge has made it possible to ask meaningful biochemical questions about the nature of the signals and the responses and about how they can lead to the formation of a spatial pattern of specified regions in two or three dimensions.

Briefly, we believe that the egg is divided into three cytoplasmic zones by the onset of the first cleavage: animal, ventrovegetal and dorsovegetal. The animal hemisphere will form epidermis in the absence of inductive signals, but also has the competence to form mesodermal and probably endodermal tissues in response to such signals. The vegetal hemisphere consists of a large 'ventral inducing' zone and a small 'dorsal inducing' zone comprising less than 90° of latitude around the dorsal meridian (Dale and Slack, 1987*b*). During the blastula stages, these two regions emit

signals which induce, respectively, an extended region of ventral mesoderm around most of the equator, and a small organizer region on the dorsal side. The signals are quite short range, their influence extending only a few cell diameters (Gurdon, 1989), but, because of simultaneous migration of cells down into the equatorial zone, about 40% of the animal hemisphere eventually becomes recruited into the mesoderm (Dale and Slack, 1987*a*). These signals are the first two of the 'three-signal model' which our group has advanced to explain mesodermal patterning, the third being a dorsalization of the mesoderm as a function of distance from the organizer (Slack and Forman, 1980; Smith and Slack, 1983; Dale and Slack, 1987*b*). This model may need to be revised as new data come in but at present we believe that it still provides the best unified account of the known facts.

This understanding naturally leads us to ask three questions: (1) What is the molecular nature of the inducing substances? (2) Are dorsal and ventral signals qualitatively or quantitatively different? (3) What is the molecular nature of the competence of the animal hemisphere cells? Two critically important clues were provided by recent experiments on signal transmission. Grunz and Tacke (1986) showed that the signals could pass through a nucleopore filter in the absense of cell processes, and Warner and Gurdon (1987) showed that the signals could pass from vegetal to animal cells even when gap junction communication had been blocked. These biological experiments greatly narrowed the possible range of mechanisms and firmly pointed towards signals that consisted of *secreted extracellular substances*. In this paper, we describe our recent work on the role of fibroblast growth factor in mesoderm induction. Work on TGFβ-like factors is described in the accompanying paper by Smith and his colleagues.

Which factors are active?

Although sources of mesoderm-inducing factors (MIFs) were discovered many years ago, they tended to excite little interest. This was for three reasons: they came from heterologous sources; they were assayed as grafted pellets, a method that precludes quantitative biochemistry; and most were very crude extracts. The best characterized was the 'vegetalizing factor' of Tiedemann (1982) isolated from late chick embryos, but even this did not inspire confidence in the wider scientific community. We started work on the subject in 1984, following our reinvestigation of the basic mesoderm induction phenomenon, and commenced by establishing an assay procedure for MIFs which was quantitative and which worked in solution. Briefly, this consists of treating animal pole explants with serial dilutions of the test substance and defining the minimum concentration required to provoke an induction as 1 unit ml^{-1}. The full procedure is described in Godsave *et al.* (1988; see also Cooke *et al.* 1987). We then attempted to extend Tiedemann's work on the chick embryo factor using our improved assay, but, following the report by Smith (1987) of inducing activity secreted by a *Xenopus* cell line, we turned our attention to an investigation of known growth factors. In our initial screen, we tested a wide range of factors and found only three that were active. These were basic fibroblast growth factor (bFGF), embryonal carcinoma derived growth factor (ECDGF) and acidic fibroblast growth factor (aFGF), all of which belonged to a small group of heparin-binding growth factors (Slack *et al.* 1987). More recently, we have examined some of the FGF-like oncogenes that have recently been discovered (Paterno *et al.* 1989). We have done this by *in vitro* transcription of cDNAs from plasmids containing SP6/T7 bacteriophage promoters followed by translation in a rabbit reticulocyte lysate. The lysate can then be assayed directly by treating ectoderm explants with a series of dilutions, and the specific activity determined by measurement of the concentration of the translated protein. So far, we have examined kFGF, which is the product of the human *ks* and *hst* oncogenes (Delli-Bovi *et al.* 1987; Taira *et al.* 1987), and INT-2, the product of the murine *int-2* oncogene (R. Smith *et al.* 1988). Both are active as mesoderm-inducing factors. The specific activity of the kFGF is very similar to that of the a and bFGF, while the specific activity of INT-2 is very much lower. Considering the factors as a group, there is a good correlation between their mesoderm-inducing activity and their mitogenic activity when tested on mammalian fibroblasts. This suggests that similar signal transduction machinery is being used for the two processes. It should be emphasized that MIFs do not have any *mitogenic* effect on *Xenopus* blastula ectoderm cells, which are already cleaving every 30 min in the absence of growth factors and are probably incapable of further stimulation.

Meanwhile, work in other laboratories has shown that some factors belonging to the TGFβ family are also active. These are TGFβ-2 (Rosa *et al.* 1988) and the XTC-MIF of Smith (Smith, 1987; J. C. Smith *et al.* 1988) and so at the time of writing we have a total of seven active factors.

Which factors are present in the embryo?

Obviously the minimum requirement for identification of an endogenous morphogen is that the substance should be present in the embryo at the developmental stage when the relevant events are happening, and in amounts that are capable of exhibiting the observed degree of biological activity. We have approached this problem directly by asking whether a MIF can be obtained from the *Xenopus* blastula, and which of the seven or more candidates it is. Our results show that it is possible to purify a MIF from *Xenopus* blastulae using heparin-affinity chromatography and that it consists of two proteins of M_r 19 and 14×10^3 which react with antibodies against bFGF (Slack and Isaacs, 1989). The quantity in blastulae is about 10 ng ml^{-1} which is sufficient to account for the ventral but not the dorsal induction. The biological properties and specific activity

of the *Xenopus* bFGF seem similar to the bovine bFGF which has been used for most of our experiments on the responses of animal cells. All the MIF activity in a crude embryo or ovary extract can be inhibited by a neutralizing antibody to bFGF, but not by antibodies to a or k FGF or TGFβ-2. Parallel work by Kimelman *et al.* (1988) has also shown the presence of bFGF mRNA and protein in *Xenopus* blastulae. Their estimate of quantity is much greater than ours but, unlike ours, it is not based on the use of quantitative biological assay methods.

We would obviously predict that the bFGF would be secreted by the cells of the vegetal hemisphere. So far, immunolocalization on embryo sections has not proved successful, probably because of the small quantities present. We have shown that the MIF released by vegetal cells in transfilter experiments can be neutralized by heparin, as can both *Xenopus* and bovine bFGF, but not by anti-bFGF antibodies. This may mean that the bFGF is secreted as part of some complex not recognised by our neutralizing antibody, but further work is necessary to prove beyond doubt that the vegetal cells really secrete bFGF. One problem in this regard is the well-known fact that bFGF lacks a classical signal sequence for secretion (Abraham *et al.* 1986), and so there remains some uncertainty about its mechanism of release from cells.

Effects of FGF on ectoderm explants

In this work, it has been found that the properties of a and bFGF in their capacity as MIFs are very similar indeed. In what follows, 'FGF' will be used to refer to either form indifferently.

Untreated explants from around the animal pole of *Xenopus* blastulae develop into solid masses of epidermal cells. It can be shown by using antibodies to epidermal markers that 100% of cells become epidermal (Fig. 1A–D). Mesoderm inductions can be provoked by FGF concentrations in excess of about 100 pM (Fig. 2A). After explants are exposed to FGF nothing much appears to happen for the first few hours, the explants round up with their blastocoelic surface inside and the cells continue to cleave just like untreated explants. However, it is the first 90 min or so of exposure that are critical. After this time, the FGF can be withdrawn without affecting the course of subsequent events. Then, while control embryos are undergoing gastrulation, the explants elongate with the original closure point at one end and the original animal pole at the other (Fig. 1E). Within a batch, the degree of elongation depends on the applied dose, but between batches there is considerable variation. After 24–36 h of culture, the induced explants start to swell and soon become transparent (Fig. 1F). These vesicles invariably contain mesodermal tissues although the quantity and type depends on the applied dose (Godsave *et al.* 1988; Slack *et al.* 1988). At low doses inductions consist of small amounts of mesenchyme and mesothelium with the occasional wisp of muscle while at higher doses we see increasing amounts of mesenchyme and increasing amounts of muscle (Fig. 1G,H; Fig. 2B). Notochord is sometimes observed following the higher dose treatments, particularly when *in vitro* translated bFGF is used, but its formation is not very predictable. This dose–response curve is significantly different from that obtained with XTC-MIF, which will induce notochord reliably at a low multiple of the minimum inducing concentration (J. C. Smith *et al.* 1988), however, it is probably rather similar to that of *Xenopus* bFGF (Fig. 3).

We have examined the location of ^{125}I-labelled FGF in explants and find that it binds mainly to those plasma membranes that are exposed at the blastocoelic surface. There is little binding to the plasma membrane of the external surface (oocyte-derived or O-membrane) and little penetration into the cell mass (Darlington, 1989). The maximal response to the high doses consists of about 20% muscle by cell composition with an additional 10–20% of mesenchyme and this probably represents all the cells that were exposed on the blastocoelic surface of the explant at the time of treatment. The fact that many cells in induced explants are still epidermal may be entirely due to the limited penetration of the FGF since our studies of single cells leads us to believe that all cells without O-membrane are potentially inducible (see below).

Competence of the ectoderm

Using animal–vegetal combinations from different stages, it has been shown that the competence of the ectoderm to respond to the natural signal(s) extends from about stage 6 (64 cells) to about stage 10½ (Jones and Woodland, 1987). We have studied the *onset* of competence to FGF in the ectoderm by exposing for a period of 90 min explants taken from different stages and this shows that competence begins at about stage 7. We have studied the *loss* of competence by permanent exposure of ectoderm explants taken from different stages and this shows that competence is lost between stages 9 and 10 (Slack *et al.* 1988). Furthermore, the *degree* of competence can be assessed by measurement of the amount of muscle formed by explants from different stages in response to a standard dose, and this shows a rise and fall with the peak at stage 8 (Darlington, 1989). So the competence for FGF seems to rise at about the same time as competence for the natural MIF(s) but falls rather earlier, since there are about 3 h between stage 9 and 10½ at 22–24 °C. Competence to respond to XTC-MIF seems to persist into gastrulation, until stage 10½–11 according to our measurements (Darlington, 1989).

Since inductions arise in response to FGF concentrations in the pM range we expected that an essential molecular component required for competence would be a specific receptor. We have probed for a receptor on explanted tissues using ^{125}I-aFGF and the cross-linking agent BS_3. This has shown that a receptor is present and appears as two gel bands of M_r about 130 and 140×10^3,

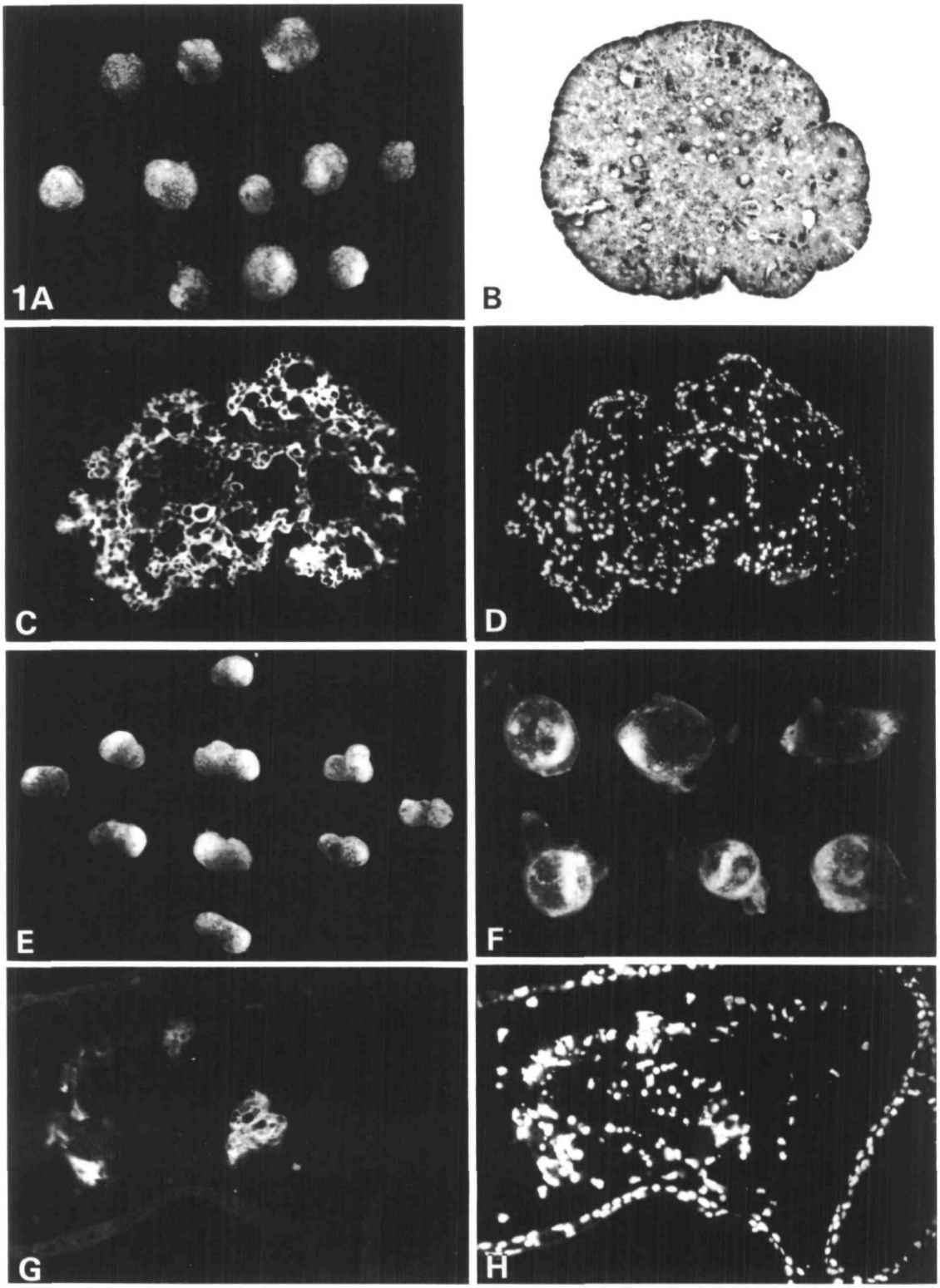

Fig. 1. Mesoderm induction by FGF. (A) Untreated ectoderm explants after 16 h. (B) Histological section of untreated ectoderm after 3 days. (C) Section stained with an antibody directed against cytokeratin XK70. All cells are stained. (D) Same section stained with DAPI to show cell nuclei. (E) FGF-treated explants after 16 h. (F) FGF-treated explants after 3 days ('vesicles'). (G) Section of induced explant stained with 12/101 anti-muscle antibody. (H) Same section stained with DAPI.

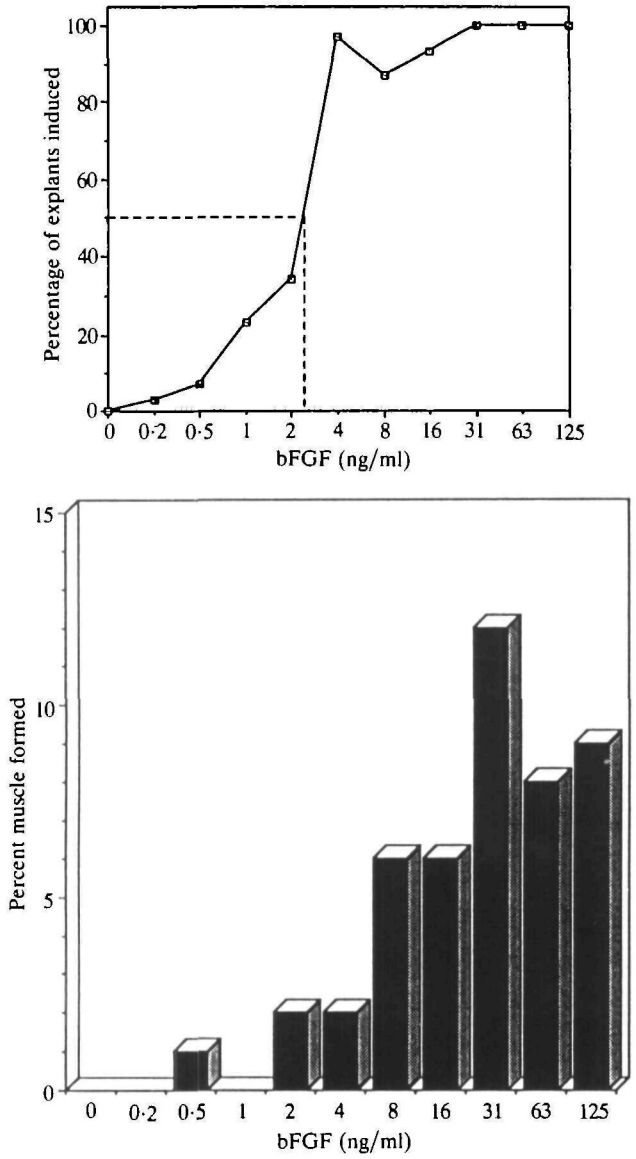

Fig. 2. FGF dose–response curves. (A) Percentage of explants induced by different concentrations of bovine bFGF. (B) Amount of muscle formed in ectoderm explants exposed to different concentrations of bFGF.

similar to the mammalian FGF receptor (Gillespie *et al.* 1989). Binding studies show that about 70–80% of bound ^{125}I-FGF can be competed out by an excess of unlabelled FGF. Assuming that this represents binding to the specific receptor then the density is about 3×10^8 molecules mm^{-2} of cell surface which is within the range of values measured for mammalian cells. The binding curve shows a half-maximal value of about 3–4 nM and a plateau at about 10 nM, which is very similar to the dose–response curve for muscle formation. This suggests that the receptor binding is a *limiting step* in the response. If it were not, then a maximal response, in this case a maximal percentage of cells induced, would be obtained at an FGF concentration below that required to saturate the receptors.

The receptor density has been studied by binding of ^{125}I-aFGF to ectoderm explants taken from different embryonic stages. The competable binding rises by a factor of 10 between the early and middle blastula, and falls again to the starting level by the onset of gastrulation. This closely parallels the rise and fall of competence to respond to FGF and suggests that competence is indeed controlled by receptor density.

Competition experiments have shown that both a and bFGF bind to the same receptor but TGFβ-2 does not. This again resembles the situation in mammalian cells and makes it probable that the extended period of competence that ectoderm explants show when treated with XTC-MIF is due to the presence of separate TGFβ receptors.

We have measured the regional distribution of FGF receptors in stage 8 blastulae by binding studies on explants (Gillespie *et al.* 1989). This shows, as predicted, that FGF receptors are present both in the marginal zone region, which normally responds to the signal *in vivo*, and in the animal pole region, which can

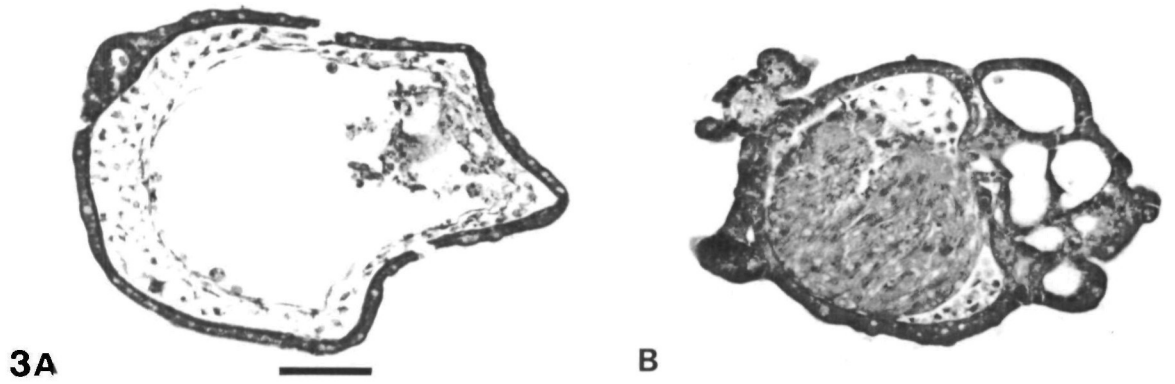

Fig. 3. Ectoderm explants induced by *Xenopus* bFGF and cultured for three days. (A) 4 units ml^{-1}. (B) 32 units ml^{-1}. Scale bar, 100 μm.

respond in experimental situations but would not normally do so *in vivo*. There is a slight excess of receptor density in the marginal zone but this is only 50% more than the animal pole value, so it would seem that the normal extent of mesoderm induction is determined by the extent of the signal and not by the presence of a more highly competent tissue in the marginal zone. There is no difference in receptor density between dorsal and ventral regions of the animal hemisphere, so this cannot account for the difference between dorsal and ventral inductions. FGF receptor is also present in the vegetal region. We do not know whether these cells need FGF for their normal development since we cannot deprive them of it in the way that we can deprive the animal cells. However, since they do not normally turn into mesoderm, we can deduce that mesodermal competence consists of something more than the presence of FGF receptors on the cell surface.

Competence of individual ectoderm cells

Some other workers have noticed that isolated ectoderm cells will not differentiate into mesodermal cell types after induction, although their differentiation into epidermis may be suppressed (Symes *et al.* 1988). This phenomenon has been called the 'community effect' (Gurdon, 1988). We have found that this requirement can be met by a few simple macromolecular additives to the culture medium. Single internal blastula ectoderm cells can be induced if they are treated with FGF and then cultured in the presence of gamma-globulin on a surface coated with fibronectin and laminin. Usually they give rise to monotypic clones of muscle or an 'epithelium' which is a non-muscle, non-epidermal cell type, possibly a form of kidney. Sometimes mixed colonies are formed with more than one mesodermal cell type. When cells are treated for only 2 h with FGF, the colonies are always monotypic (Godsave and Slack, 1989). We are presently using this culture system to examine the specification of single cells isolated from different parts of the marginal zone of normal embryos, and have shown that mesodermal clones can be obtained from the marginal zone of midblastulae.

Further experiments involving the induction of single cells have shown that cells bearing the oocyte-derived membrane (O-membrane) are non-inducible (Darlington, 1989). The most informative protocol has involved (1) labelling of donor embryos by injection with the lineage label rhodamine-dextran-amine (RDA), (2) isolating single labelled cells in Ca^{2+}-free medium, (3) wrapping these in ectodermal jackets from unlabelled embryos, (4) inducing the whole sandwich with FGF or another MIF before it has sealed. When inner cells wholly surrounded by cleavage membrane (C-membrane) are used then many progeny of the labelled cell are found in the induction. However, when cells bearing O-membrane are used only a very few progeny are found to be induced. Close examination of these few shows that all of them are themselves wholly surrounded by C-membrane, and must therefore have arisen from the original cell by tangential cleavage. So they do not represent exceptions but rather they are important positive controls, showing that the culture conditions do not militate against mesoderm differentiation. A further control in these experiments is provided by the fact that the cells that do not form mesoderm *do* form epidermis, showing that the failure to form mesoderm is not due to some damage inflicted on the cells in the course of the manipulations.

The nature of the dorsal induction

It is generally agreed that the signal near the dorsal meridian differs from that around the remainder of the blastula circumference. Some workers have tended to think that it is qualitatively similar but more intense while others have leaned towards the view that it is qualitatively different. If we accept that bFGF is *the* ventral morphogen, then the quantitative view seems unlikely since notochord inductions are not reliably produced even by very high concentrations of FGF, and the uniform distribution of FGF receptor shows that the dorsal and ventral ectoderm will respond alike to similar concentrations of FGF. However, it has been shown that the effect of FGF can be modified by other factors. There is strong synergism between FGF and TGFβ-1 (Kimelman and Kirschner, 1987) and between FGF and lithium ion (Slack *et al.* 1988). Neither TGFβ-1 nor Li are active as mesoderm-inducing factors on their own and the synergism is usually manifested as an excess formation of muscle rather than by induction of notochord. TGFβ-2 does have mesoderm-inducing activity on its own, and like FGF does not usually induce notochord. However, the synergism between FGF and TGFβ-2 is strong enough to give reliable induction of notochord (E. Amaya, pers. comm.). We have seen above that the receptors for FGF and TGFβ on *Xenopus* ectoderm are distinct but the synergistic effects suggest that there is a common intermediate at some level in the signal transduction pathway. This intermediate is presumably one whose level can be elevated by Li^+.

A reasonable working hypothesis based on these data might be that bFGF is the ventral morphogen and bFGF+TGFβ-2 the dorsal morphogen. We would further suppose that the FGF system is prelocalized in the vegetal hemisphere of the egg while the TGFβ system is activated on the dorsal side only as a result of the postfertilization cytoplasmic movements (see Fig. 4 and Gerhart *et al.* this volume). This would then explain the effects of UV radiation and Li^+ on whole embryos. If the vegetal hemisphere of the egg is irradiated with a sufficient dose of UV light then the cytoplasmic movements are inhibited and a radially symmetrical ventral embryo is formed (Grant and Wacaster, 1972; Cooke and Smith, 1987). The simplest interpretation of this is that the FGF system is normally present in the vegetal hemisphere all around the circumference and is unaffected by the treatment while the TGFβ system would depend on the postfertilization cytoplasmic movements

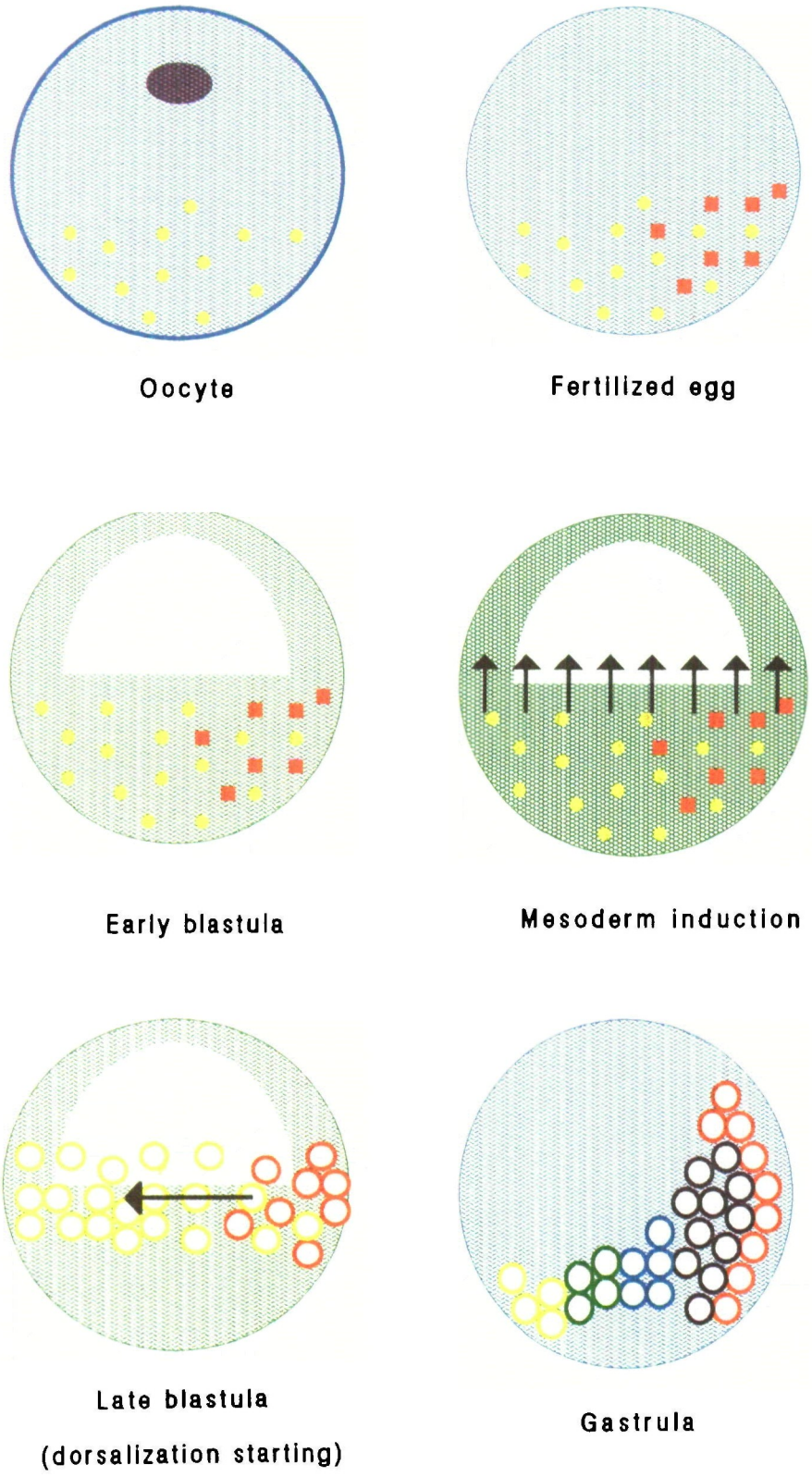

Fig. 4. Diagram of current version of the three-signal model. The yellow dots are sources of bFGF, prelocalized in the oocyte. The red squares are sources of TGFβ-2 (or XTC-MIF) which become activated and localized on the dorsal side following fertilization. The green colour represents FGF receptors rising and falling during the blastula stages. The arrows represent short-range diffusion of the morphogens. The open circles represent cells: red for organizer type, yellow for ventral mesoderm type, other colours for intermediate mesodermal types formed by dorsalization.

which are blocked by the UV dose. Li^+ treatment of the early embryo produces a symmetrical dorsalization (Kao *et al.* 1986; Cooke and Smith, 1988). Here the postfertilization movements have already happened but we presume that the Li can elevate the concentration of a signal transduction intermediate and so mimic the effect of a uniform dorsal stimulus. It has been shown that lithium will dorsalize isolated ventral marginal explants to the level of large muscle masses (Slack *et al.* 1988; Kao and Elinson, 1988).

In fact, we have no evidence at present that the *Xenopus* homologue of TGFβ-2 is present in the early embryo and it may be that some other TGFβ-like molecule is doing the job. An obvious candidate is the XTC-MIF of Smith since this has chemical properties resembling TGFβ and is currently the most active of all the MIFs and the only one that will induce notochord on its own. Another possibility is the Vg1 product. Here we know that the mRNA is present in the embryo and localized in the vegetal hemisphere (Weeks and Melton, 1987; Yisrael *et al.*, this volume). However, there does not appear to be any preferential localization on the dorsal side, and perhaps more seriously there is as yet no indication of any biological activity shown by the protein. Clearly more data is needed in this area and in particular data on the presence, distribution and activity of TGFβ-like molecules in the early *Xenopus* embryo.

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