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Specification of the body plan during *Xenopus* gastrulation: dorsoventral and anteroposterior patterning of the mesoderm

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Summary

Although the mesoderm itself is induced at the blastula stage, its subdivision mainly occurs in response to further inductive signals during gastrulation. In the late blastula, most of the mesoderm has a ventral-type commitment except for the small organizer region which extends about 30° on each side of the dorsal midline. During gastrulation, dorsal convergence movements bring the cells of the lateroventral marginal zone up near the dorsal midline and into the range of the dorsalizing signal emitted by the organizer. This dorsalizing signal operates throughout gastrulation, can cross a Nuclepore membrane, and is not mimicked by lithium, FGFs or activin.

Anteroposterior specification also takes place during gastrulation and is probably controlled by a dominant region at the posterior end of the forming axis.

We have studied the expression patterns in *Xenopus* of three members of the FGF family: bFGF, int-2 and a newly discovered species, eFGF. These all have mesoderm inducing activity on isolated animal caps, but are likely also to be involved with the later interactions. RNAase protections and in situ hybridizations show that the int-2 and eFGF mRNAs are concentrated at the posterior end, while bFGF is expressed as a posterior to anterior gradient from tailbud to head.

Studies of embryos in which bFGF is overexpressed from synthetic mRNA show that biological activity is far greater when a functional signal sequence is provided. This suggests that int-2 and eFGF, which possess signal sequences, are better candidates for inducing factors in vivo than is bFGF.

Key words: *Xenopus*, gastrulation, mesoderm induction, dorsoventral specification, anteroposterior specification, fibroblast growth factors, activins.

Introduction

Gastrulation is a time of extensive morphogenetic movements and in the vertebrate embryo it is also a time of extensive regional specification. The formation of the *Xenopus* body plan starts with cortical rotation in the egg and mesoderm induction in the blastula, but the main events of anteroposterior and dorsoventral specification both occur during gastrulation (Fig.1). So, in contrast to *Drosophila*, where the principal territories of the body plan are set up before gastrulation, in the vertebrates we have to understand the inductive interactions which bring about specification in the context of the simultaneous cell and tissue movements.

The main advance in the understanding of early *Xenopus* development in recent years has been the identification of a number of inducing factors belonging to the FGF, activin and wnt families. The FGFs and activins were first identified as mesoderm inducing factors, but we now think it is likely that they also have a role in the later interactions. In this paper we shall briefly review the embryology of dorsoventral and anteroposterior specification in *Xenopus* and consider which factors are candidates for which of the biological functions under consideration.

Dorsoventral specification

The initial step in dorsoventral (DV) specification is the cortical rotation which occurs following fertilization (reviewed by Gerhart et al., 1989). This is in some way necessary for the establishment of a "DV" centre, also called a "Nieuwkoop centre", in the dorsovegetal quadrant. During the blastula stages, the DV centre induces a small territory on its animal side to become the *organizer*, while around the remainder of the equatorial circumference a signal is emitted from the vegetal cells that induces a ring of "ventral-type" mesoderm from the equatorial part of the animal hemisphere (Gimlich, 1986; Dale and Slack 1987b; Stewart and Gerhart, 1990). Eggs that were irradiated with ultraviolet light shortly after fertilization do not undergo the cortical rotation and form no DV centre. They gastrulate in a radially symmetrical manner with the whole circumference of the marginal zone behaving like the ventral half of a normal embryo. Since they form abundant blood and loose mesenchyme around their circumference it seems that their ventral mesoderm inducing signal functions normally and is probably present around the whole circumference also in normal embryos. Although the regional specificity of mesoderm induction was originally thought to be vested entirely

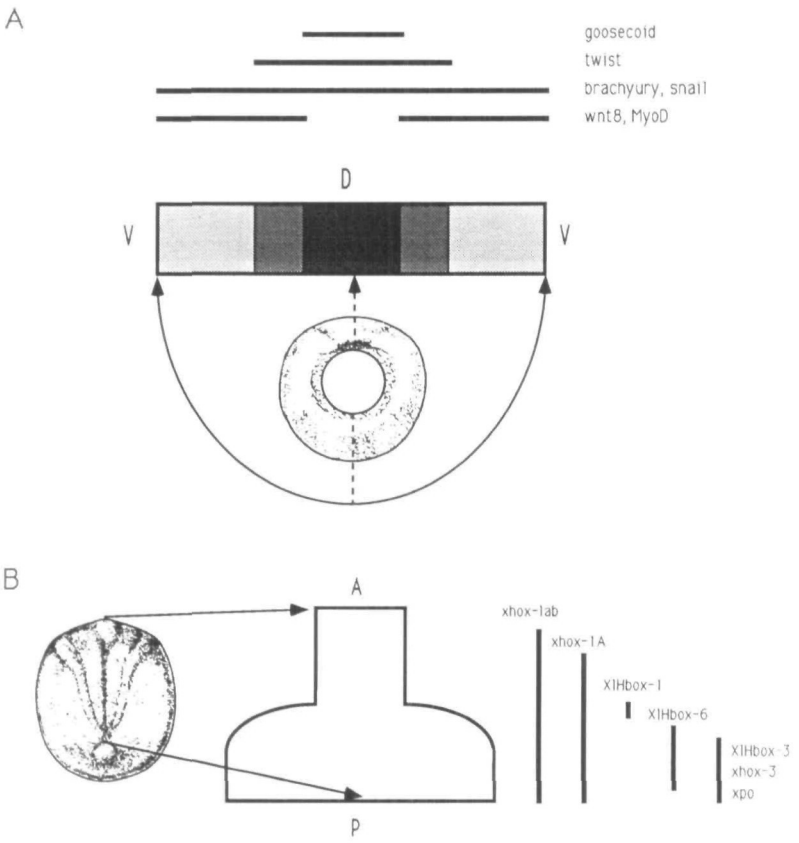

Fig. 1. Regionalisation of the mesoderm in the *Xenopus* gastrula. (A) Expression domains during gastrulation of genes with different dorsoventral domains. (B) Expression domains in the neurula of genes with different anteroposterior domains. Sample references to the expression patterns are as follows:
goosecoid: Cho et al. (1991a)
twist: Hopwood et al. (1989a)
brachyury: Smith et al. (1991)
snail: Sargent and Bennett (1990)
wnt-8: Christian et al. (1991); Smith and Harland (1991)
MyoD: Hopwood et al. (1989b); Frank and Harland (1991)
xhox-lab: Sive and Cheng (1991)
xhox-1A: Harvey et al. (1986)
XlHbox-1: Carrasco and Malacinski (1987); Oliver et al. (1988)
XlHbox-6:Sharpe et al. (1987)
XlHbox-3 (Xhox-36): Condie and Harland (1987)
xhox-3: Ruiz i Altaba et al. (1991)
xpo: Sato and Sargent (1991)

in the signal, it has been shown recently that there is also a difference of competence, especially of the region just above the equator on the dorsal side, which is more prone to become organizer than the rest of the animal hemisphere (Sokol and Melton, 1991).

So by the middle blastula stage the specification map of the embryos shows a small (60-90°) region forming notochord and a large (270-300°) region forming loose mesenchyme and blood cells. A specification map is compiled by explanting small pieces of tissue and allowing them to self differentiate in culture. It is a measure of the commitment achieved by the tissue up to the time of explantation. By contrast a fate map of an embryo is compiled by labelling each region in situ and allowing the intact embryo to develop further, and it shows what each region will become in normal undisturbed development. The fate map of the cleavage and blastula stages is not the same as the specification map. It shows that a substantial proportion of the axial tissues, for example about 60% of the myotomal muscle, is derived from the ventral half (Dale and Slack, 1987a). This means that much of the mesoderm which initially has a ventral character must be promoted to axial status at some later stage.

The available evidence suggests that this process, which we call *dorsalization*, occurs during gastrulation. Juxtaposition of dorsal and ventral tissues from early gastrulae has long been known to cause dorsalization of the ventral component, resulting in the formation of muscle masses and pronephric tubules instead of loose mesenchyme and blood cells (Slack and Forman, 1980; Dale and Slack, 1987b; Fig. 2). We have now shown, using equivalent combinations made at different stages throughout gastrulation, that dorsalization can occur at high frequency between stage 10 and stage 12. We have also carried out heterochronic combinations and it may be deduced from these that the dorsal midline tissue continues to emit a dorsalizing signal until after closure of the blastopore, while the competence of the ventral tissue to respond falls sharply after stage 12.

These experiments show that dorsalization *can* occur up until the end of gastrulation, but does it actually do so? The best evidence that it does (apart from the comparison between fate and specification maps referred to above) is obtained by looking at the relative sizes of axis and blood forming territories in normal embryos compared to embryos in which gastrulation movements have been inhibited. One way of inhibiting gastrulation movements is by injection with suramin (Gerhart et al., 1989) although other methods lead to the same result. In suramin treated embryos, not only is there a truncation at the anterior end, but in the trunk region the axis is much smaller and the blood forming territory much larger than usual (Fig.3). Since a treatment at the beginning of gastrulation can prevent dorsalization, the interaction must occur after this stage. We also know that competence of ventral tissue to become dorsalized is lost after stage 12 (see above) so it follows that dorsalization must normally be occurring during gastrulation. The movements of gastrulation involve massive dorsal convergence of marginal zone cells, which bring about three quarters of the mesoderm up into the axial region. This means that the signal may only need to have a very short range, of a few cell diameters, and it is presumably those cells that come close to the dorsal midline that receive the signal and

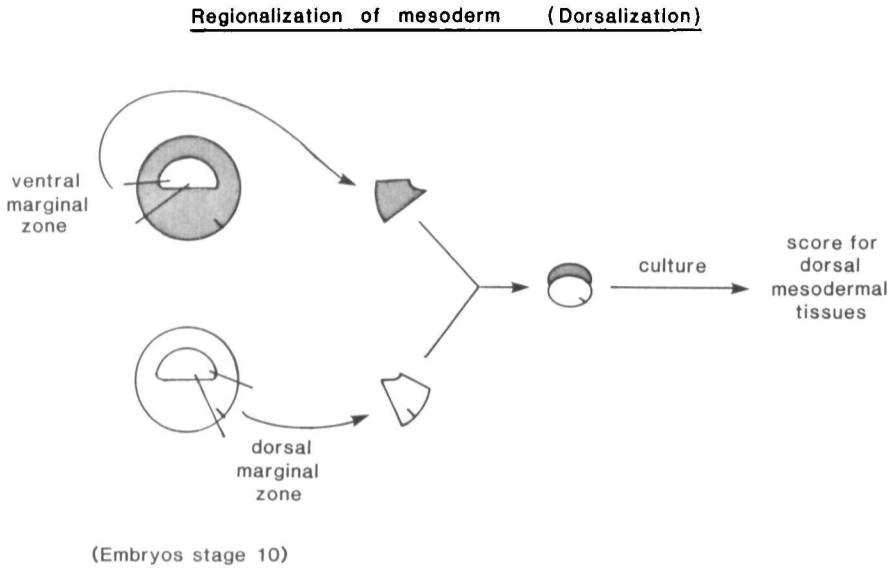

Fig. 2. Design for experiments on dorsalization of the mesoderm: FDA-labelled ventral marginal zone explants are combined with unlabelled dorsal explants and cultured for two days. The formation of labelled muscle blocks or pronephric tubules is indicative of dorsalization.

become dorsalized, while those that remain in the ventrolateral region do not see the signal and remain ventral in character.

We have recently examined the dorsalizing signal using a transfilter apparatus of the type described by Slack (1991). This shows that the signal can be transmitted across a liquid gap, but with lower efficiency than the mesoderm inducing signals. We have also tested a variety of cytokines, including activin A and FGFs, on gastrula stage ventral explants to see if any of them can provoke dorsalization, but none has done so, even when they have successfully brought about mesoderm induction in blastula stage animal caps treated simultaneously. Lithium ions can dorsalize ventral explants from the middle blastula as previously reported (Slack et al., 1988), but can no longer do so by the late blastula or gastrula stages. At present therefore we have no idea about the nature of the dorsalizing signal, except that it is unlikely to be a member of the FGF or activin classes.

Anteroposterior specification

There is a high degree of cellular intercalation occurring on the dorsal side during gastrulation (Wilson and Keller, 1991). Because of this any small group of cells in the dorsal lip region will become stretched out and scrambled by the cell mixing and so it seems unlikely that any anteroposterior levels could be specified before gastrulation. The evidence we have from several lines of work suggests that anteroposterior specification and gastrulation go hand in hand (reviewed by Gerhart, 1989; Slack and Tannahill, 1992).

In general, experiments in which anterior and posterior tissues are juxtaposed seem to result in the anterior member becoming posteriorized while the posterior member remains unchanged (reviewed by Slack and Tannahill, 1992). Much of the data underlying this statement are quite old, the experiments being performed on urodeles and without adequate labels to distinguish graft from host cells. Recently we have repeated some of these on axolotls using FDA-labelled grafts and the indication so far is that the results are indeed correct. For example if an early dorsal lip is grafted into the position of a late dorsal lip, it becomes integrated into the axial structures of the posterior trunk and tail (Fig.4). On the other hand, a late lip grafted into an early gastrula does not populate the head. It still populates the trunk and tail, and the head is left severely malformed. The idea of posterior dominance is consistent with the widespread belief that the genes of the Antennapedia-like homeobox clusters (HOX genes) are coding factors for different anteroposterior levels, since these genes are activated in a serial threshold arrangement, all being on at the posterior end and each territory in the posterior to anterior direction being specified by the loss of one more gene product. As to the actual mechanism of generation of a sequence of posterior to anterior states, two possibilities have been the subject of recent informal discussions, which can be called for short the "timing" model and the "signalling" model.

In the timing model, the early dorsal lip is seen as possessing an anterior specification and of acquiring a progressively more posterior character with time. For example some substance, M, could accumulate in the lip region and as its concentration rises so more and more posterior genes would be activated. In those cohorts of cells that involuted away from the lip, the altered environment of the embryo interior would stop this accumulation and "freeze" the tissue at that level of posterior specification achieved by the time of leaving the lip. The end result will be a series of territories arranged from anterior to posterior in positions which have an ordered sequence of anterior to posterior states of specification.

In the signalling model, the lip is seen as permanently posterior in character. It emits a morphogen, M, that forms a posterior to anterior gradient across the involuted tissue. Each of the AP coding genes is turned on at a different threshold concentration. Cohorts of cells respond to this signal as a function of distance and hence become progressively more anterior in character as they invaginate away from the lip. Their states do not become irreversibly determined until the end of gastrulation.

In both models we are obliged to assume that something changes on invagination as a result of exposure to the internal environment, which might mean exposure to the blastocoelic fluid, or contact with the blastocoel roof, or both. The main difference between the models is that in the second the anteroposterior level remains labile in the dorsal mesoderm for some time after invagination, whereas in the former it becomes fixed straight away. We feel that more embryological work is required to define this process more closely and that this is an essential adjunct to the molecular work to be described below.

"Mesoderm inducing" factors

When the first pure substances were shown to have mesoderm inducing activity, it was confidently expected that they would indeed be performing this task in vivo. However it has not yet been possible to prove that either bFGF or the activins definitely have a role in this process. No mRNA has been detected for activin A or B in early stage *Xenopus* embryos (Thomsen et al., 1990), although some activin-like protein has been found (Asashima et al., 1991). bFGF is expressed as mRNA and protein in the early embryo (Kimelman et al., 1988; Slack and Isaacs, 1989) but we have concluded after a series of overexpression experiments that it cannot be secreted from cells (Thompson and Slack, 1992). In these experiments, synthetic mRNA for bFGF is injected into fertilized eggs, they are allowed to develop to the blastula stage and then animal caps are explanted (Fig.5). Despite the synthesis of large amounts of bFGF protein, and its concentration in cell nuclei, only a limited degree of autoinduction is found in such caps. A similar study by Kimelman and Maas (1992) also showed only limited activity from large doses of RNA in comparable "ventral type" caps. However the biological activity goes up by over 100-fold if the bFGF is provided with a signal sequence from the immunoglobulin gene, or if another member of the family, which does possess a signal sequence, such as human kFGF, is used (Thompson and Slack, 1992).

In an attempt to test directly the role of activins and bFGF, a series of transfilter experiments were carried out in which the inducing and responding tissues were separated by an assembly of membranes about 100 μm wide (Slack, 1991). A high frequency of control inductions was obtained although these are of a ventral character, with a variable content of muscle, so it may be that the DV signal cannot cross the liquid gap. Inclusion in the liquid gap of high concentrations of follistatin, a naturally occurring inhibitor of activin, or of neutralizing antibody to bFGF, failed to inhibit the transfilter inductions. This suggests that these substances are not the factors released from the vegetal cells, although they may still have a role at a subsequent stage of mesoderm induction within the responding tissue.

Because bFGF seemed not to be secreted from the vegetal cells, and indeed the overexpression experiments suggest that it cannot be secreted at all, we turned our attention away from bFGF and towards a search, in *Xenopus*, for other members of the FGF family, as will be described below.

If the activins and bFGF are not normally secreted from vegetal cells, why do they show activity when applied to animal caps? The explanation probably lies in the fact that the receptors are maternally coded and are present in an active form on the cell surfaces of the early stages (Gillespie et al., 1989; Musci et al., 1990; Friesel and Dawid,

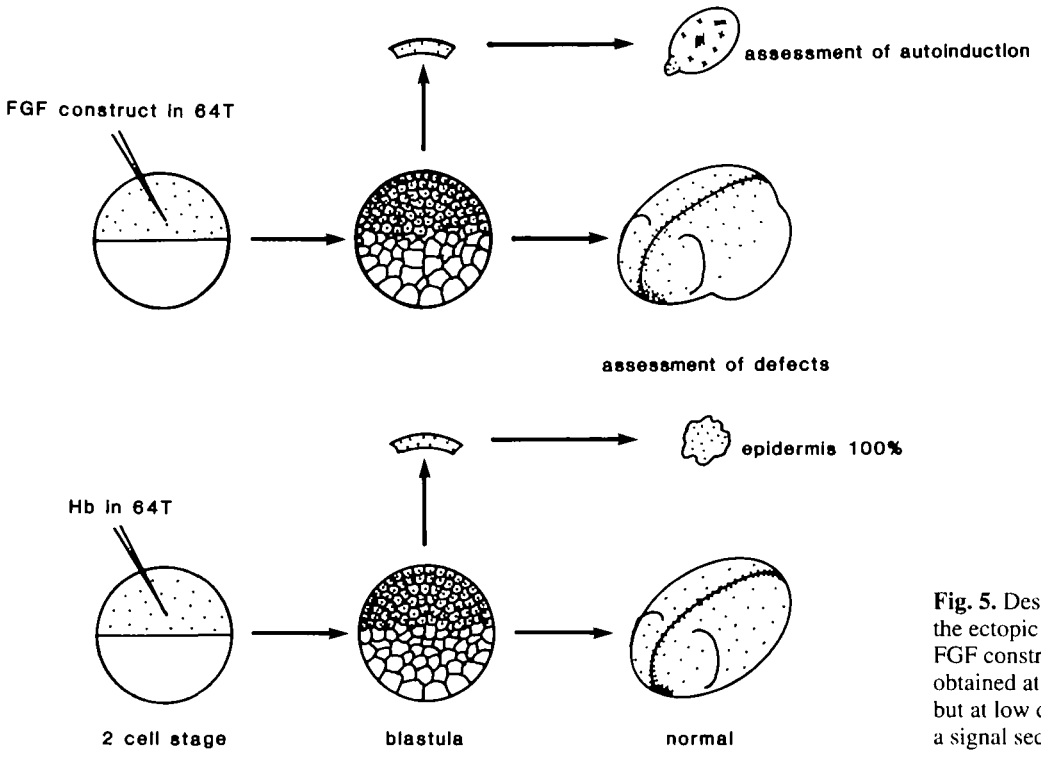

Fig. 5. Design for experiments involving the ectopic overexpression of various FGF constructs. Autoinductions are only obtained at high doses of bFGF mRNA, but at low doses of constructs containing a signal sequence.

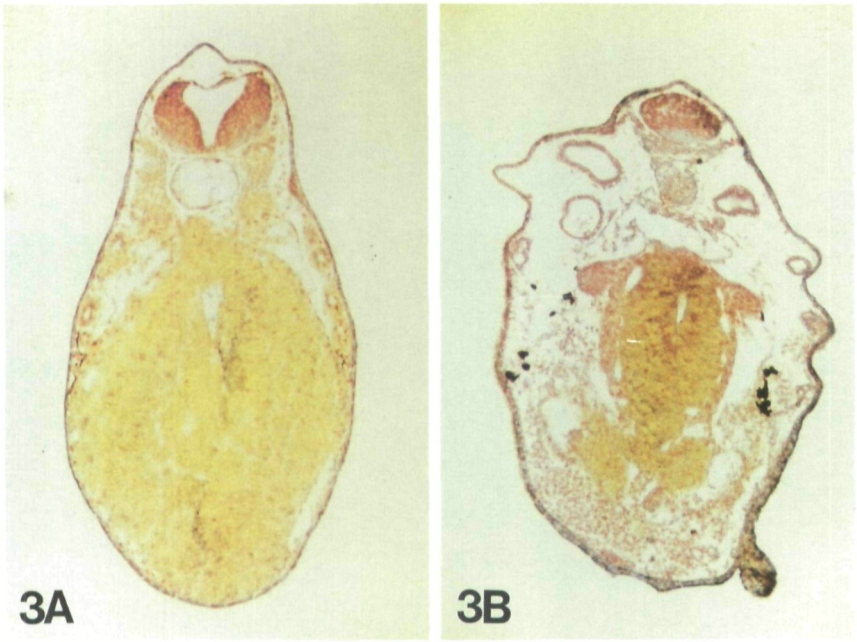

Fig. 3. Effect on dorsoventral proportions of blocking cell movements during gastrulation. (A) Normal embryo, TS through the level of the pronephros. (B) An embryo injected with suramin at the early gastrula stage. The proportion of blood cells relative to axial mesoderm is very considerably increased.

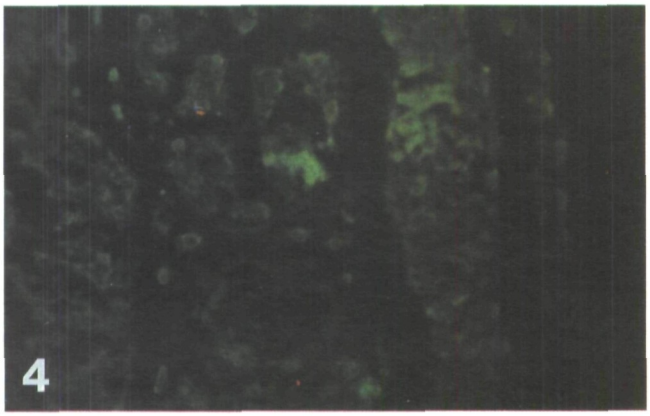

Fig. 4. Effect of grafting the dorsal lip of an early gastrula to the dorsal lip of a late gastrula. This experiment was performed on axolotl embryos and the graft was FDA labelled. The graft becomes integrated into the tissues of the posterior axis.

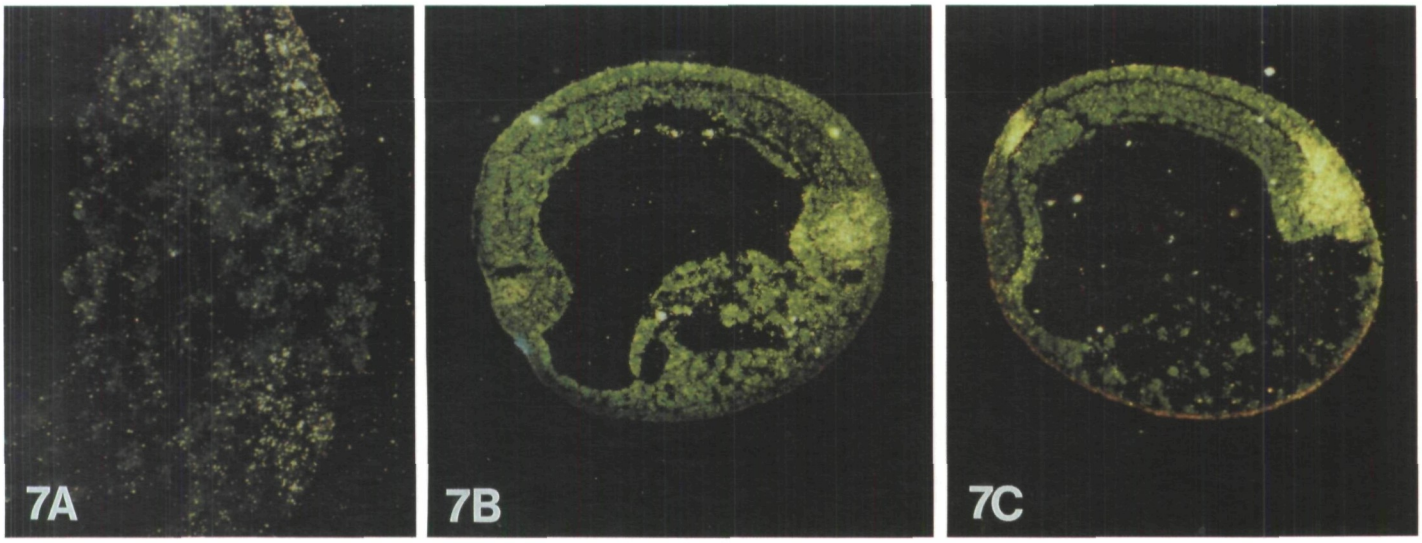

Fig. 7. In situ hybridization of endogenous mesoderm inducing factors. Anterior lies to the left. (A) eFGF in the middle gastrula stage showing activity in the mesoderm near the blastopore. (B) eFGF in the early neurula, parasagittal section, showing activity in the posterior mesoderm. (C) int-2 in the early neurula showing activity in the posterior mesoderm and in the anterior of the neural plate.

1991; Kondo et al., 1991). We know that the number of second messages in the signal transduction pathways is quite limited and so if a stimulus is received by blastula cells that elevates (say) tyrosine kinase activity then a whole cascade of protein phosphorylations will be started which will push the cells down the mesoderm pathway (Whitman and Melton, 1989; Gillespie et al., 1992). The FGF receptors are all tyrosine kinases and the activin receptors are SerThr kinases, so stimulation by the two classes of factor is likely to bring about distinct but overlapping patterns of protein phosphorylation. This may account for distinct but overlapping biological response, briefly summarised by saying that activin gives dorsal type inductions while FGF gives ventral type inductions (Smith, 1989; Green et al., 1990).

There is now reasonable agreement on the criteria that need to be satisfied to identify the true mesoderm inducing factor or factors. Firstly they must be expressed at the right stage and in sufficient quantity. Secondly the purified protein must show the expected biological activity. Thirdly inhibition of the factor should cause inhibition of the process in vivo. These have not yet been satisfied for bFGF or activin, although are closely approached for eFGF (see below). Recently it has been shown that synthetic mRNA from the *wnt-8* gene will mimic the DV signal if injected into vegetal blastomeres (Sokol et al., 1991; Smith and Harland, 1991). The *wnt-8* gene itself is not expressed until gastrulation, and then in the ventrolateral part of the marginal zone, so it cannot be regarded as a credible candidate itself. Even if there are maternally coded *wnt* mRNAs with similar activity, more experiments would obviously need to be done to satisfy the conditions listed. A similar list of criteria would hold for any putative inductive signal involved in later events.

Expression patterns of active factors

Regardless of the exact role that activins and FGFs ultimately turn out to have with regard to mesoderm induction, they are quite likely also to be involved in later inductive interactions necessary to establish the body plan, such as those described above. In order to make a reasonable guess about their functions the first step is to establish the developmental expression pattern of each factor. We have in our laboratory attempted to do this using RNAase protections and in situ hybridizations to detect mRNA. We have studied four factors. Firstly there is bFGF, the prototype member of the FGF family amd the one first shown to be a mesoderm inducing factor (Slack et al., 1987). Then there is int-2, originally discovered as an insertion site for murine mammary tumour virus and later shown to have mesoderm inducing activity (Paterno et al., 1989). Thirdly, there is eFGF, which was cloned in our laboratory as part of a search for potentially secretable FGFs (Isaacs et al., 1992). It is a molecule about equally similar to human kFGF and FGF-6, it has a signal sequence and protein expressed in bacteria has mesoderm inducing activity. Fourthly we have also included activin B, originally cloned by Thomsen et al. (1990), which is very similar in biological activity to activin A (Smith et al., 1990).

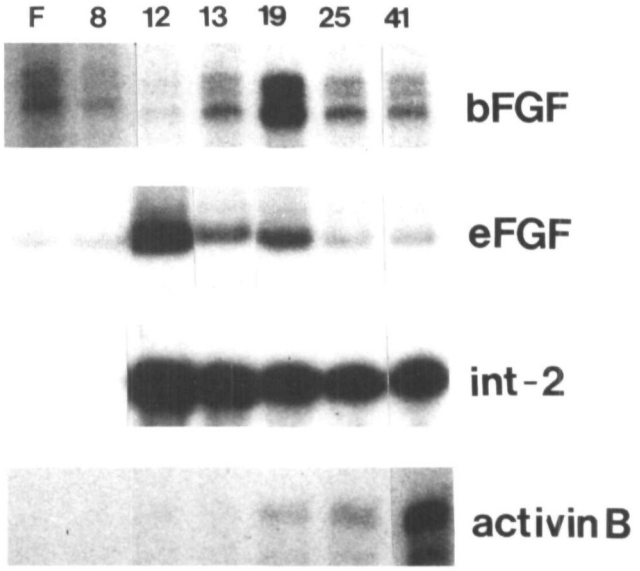

Fig. 6. Developmental time courses of four endogenous mesoderm inducing factors: bFGF, eFGF, int-2 and activin B. The figure shows parallel RNAase protections at different stages. The RNA loading was 20 μg for the FGFs and 40 μg for the activin B.

In whole embryos bFGF and eFGF are both expressed maternally although at low levels. Int-2 and eFGF (zygotic) come on in the early gastrula, bFGF (zygotic) in the early neurula, and activin B in the late neurula (Fig.6). Dissections of blastulae show that neither bFGF nor eFGF are localized at this stage. In the gastrula, both eFGF and int-2 are expressed first in the blastopore lip region (Fig.7). They remain on in the blastopore lip but not in the invaginated mesoderm as gastrulation proceeds, suggesting that they must be turned off in the mesoderm that has migrated in away from the lip. Int-2 also comes on as a patch in the prospective mid/hindbrain region of the forming neural plate, and we have shown that this is an early response to neural induction (Tannahill et al., 1992). In the neurula and tailbud stages both factors show a sharp restriction to the extreme posterior of the mesoderm, later becoming the tailbud.

The zygotic expression of bFGF and activin B both commence after the end of gastrulation. bFGF is expressed preferentially in the posterior but much less sharply than eFGF and int-2, so there is a gradient from the tail to the head end (Fig.8A). Activin B on the other hand is initially more abundant at the head end and later is expressed also in the posterior (Fig.8B).

Evidence for a role in AP or DV patterning

Several studies in the last few years have implicated the FGFs and activins in anteroposterior specification. However there have been some problems of interpretation because the experiments do not involve direct respecification of gastrula tissues. Ruiz i Altaba and Melton (1989a) have examined the behaviour of animal caps, induced with FGF or activin, and then implanted into early gastrulae by the "Ein-

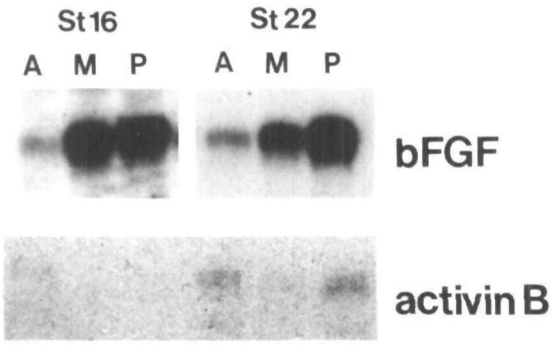

Fig. 8. Anterior-posterior distribution of messenger RNA for bFGF and activin B. RNAase protections are shown of anterior, middle and posterior thirds from the stages indicated. 20 μg of RNA was used for each lane.

steckung" procedure. Activin-treated caps tend to induce heads while FGF-treated caps tend to induce tails. We have always been puzzled by the fact that isolated FGF-treated caps develop into ventral structures, wheras posterior ones were obtained in these Einsteck experiments. It now seems that the difference lies in the exposure to additional signals after implantation. We have found that when ventral marginal zone (VMZ) explants are implanted into embryos they cause the formation of extra tails, although in isolation, like FGF induced caps, they form ventral tissues. Ruiz i Altaba and Melton have also studied a gene called *Xhox-3*, which is an *eve* type homeobox gene expressed in the posterior mesoderm (Ruiz i Altaba and Melton, 1989b; Ruiz i Altaba et al., 1991). It is turned on to a higher degree in animal caps by FGF than by activin, overexpression causes reduction of the head, and injection of an antibody causes, in a proportion of cases, defects in the posterior.

Cho and De Robertis (1990) have investigated the activation of HOX cluster genes in animal caps treated with activin or FGF. *XlHbox1*, normally expressed in the anterior trunk region, is preferentially activated by activin, and XlHbox6, normally expressed in the mid- and hind-trunk region, is preferentially activated by FGF. In a further study, Cho et al. (1991a) found that overexpression of *XlHbox6* alone was sufficient to cause supernumerary tail formation after subsequent Einsteckung procedure. It could even override the head forming effect of activin induction.

In both these sets of experiments the assay is rather indirect since the formation of axial structures in Einsteckung experiments involves participation by both the graft and the host tissue, and requires some further inductive signals from the host. However for both *XlHbox6* and *Xhox-3* there is a *prima facie* case that the genes are involved in anteroposterior specification. In neither case does overexpression in isolated caps result in mesoderm formation so they seem to be controlling positional coding rather than tissue type. Both are activated by FGF but we do not know how direct this relationship is, and it is possible that there are other intervening genes to be discovered.

An experiment that directly addresses the role of the FGF family was performed by Amaya et al. (1991). They made a version of the FGF receptor lacking the cytoplasmic domain. This forms unproductive dimers with the endogenous receptor and prevents response to exogenous FGF. Overexpression of this construct in intact embryos should theoretically lead to failure of those processes that depend on any member of the FGF family. Although a detailed study of the morphology of the affected embryos has not yet been published, it seems that they have less mesoderm than expected, the dorsoventral arrangement of tissues is deranged, and they may lack posterior parts of the axis.

If we put together the expression patterns reported above with the functional experiments reviewed in the present section, it does look rather probable that, in addition to their involvement in mesoderm induction, an important role of the FGF family is concerned with anteroposterior specification. One possibility is that the FGFs are posterior morphogens and that a gradient is set up by secretion from the blastopore lip region. *XlHbox6* and *Xhox3* and probably other genes as well would then be turned on above a certain concentration threshold and they would be responsible for activating the appropriate terminal differentiation genes for the trunk and tail. Another possibility is that the FGF type factors are permissive for the maintenance of an uncommitted state, and that once cells leave the blastopore lip region, or later the tailbud, they become specified by reference to other signals. In either case it is likely that we shall have to contend with a certain redundancy of function since at least eFGF and int-2, which have overlapping biological activities, are expressed in a very similar tight zone in the posterior.

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