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Slow emergence of a multithreshold response to activin requires cell-contact-dependent sharpening but not prepattern

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SUMMARY

The growth factor activin elicits mesodermal fates when applied to prospective ectodermal cells of the *Xenopus* blastula stage embryo. Previous experiments with dissociated cells showed that there are at least five different responses separated by closely spaced, sharp dose thresholds. Here we investigate this multithreshold activin response further using probes for genes expressed at early gastrula stages, namely *Xbra, goosecoid, noggin, Xwnt-8* and *Mix.1*. We show that initial dose-response profiles are broad and smooth in contrast to the later threshold-bound patterns. For *Xbra, goosecoid* and *noggin*, the later expression ranges are subsets of earlier ones. Unexpectedly, *Xwnt-8* is initially induced at high doses only, but later appears only in cells that have received a low dose of activin. Keeping the cells dissociated after activin treatment, rather than allowing them to reaggregate, prevents sustained expression of *Xbra* and *Xwnt-8* but allows that of *goosecoid* and *noggin*. However, cell contact is required for sharpening the dose-response threshold of *goosecoid*. Finally, we show that a previously reported dorsoventral prepattern in the animal cap is also cell-contact dependent and it is not required for the multithreshold response to activin.

Key words: mesoderm induction, *goosecoid*, *noggin*, *Xwnt-8*, *Mix.1*, *Xenopus*

INTRODUCTION

In one of the earliest patterning events in amphibians, known as mesoderm induction, cells of the vegetal half induce overlying animal cells to make mesoderm (reviewed in Smith, 1993; Kimelman et al., 1992; Sive, 1993). The peptide growth factor activin has been implicated in this process by its ability to induce mesoderm in animal pole explants (Smith et al., 1990; van den Eijnden-van Raaij et al., 1990; Ariizumi et al., 1991), by its presence in the embryo (Asashima et al., 1991; Thomsen et al., 1990) and by the effects in vivo of interference with a putative activin receptor (Hemmati-Brivanlou and Melton, 1992). The specific type of mesoderm induced in explants is critically dependent on the dose of factor received by animal cap cells (Green et al., 1990). Provided the exposure of cells is well controlled by dispersing the cells during treatment, cells from animal caps can differentiate to distinct fates by discriminating between activin doses differing by as little as 30% (Green and Smith, 1990). Furthermore, activin can elicit at least five different responses dependent on dose (Green et al., 1992). Such multiple threshold-delimited responses are what one would expect of a morphogen patterning system in which a smoothly graded distribution of a morphogen would lead to a discontinuous pattern (Wolpert, 1969; Green and Smith, 1991).

Nothing is known about the mechanism by which multithreshold responses arise in *Xenopus* cells. In the nearest parallel, the developing fruit fly *Drosophila*, gradients of morphogens do lead to discontinuous patterns of tissues, but the gradients are intracellular (St Johnston and Nusslein-Volhard, 1992; Ip et al., 1992). Aspects of mechanisms seen in *Drosophila* would apply in *Xenopus* if extracellular concentrations of growth factors are transduced quantitatively to intracellular concentrations of active DNA-binding proteins. However, at another extreme, the multiple thresholds may rely on a cascade of binary decisions resulting from multiple rounds of cell-cell interactions (see Smith et al., 1989; Gurdon, 1988; Green and Cooke, 1991).

Multiple activin responses correspond to the sequence of tissues on the dorsoventral axis (Green et al., 1992). However, at stages when cells are competent to respond to activin, embryological experiments have defined only two domains within prospective mesoderm, 'dorsal' (a 60° sector of the marginal zone) and 'non-dorsal' (the remaining 300° sector) (Dale and Slack, 1987; Stewart and Gerhart, 1990). Increasing numbers of genes have been cloned that are expressed in restricted domains at blastula stages and these tell a similar story. Gene expression patterns have so far fallen into three classes, dorsal (such as *goosecoid* and *noggin* (Blumberg et al., 1991; Smith and Harland, 1992)), non-dorsal (*Xwnt-8*, (Christian et al., 1991)) and dorsoventrally uniform (*Mix.1*, *Xbra* (Rosa, 1989; Smith et al., 1991). These data reinforce the

Three Signal Model (Smith and Slack, 1983) in which two initial specifications within the blastula marginal zone are elaborated into the multiple states of the dorsoventral pattern during gastrula stages (a process called 'dorsalization'). However, animal cap cells are not responsive to activin at these later stages (Green et al., 1990) and activin is unable to mimic dorsalization signals (Lettice and Slack, 1993; Smith et al., 1993). It is thus hard to see the relationship between the sequence of events in vivo and the multithreshold activin responses of animal cap cells, despite the latter's experimental robustness and conceptual appeal as an explanation for pattern formation. There is a way of reconciling the two sets of data: if initial responses to activin are heterogeneous, consisting of mixtures of cells with either a dorsal or a ventral response in a dose-dependent ratio, then they could evolve into the multiple responses by cell-cell signalling akin to dorsalization (Smith et al., 1989). A simple version of this model was ruled out by cell mixing experiments (Green and Smith, 1990) but other versions are possible. Such versions all require a starting population of cells with a heterogeneous response to activin, and indeed a dorsoventral prepattern in the animal cap's activin responsiveness that could be responsible for such heterogeneity has been observed (Sokol and Melton, 1991; Ruiz i Altaba and Jessell, 1991; Bolce et al., 1992; Christian and Moon, 1993; Kinoshita et al., 1993). In addition, all models invoking such binary cascades require both time for secondary signals to be sent and received and that cell-cell contact be maintained.

In this paper, we set out to examine how the multithreshold activin response arises in terms of time, cell-cell contact and cell heterogeneity (prepattern). We exploit the availability of probes to several early-response genes to show that the thresholds do not appear immediately, but emerge from smooth dose-response profiles. We show that cell-cell contact is required for maintenance of some responses and sharpening of at least one threshold. Finally, we exclude possible mechanisms relying on a known source of heterogeneity, namely the UV-sensitive dorsoventral animal cap prepattern, and show that the prepattern depends on some dispersible ventralizing factor.

MATERIALS AND METHODS

Embryos and dissections

Embryological methods were as in Green et al. (1992). Briefly, animal caps were excised in calcium- magnesium-free medium (CMFM) (Sargent et al., 1986) at blastula stage 8. Incubation of caps in 2-3 changes of fresh CMFM over about 30 minutes combined with gentle pipetting allowed disaggregation of inner layer cells and removal of largely intact outer layers. To prevent cells sticking, pipettes had all been treated with a wash of 0.4% poly-HEMA (poly-hydroxy-ethyl-methacrylate, Aldrich) in ethanol/acetone (1:1) solution and allowed to dry. Cells from 250-300 caps were pooled and one-eighth aliquots taken for incubation in various dilutions of activin in CMFM containing 0.1% bovine serum albumin fraction V (Sigma). After 1 hour cells were centrifuged in poly-HEMA-treated microfuge tubes for 3 minutes at 165 *g*. Cell pellets were gently resuspended in 1.5 ml CMFM, centrifuged again, resuspended in 75% NAM and finally centrifuged again to optimize reaggregation. This procedure was always completed well before stage 10 (onset of gastrulation in control embryos).

For analysis at gastrula stages, reaggregates were incubated until control embryos had reached stage 10.5 (about 2 hours after reaggregation). They were then bisected with a sharpened tungsten needle. One half aggregate was put into a microfuge tube containing 0.5 ml 75% NAM for incubation to neurula stage while the other was frozen in a minimal volume of medium for later RNA extraction.

Where cells were to be kept in an unreaggregated (dispersed) state, the scheme was as follows: cells were centrifuged, resuspended in 1.5 ml CMFM, centrifuged again and resuspended in 1 ml CMFM. Half of the cells (i.e. 0.5 ml of suspension) were kept dispersed by transfer into an agarose-lined or unlined 35 mm plastic Petri dish containing 2 ml CMFM and swirled occasionally to maintain dispersal. As a positive control for the multithreshold response, the remaining 0.5 ml cell suspension was reaggregated by adding 1 ml 75% NAM followed by centrifugation.

Activin

Recombinant human activin A was used as in Green et al. (1992) as a conditioned medium from CHO cells transfected with the human beta-A gene (gift of Genetics Institute, Cambridge, MA). It was purified virtually to homogeneity as described in Green et al. (1992) on a phenyl-Sepharose CL-4B column (Pharmacia) followed by two reverse-phase HPLC steps. Activity of the factors was assayed by the animal cap assay (Cooke et al., 1987), with one unit per ml being defined as the minimum concentration necessary for induction to occur.

UV ventralization

Eggs were dejellied 5-10 minutes after fertilization in 2% cysteine (adjusted to pH 8 with sodium hydroxide) and placed in 75% NAM in a quartz-bottomed dish resting on a Mineralite hand-held ultraviolet lamp resting face up. The embryos were spread out so that each could roll around to have its vegetal pole pointing directly downwards. Exposure to short-wave UV light was either for 4 minutes (lamp filter in place, dish about 2 cm above filter) or 75 seconds (lamp filter removed, dish 3-4 mm from bare tube), with the conditions for either conformation previously calibrated on two batches of embryos. Care was taken to 'prewarm' the lamp (i.e. it was switched on for a minute or two) before each use, and not to move the embryos or dish until well into the second cell cycle. These measures ensured optimal reproducibility between and homogeneity within batches. Control UV-treated intact embryos were scored at tadpole stage according to the DAI scale of Kao and Elinson (1988). Batches having DAI averages for at least 20 embryos over 1.0 were discarded.

RNA extraction and RNAse protections

Embryos, aggregates and cells were frozen in a −80°C freezer in a minimum volume of medium. RNA was extracted as in Smith et al. (1991), homogenizing in an SDS-containing buffer, or sometimes, as described by Sargent et al. (1986), vortexing in a buffer containing guanidinium isothiocyanate. For the latter, the final lithium chloride precipitation was omitted.

RNAse protection procedure was as in Green et al. (1990). Note that only RNAse T1 was used in the digestion steps. Probes were as follows: *XlHbox6* (*Xenopus* HOX2.5), *Xbra*, actin and *goosecoid* probes as in Green et al. (1992). *EF1α* (Krieg et al., 1989) was as in Sargent and Bennett (1990), a 94 nt *Hin*f fragment (nucleotides 100-194) cloned into pSP72. *Xwnt-8* (wingless/int1-related gene) was the 1.42 kb *Eco*RI fragment (clone #7.2) of Christian et al. (1991) cloned into pGEM1, linearized with *Ava*I, probe length 271 nt, protected length 257. *Noggin* (dorsal-specific, dorsalizing gene) (Smith and Harland, 1992), was a cDNA with an 857 bp deletion from the 3′ end cloned by Bill Smith into pGEM5Zf(-) (clone A3, plasmid p5.5), probe length 270 nt, protected length 232 nt. *Mix.1* (activin induced gene) (Rosa, 1989) was a 184 bp EcoR1-Pst1 5′-end fragment cloned by J. B. A. G. into BluescriptSK+, probe length 277, protected length 184.

Quantitation of RNAse protections was performed using a Molecular Dynamics PhosphorImager. Digital images of phosphor screens previously exposed to the RNAse protection gels were

analyzed using the manufacturer's software. Counts were integrated in a standard area covering the central 50% of the bands and local background in similar neighbouring areas was subtracted. Counts in each sample were normalized relative to *EF1α* counts in that sample.

Histological procedures

Histological procedures for detection of notochord by morphology and antibody staining were as in Green et al. (1992) following the method for acrylamide-embedded frozen sections of Hausen and Dreyer (1981). Staining was with anti-notochord-sheath monoclonal antibody MZ15 (Smith and Watt, 1985) followed by fluorescein-labelled secondary antibody. Counterstaining was by a 10 minute incubation with Eriochrome black (Aldrich) (40 µl 1% Eriochrome black solution in ethanol added freshly to 10 ml PBS).

RESULTS

Multithreshold responses appear well after early gene induction

An increasing number of genes have been cloned that are expressed in prospective mesoderm at blastula and gastrula stages, the stages during which the critical events of induction and patterning take place. We examined the activin dose-response of five 'early' genes. *goosecoid* (*gsc*) is a homeobox-containing gene that is expressed in the gastrula dorsal lip (Blumberg et al., 1991; Cho et al., 1991). *Noggin* is also expressed dorsally and has been implicated in both dorsalization and neural induction (Smith et al., 1993; Lamb et al., 1993). *Xwnt-8*, a member of the wingless-int1-related family, is initially expressed in a large region of the gastrula marginal zone not expressing *goosecoid* (i.e. the non-dorsal marginal zone) and is subsequently restricted to a narrower ventral domain as gastrulation proceeds (Christian et al., 1991; Christian and Moon, 1993; Smith and Harland, 1991). *Xbra*, the *Xenopus* homologue of the mouse *Brachyury* or *T* (Tail-less) gene, is expressed throughout the marginal zone even before gastrulation begins and persists in prospective and differentiating notochord during gastrula and neurula stages (Smith et al., 1991). Finally, *Mix.1* is expressed throughout the vegetal hemisphere of blastulae and disappears from mesoderm during gastrulation (Rosa, 1989). Induction of *Mix.1*, *Xbra* and *gsc* have been shown to be independent of protein synthesis (Rosa, 1989; Smith et al., 1991; Cho et al., 1991). In addition we probed for expression of the 'late' genes *XlHbox6* (a posteriorly restricted HOX gene (Wright et al., 1990)) and muscle-specific 'cardiac' actin (Gurdon et al., 1985) as positive controls for the multithreshold response seen in previous experiments (Green et al., 1992).

Mid-blastula animal caps were excised, inner layer cells dissociated and incubated in activin for an hour. The cells were then washed and reaggregated (Green et al., 1992). Aggregates were bisected when control embryos had reached early gastrula stage 10.5, typically 2.5 hours after reaggregation. RNA from one half-aggregate was analyzed immediately and the other half-aggregate was incubated overnight to late neurula/early tailbud stage (stage 17) for comparison to previous experiments. RNA was analyzed using multiple-probe RNAse protection, including a constitutively expressed gene *EF1α* (Krieg et al., 1989) as a quantitative internal control.

Fig. 1 shows a typical RNAse protection and its quantitation. An overall observation is that 'early' (gastrula) patterns, from RNA analyzed two to three hours after induction, are very different from 'late' (neurula) patterns in RNA extracted after an overnight incubation. First we will describe the neurula (stage 17) patterns to put them in the context of our previous work on that stage.

The neurula RNA shows that *XlHbox6*, muscle actin and *goosecoid* are induced in low, medium and high activin dose ranges respectively, while *Xbra* RNA appears in low as well as medium-high doses as previously reported (Green et al., 1992). Note that the *XlHbox6* expression is here of mesodermal character and not neural since neural character is not induced by activin under these conditions (Green et al., 1990). The weakness of *goosecoid* signal observed at the highest activin dose in Fig. 1 was not always seen, but had occasionally been observed in previous experiments. This could reflect a real declining trend in the *goosecoid* profile, with egg-batch differences in sensitivity giving varied dose cut-offs. *Xwnt-8* is induced at the same doses as *XlHbox6*, consistent with its non-dorsal (posteroventral) character. The *noggin* gene has a somewhat complex profile: it is induced at the same doses as *goosecoid* as well as slightly lower doses. There is a second *noggin* peak at low doses (slightly exaggerated in this gel by smearing of the *Xwnt-8* band). This double peak is much broader and less sharply bounded than that of *Xbra*. *Noggin* expression at high activin doses is consistent with the neuralizing ability of cells induced with these doses of activin (Green et al., 1990), given the dorsal character of *noggin*-expressing cells in vivo (Smith and Harland, 1992) and the neuralizing effect of *noggin* protein itself (Lamb et al., 1993). *Mix.1* is expressed in neurectoderm but not in mesoderm at this stage (Rosa, 1989), and its absence in this experiment confirms the absence of neural induction (Green et al., 1990).

The early gastrula responses appear monotonic and thresholds are not observed for most genes (Fig. 1). High doses of activin induce all the early genes examined, including *Xwnt-8*. The latter is surprising since in vivo expression of *goosecoid* has been shown to exclude expression of *Xwnt-8* (Christian and Moon, 1993). Comparison of the gastrula (stage 10.5) and neurula (stage 17) profiles shows that cells that have previously expressed *Xwnt-8* cease to do so while cells that initially have little or no *Xwnt-8* RNA subsequently acquire very large amounts. Also surprising is the appearance of *noggin* gene expression in the zero-activin sample. This could be maternal *noggin*, but cell dissociation itself might have some de-repressing effect on this gene (see Discussion). The *Mix.1*. gene is more strongly expressed than any the above genes. Quantitation of the RNAse protections normalized using the *EF1α* signal (see Methods for details), showed that the amount of *Mix.1* message is 5- to 10-fold greater than for the others. *Mix.1* expression also increases dramatically and apparently relentlessly with dose. Of all the dose-response profiles examined, that of the *Brachyury* gene was the most variable between experiments. However, in general it did not show the gradual rise with dose seen for the other genes. Instead, a sharper rise (more like a threshold) and a plateau at higher doses were observed. Note that this 'threshold' at stage 10.5 does not correspond to the later, low threshold observed at stage 17 for *Xbra*. *Goosecoid* induction initially rises steadily with activin dose, with a fall in the top dose. The fall is variable from experiment to experiment, as discussed above.

The overall picture that emerges is that, while neurula stage

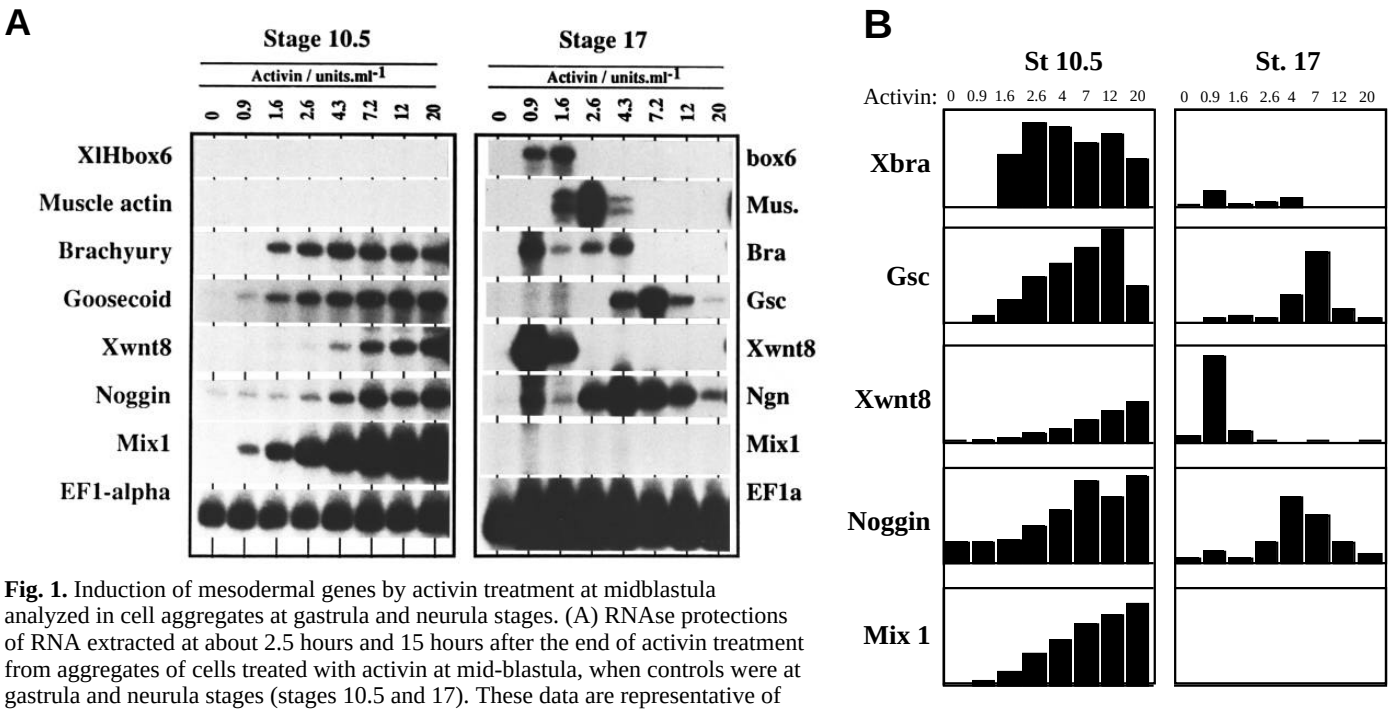

Fig. 1. Induction of mesodermal genes by activin treatment at midblastula analyzed in cell aggregates at gastrula and neurula stages. (A) RNAse protections of RNA extracted at about 2.5 hours and 15 hours after the end of activin treatment from aggregates of cells treated with activin at mid-blastula, when controls were at gastrula and neurula stages (stages 10.5 and 17). These data are representative of four such experiments. The figure is compiled from three separate assays on RNA from one experiment, one for actin, one for *XlHbox6* and *goosecoid* and one for *Xbra*, *Xwnt8*, *noggin* and *Mix.1* RNA. See methods for details of probes. One unit/ml of activin is defined as the minimum concentration required to induce mesoderm in an animal cap explant (approximately 0.1 ng/ml or 5 pM protein). (B) Quantitation of RNAse protections. Bar height is number of counts for a gene divided by the number of counts for *EF1-α* in the same sample. *EF1-α* increases on average about 4-fold between time points analyzed. For each gene maximal expression is plotted as full height (in fact, maximal expression of Mix1 is significantly higher than that of the other genes - see text).

profiles show a well-defined dorsoventral sequence of markers consistent with that seen in vivo, early (i.e. gastrula) dose-response profiles are very different, with few if any thresholds observed initially. This still leaves wide open the question of mechanism, particularly whether the emergence of the threshold response is cell-autonomous or requires a cascade of secondary cell-cell interactions.

Aggregation is required for threshold sharpening

To investigate whether the multithreshold activin response requires cell-cell interactions, we performed the simple variation on the above experiment of not reaggregating cells after activin treatment but maintaining them in a dispersed state until neurula stage. Previous similar experiments (Symes et al., 1988) had shown that such cells differentiate to give epidermis-specific antigens and that they are diverted from such differentiation by early treatment with activin. However, the fate of these activin-treated cells was not known. Here we tested for a range of mesodermal markers with results shown in Fig. 2. Tantalizingly, none of the genes *XlHbox6*, *Xwnt-8* and *Xbra* continues to be expressed in the cells that are kept dispersed until neurula stage. Absence of *Xbra* RNA is surprising because it has been shown that induction of *Xbra* is initially cell-contact independent (Smith et al., 1991). *Mix.1* is also not expressed at this stage (not shown), which is unsurprising, given that *Mix.1* is not expressed in embryos or induced caps at this stage either.

By contrast, both of the dorsal-specific genes *goosecoid* and *noggin* are induced and continue to be expressed in cells kept dispersed. It is therefore clear that both *goosecoid* and *noggin* expression in response to activin is cell autonomous. Both the *goosecoid* and *noggin* profiles in the dispersed cells are different from those seen in Fig. 1 for the gastrula stage: over time there is some restriction of the response with respect to dose despite the dispersal of cells. *Noggin* expression is affected by aggregation, but the effect is subtle: a slight downward shift in a rather broad induction optimum and disappearance of the weak secondary optimum at low activin dose. In the case of *goosecoid*, the expression profile in unreaggregated cells is less sharply threshold-bound than in aggregates. In other words, aggregation is required for sharpening of the threshold implying that there are threshold-sharpening mechanisms that require cell-cell signalling.

Dissociated cells from ventralized embryos respond like cells from normal embryos

Since much of the response of animal caps to activin is cell-contact dependent, we decided to see if this were also true of the previously demonstrated dorsoventral prepattern of the animal cap (Sokol and Melton, 1991; Ruiz i Altaba and Jessell, 1991; Bolce et al., 1992; Christian and Moon, 1993; Kinoshita et al., 1993). Activin induces dorsal markers (such as notochord) in dorsal halves of caps but cannot in ventral cap halves or caps from UV-ventralized embryos. We reasoned that, if the prepattern were cell-contact dependent, we should be able to induce dorsal markers in dissociated cells from UV-

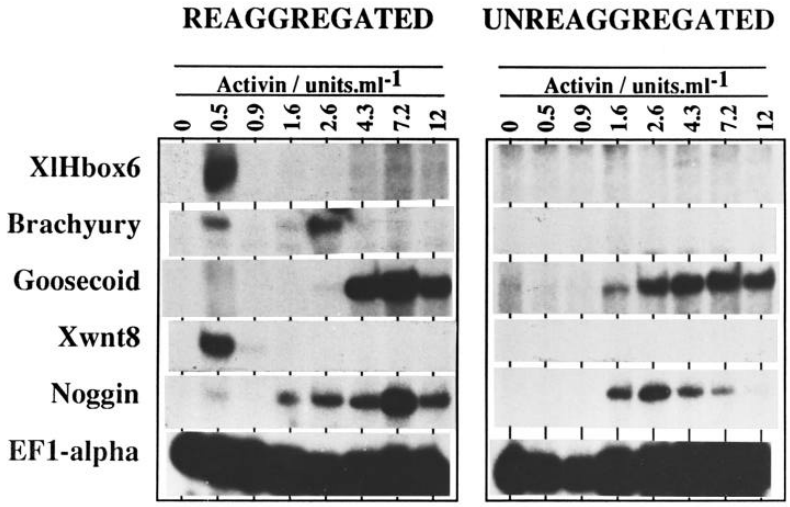

Fig. 2. Effect on induction dose-response profiles of reaggregation versus maintained dispersal of cells treated at midblastula with activin analyzed at neurula stage. Cells were dissociated and treated with activin for 1 hour, and washed by gentle centrifugation and resuspension. Half of each cell suspension was reaggregated by centrifugation in calcium-containing medium while the other half was kept dispersed by regular gentle pipetting in CMFM. RNAse protection assays were performed as in Fig. 1 on RNA extracted at control neurula stage.

ventralized embryos. Alternatively, UV-ventralization might abolish dorsal parts of the activin response in a cell dissociation experiment, just as it does in intact animal caps. Furthermore, heterogeneous induction of dorsally and ventrally prepatterned cells (and subsequent interactions between them) might be needed for the full spectrum of responses, just as the mesodermal pattern in vivo depends on interactions between dorsal and non-dorsal sectors of the marginal zone.

We therefore took cells from animal caps of normal or UV-ventralized embryos, treated them with doses of activin and reaggregated them to allow differentiation as before. We used only UV-treated embryos from batches with extreme ventral phenotypes (DAI below 1.0) in which notochord induction by activin in animal caps is essentially eliminated (data not shown; see also Sokol and Melton, 1991 and Bolce et al., 1992). Fig. 3 shows that the ventralized cells from UV-treated embryos can be induced to make notochord and express the dorsal marker *goosecoid*. UV-treatment leaves the multithreshold activin response largely unaffected. Slight shifts in the threshold of *goosecoid* induction and in intensities of *XlHbox6* and *Xbra* induction do occur, but these are within experimental variability. So, while dorsoventral prepattern in the animal cap can influence the response to activin, the results here show that its role in the multithreshold response is minimal. In addition, they also reveal something about the nature of the prepattern: since activin cannot induce *goosecoid* and notochord in ventral and ventralized caps, but can in cells that have been dissociated, it must be the process of dissociation that derepresses or deinhibits the dorsal response in ventral cells. In other words, the prepattern must be due to a diffusible/dispersible ventralizing factor on the ventral side rather than predorsalization on the dorsal side.

DISCUSSION

Early and late gene expression profiles induced by activin are different

The above experiments show that, in contrast to the neurula pattern, the immediate response of *Xenopus* blastula cells to activin treatment is not apparently a multithreshold one. The broad sloping profiles of most of the genes examined suggest a mechanism in which the early dose-dependent differences are subtle and quantitative and are refined through multiple inter-

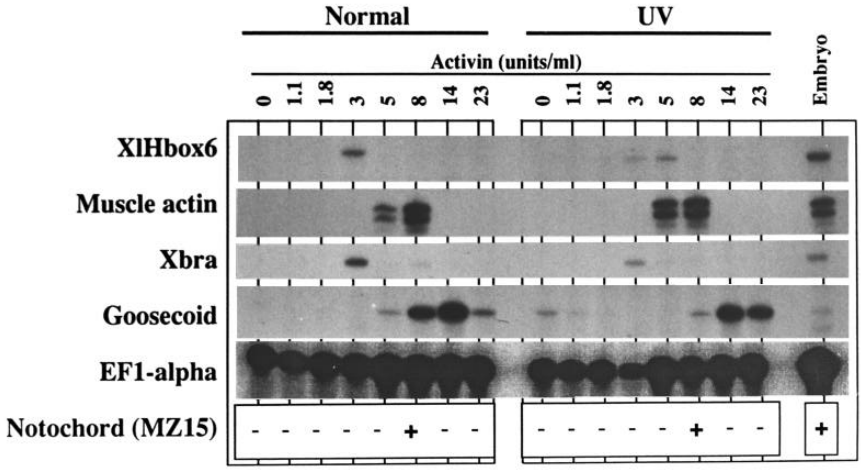

Fig. 3. Comparison of activin dose-response profiles in cells from normal and UV-ventralized embryos. RNAse protections of RNA extracted from halved aggregates of activin-treated cells when controls were at neurula stage as in Fig. 1. Notochord differentiation was assayed in the other half of these aggregates at control stage 25 by staining sections with MZ15 antibody and inspecting sections for vacuolar notochord morphology. A single '+' sign indicates approximately the same amount and degree of notochord differentiation for UV and control aggregates. UV-ventralized cells came from the same batch of embryos as the controls and were treated with UV light in the first cell cycle. Degree of ventralization was assayed according to Kao and Elinson (1988) to give a DAI average of 0.9 (DAI zero is completely ventralized, DAI 5 is normal). Antibody staining was assayed in two similar experiments, RNA was analyzed in another three similar experiments with essentially the same outcomes.

actions between genes and/or cells. However, the relatively sharp dose-response profile for *Brachyury* (*Xbra*) shows that something approaching a threshold can be generated quickly and as part of a response that is known to be cell autonomous (Smith et al., 1991). It may also be that at early stages there are thresholds for genes for which we do not have probes. It seems likely that the *Xbra* gene promoter might be similar to that of the hunchback gene in *Drosophila* in which a threshold response is achieved through the clustering and cooperativity of transcription factor binding sites (Driever and Nusslein-Volhard, 1989; Ip et al., 1992). For this to be true, the extracellular concentration of activin must be transduced quantitatively into intracellular concentration of active transcription factor. Such proportional transduction is hinted at by the remarkably linear response profile of the *Mix.1* gene, even though the *Mix.1* gene product cannot itself be responsible for the early *Xbra* transcription (since the latter is protein-synthesis-independent (Smith et al., 1991)). Some caution should also be expressed about what the *Mix.1* and similar profiles represent: they could result from a gradually increasing response of each cell, or, alternatively, maximal expression of an increasing proportion of cells. Distinguishing between these possibilities will require expression analysis of individual cells. Doing this with sufficient quantitative accuracy, especially overcoming problems of normalization and non-linearity of detection of standard in situ hybridization techniques, is just coming within the reach of feasibility (see (Bögler et al., 1993)).

Evidence for cell-cell signalling role in the multithreshold response

The work of Gurdon and Sargent (Gurdon et al., 1984; Sargent et al., 1986) showed that expression of muscle actin is abolished when cells are dissociated in calcium-free medium. Furthermore, such cells can develop in a number of ways while still dissociated (Sargent et al., 1986; Grainger and Gurdon, 1989). Symes et al. (1988) showed that dispersed cells could still be diverted from an epidermal fate by treatment with activin. In this paper, we show that such dispersed and activin-treated cells can be positively induced to express dorsal mesoderm-specific genes *goosecoid* and *noggin*. Thus, there are at least some sustained positive mesodermal responses to activin that are truly cell-contact (and incidentally also substrate-) independent.

Of the genes whose expression we analyzed, both of the two cell-autonomously inducible ones were dorsal-specific. By comparison, interference with the FGF receptor has little or no effect on dorsal-specific gene expression even though it prevents activin induction of several mesodermal genes (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). However, *Xwnt-8* expression is not sensitive to FGF receptor interference while it is sensitive to cell dispersal as shown above. Thus, although FGF might be one of the intercellular signals that is lost through cell dispersion, it cannot be the only one.

Interestingly, while *goosecoid* and *noggin* expression persist despite cell dispersal, neither of their dose-response profiles becomes sharply threshold delimited. For *goosecoid*, this is in contrast to the sharp threshold seen in reaggregated cells. One plausible explanation for such cell-contact-dependent sharpening is the Community Effect in which cells tend towards the fates of their neighbours and away from other fates (Gurdon, 1988; Gurdon et al., 1993). Thus, in this case, cells at the edge of the *goosecoid*-inducing dose range would be mostly non-*goosecoid* cells and would suppress *goosecoid* expression in the errant minority, thus sharpening the dose threshold. It is also clear, however, that in dissociated cells both *noggin* and *goosecoid* expression patterns are more restricted with respect to dose at neurula stage than at gastrula stage. Thus, there must also be cell-autonomous thresholding mechanisms. In addition to threshold-sharpening types of signal, these experiments identify the need for maintenance signals: cell-contact-dependent signals that are needed to maintain previously induced responses such as *Xbra*.

The role of *noggin*

Induction properties of *noggin* are of particular interest given this molecule's remarkable abilities in both dorsalization (Smith et al., 1993) and neural induction (Lamb et al., 1993), and deserve particular discussion. Firstly, activin can induce *noggin* gene expression allowing for the possibility that activin acts upstream of *noggin* in the course of development. Secondly, the low level of *noggin* expression seen simply as a result of dissociating cells may explain a 'background' level of neural gene expression seen in other cell dissociation experiments (Green et al., 1990; R. Cornell, personal communication) and autoneuralization effects of prolonged dissociation (Grunz and Tacke, 1989). It implies that *noggin* expression is normally suppressed in intact tissues and that in some sense this might make neuralization a kind of default pathway. Curiously, low levels of activin are a candidate for suppression of 'default' neural differentiation (Hemmati-Brivanlou and Melton, 1992), though we have not observed any suppression of spontaneous (i.e. early) *noggin* expression by activin.

UV-sensitive dorsoventral prepattern due to a dispersible ventralizing factor

The body of literature on dorsoventral or UV-sensitive prepatterns in the animal cap's responsiveness to activin is now quite substantial (Sokol and Melton, 1991; Ruiz i Altaba and Jessell, 1991; Bolce et al., 1992; Christian and Moon, 1993; Kinoshita et al., 1993), especially considering that such prepattern does not seem to be necessary for normal development (Nieuwkoop, 1969). The experiments in this paper show that the UV-sensitive prepattern is not necessary for multiple thresholds as such, though it may have a slight modulating influence. Since dissociated cells can respond to activin-like dorsal cap halves (by expressing dorsal-specific genes and tissues), the prepattern in intact caps is most likely due to a dissociable ventralizing factor on the ventral side of the cap. An obvious candidate molecule for such a factor is bone morphogenetic factor 4 (BMP4, also known as DVR4 for Decapentaplegic-, Vg1-related 4) which has been shown to be able to ventralize the effects of activin induction (Dale et al., 1992; Jones et al., 1992). This would fit with the notion that non-dorsal regions of the blastula and gastrula stage embryo are not merely a passive field into which the dorsalizing signal weakly penetrates but an active source or reservoir of anti-dorsal signals (see Sive, 1993). Anti-dorsal signals may not be critical in normal development in the animal cap, but they may well have a regulative role in normal mesodermal patterning in the marginal zone (Cooke, 1983; Green and Cooke, 1991).

Gradients in vivo?

While the responses to activin seen in our experiments eventually come to bear an obvious resemblance to dorsoventral mesodermal pattern in vivo, they do not do so initially. Early in vitro profiles are gently graded while in vivo expression of many genes appears to be spatially quite well restricted as soon as it is detectable. Of course, 'quite well restricted' may be the point: there could be important gradations of expression in the small region within and at the borders of the Spemann organizer. These have not been reported, but they could be quite subtle. In contrast, as we have argued before (Green et al., 1992), the dorsoventral uniformity of *Mix.1* expression (Rosa, 1989) seems to indicate a dorsoventral uniformity of activin-like induction. The tight relationship between activin dose and *Mix.1* response shown in this paper reinforces that interpretation.

Therein lies a paradox: if the in vivo distribution of activin(-like) induction is dorsoventrally uniform, why are cells able to respond so discriminatingly and variously to the specific dose of activin? Cells do not respond to another mesoderm inducer, FGF, in this way (Green et al., 1992), so one cannot say that thresholds and restriction of expression patterns are somehow innate to induced mesoderm. One might resolve this paradox if the threshold response were really due to 'activin-plus-X' where 'X' is another factor. In our experiments, activin is varied and X would be constant (or induced by activin in a proportional way); in vivo, activin would be dorsoventrally uniform while X would be dorsoventrally graded. Thus, activin in vivo would be an essential co-inducer of X, or X would be a modifier of activin induction. Such a combinatorial model is by no means a new idea (Kimelman et al., 1992; Moon and Christian, 1992; Sive, 1993). *Noggin* and *Wnt* gene products are obvious candidates for 'factor X'. Noggin in particular has suggestive properties: it is a secreted protein and it can effect dorsalization (Smith et al., 1993) and, as we have shown, activin induces *noggin* RNA in an approximately proportional way. Testing this idea of quantitative cooperation between activin and *noggin* is a subject of current work

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