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Concentration-dependent patterning of the *Xenopus* ectoderm by BMP4 and its signal transducer Smad1

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SUMMARY

Morphogens are thought to establish pattern in early embryos by specifying several cell fates along a gradient of concentration; a well-studied example is the *Drosophila* protein decapentaplegic (DPP) acting in the wing disc. Recent work has established that bone morphogenetic protein 4 (BMP4), the vertebrate homologue of DPP, controls the fundamental choice between neural and epidermal fates in the vertebrate ectoderm, under the control of antagonists secreted by the organizer region of the mesoderm. We now show that BMP4 can act as a morphogen, evoking distinct responses in *Xenopus* ectodermal cells at high and low concentrations, in a pattern consistent with the positions of the corresponding cell types in the embryo. Moreover, this complex cellular response to extracellular BMP4 concentration does not require subsequent cell-cell communication and is thus direct, as required of a classical morphogen. We also show that the same series of cell types – epidermis, cement gland and neural tissue – can be produced by progressively inhibiting endogenous BMP signaling with specific antagonists, including the organizer factor noggin. Finally, expression of increasing doses of the signal transduction molecule Smad1 accurately reproduces the response to BMP4 protein. Since Smads have been shown to act in the nucleus, this finding implies a direct translation of extracellular morphogen concentration into transcription factor activity. We propose that a graded distribution of BMP activity controls the specification of several cell types in the gastrula ectoderm and that this extracellular gradient acts by establishing an intracellular and then nuclear gradient of Smad activity.

Key words: BMP4, morphogen, *Xenopus*, ectodermal patterning, Smad

INTRODUCTION

In the early embryo, the specification of cell fates relies in large part on secreted signaling molecules. In many cases, these signals appear to mediate traditional inductive interactions, in which cells or groups of cells assume one fate in the presence of a signal from neighboring cells and another in its absence. It has been repeatedly suggested, however, that an extracellular signaling molecule, or morphogen, distributed in a gradient, could establish a more complex pattern in one step by specifying different fates at different concentrations (Turing, 1952; Lawrence, 1966; Wolpert, 1969). It has proven difficult in practice to prove the existence of morphogens in embryos, however, and claims of success have been vigorously contested. Much of the controversy has centered on whether signaling molecules can act at a distance (Gurdon et al., 1994, 1995; Nellen et al., 1996; Reilly and Melton, 1996), while a perhaps more fundamental attribute of a morphogen, the capacity to directly specify multiple cell fates, has been relatively neglected. Moreover, the signal transduction mechanisms that might permit such complex interpretation of ligand concentration remain largely mysterious. In at least two cases, however, mesoderm induction by activin in amphibians and wing imaginal disc patterning by DPP in flies, a particularly strong case has been made that a member of the TGFβ family of growth factors can elicit distinct cell responses at different concentrations (Green et al., 1992; Green and Smith, 1990; Gurdon et al., 1994, 1995; Nellen et al., 1996).

We have recently shown that BMP4, a vertebrate homologue of DPP, can direct dissociated *Xenopus* ectoderm to form epidermis instead of neural tissue (Wilson and Hemmati-Brivanlou, 1995). Several types of data now support the idea that a BMP mediates the choice between neural and epidermal fates in the amphibian ectoderm (Hawley et al., 1995; Sasai et al., 1995; Suzuki et al., 1995; Xu et al., 1995; reviewed in Weinstein and Hemmati-Brivanlou, 1997). Moreover, recent work suggests that secreted neuralizing signals from Spemann's organizer – noggin, chordin and possibly follistatin – act by antagonizing BMP action in the ectoderm and thus allowing neural tissue to form (Piccolo et al., 1996; Yamashita et al., 1995; Zimmerman et al., 1996). We now present evidence that the response of gastrula ectoderm to BMP4 is more complex than a simple binary choice of fates and that BMP may act in a concentration-dependent manner to specify ectodermal fates lying at the edge of the neural fate as well as epidermis. Moreover, we show that this pattern of cell responses can be replicated both by dispersion for different periods of time and by expression of different concentrations of neuralizing agents believed to interfere with endogenous BMP signaling, noggin and a dominant negative version of a BMP receptor. We propose that the action of BMP antagonists secreted by the organizer creates a graded distribution of BMP

signaling in the gastrula ectoderm. Where signaling is high, epidermis is induced, while neural tissue forms where BMP is strongly inhibited. Where inhibition is partial, and cells experience intermediate levels of BMP action, cement gland and possibly neural crest and placodal cell types are specified. Finally, we show that overexpression of the recently discovered BMP signal transducer Smad1 (Graff et al., 1996; Thomsen, 1996) can reproduce in detail the response of dissociated ectoderm to BMP4 protein, inducing cement gland and epidermal markers in a concentration-dependent fashion. We infer from this finding that interpretation of the morphogen signal into discrete responses may happen in the nucleus.

MATERIALS AND METHODS

Embryological methods

Eggs were obtained and fertilized in vitro by standard methods and staged according to Nieuwkoop and Faber (1967). Noggin, tBR and Smad1 RNAs were injected into the animal hemispheres of both blastomeres at the 2-cell stage in 0.5× MMR with 3.5% Ficoll. Intact animal caps were cut at late blastula stage (stage 8-9) and cultured in 0.5× MMR; caps for dissociation were cut at late stage 9 in calcium- and magnesium-free medium (CMFM: Sargent et al., 1986), and dispersed in calcium- and magnesium-free modified Barth's solution with 1 mg/ml BSA, as described previously (Wilson and Hemmati-Brivanlou, 1995; Wilson and Melton, 1994). Cells were redispersed occasionally by swirling and reaggregated by transfer to BSA-coated Eppendorf tubes containing 0.75× modified Barth's solution and brief centrifugation at 1000 revs/minute. Unreaggregated cells were collected for RNA extraction in the same way.

Ventralized embryos were produced by exposing dejellied eggs to UV radiation in an upside-down Stratalinker (Stratagene) 25-30 minutes after fertilization, at a setting of 1500 (150 mJoules) (Bolce et al., 1992). At late blastula stages, dead and abnormally developing embryos were removed from UV-irradiated and control populations, and experimental embryos and intact siblings (to evaluate the degree of ventralization) selected at random from the remaining animals. The dorsal-anterior index (DAI) was evaluated at stage 39 according to Kao and Elinson (1988).

RNA extraction and RT-PCR

RNA was extracted from cells, caps and embryos by proteinase K digestion in lysis buffer followed by DNase treatment, as described previously (Wilson and Melton, 1994). Each DNase reaction contained 2 units of RNase-free DNase from Boehringer Mannheim. Reverse transcription and PCR were also carried out as in Wilson and Melton (1994), with the following modifications. Reverse transcription was primed with random hexamers instead of oligo(dT), 100 units MMLV RTase from Gibco/BRL were used and reactions were incubated at 42°C. PCR cycles were shortened to 30 seconds at 93°C, 60 seconds at 55-60°C and 30 seconds at 72°C. Reactions contained 5 μl of a 60% sucrose solution with 1 mM cresol red and were loaded onto gels directly.

PCR primer sequences

EF1α (Krieg et al., 1989)
U: CAG ATT GGT GCT GGA TAT GC
D: AC TGC CTT GAT GAC TCC TAG
Epidermal keratin (Jonas et al., 1985)
U: CAC CAG AAC ACA GAG TAC
D: CAA CCT TCC CAT CAA CCA
NCAM (Kintner and Melton, 1987)
U: CAC AGT TCC ACC AAA TGC
D: GGA ATC AAG CGG TAC AGA
XAG (Blitz and Cho, 1995)
U: GAG TTG CTT CTC TGG CA
D: CTG ACT GTC CGA TCA GAC
Xbra (Smith et al., 1991)
U: GGA TCG TTA TCA CCT CTG
D: GTG TAG TCT GTA GCA GCA
XCG (H. Sive, pers. comm.)
U: TAC TAC TGG GGC AGC TTC
D: TTC CGT GTG AGG ATT CAG

Plasmids and in vitro transcription

RNA for injection was produced in vitro from linearized plasmid using the Message Machine kit from Ambion. Noggin in pSP64T was obtained from Richard Harland (Smith and Harland, 1992); tBR in pSP64T was constructed as previously described (Suzuki et al., 1995); human Smad1 (Liu et al., 1996) was subcloned into pSP64TEN, a modified version of pSP64T (S. Kong and A. Hemmati-Brivanlou, unpublished).

RESULTS

We have shown previously that BMP4 can induce epidermis in dissociated *Xenopus* ectoderm, suppressing the neuralization caused by prolonged dispersal (Wilson and Hemmati-Brivanlou, 1995). We now show that the action of BMP4 in this assay is concentration-dependent and that low concentrations promote specification of a third ectodermal cell type, cement gland. The cement gland is a larval adhesive organ, which forms just anterior to the neural plate in *Xenopus* (Sive and Bradley, 1996). Early gastrula animal cap explants were dissociated for 4 hours in the presence of recombinant BMP4 protein at a range of concentrations. Fig. 1 shows that in the absence of BMP and at the lowest doses, dissociated ectoderm is neuralized as expected, expressing the general neural marker NCAM (Kintner and Melton, 1987) after reaggregation and culture to late neurula (stage 18). As previously reported, BMP4 suppresses NCAM and induces epidermal keratin, with highest expression at the highest levels of added growth factor. At 100 ng/ml the ectoderm appears to be completely epidermalized. At low BMP concentrations, however, the cement gland markers *XCG* and *XAG* (Sive and Bradley, 1996) are strongly induced, as is a third gene, *XA*, expressed in the cement and hatching glands (Sive and Bradley, 1996) (data not shown). These markers reach a peak at about 1 ng/ml BMP4 protein and decline rapidly at higher inducer levels. At the concentrations of BMP4 used here, mesoderm is not induced, as evidenced by the absence of *Xbra* (*brachyury*) expression (Smith et al., 1991). There is overlap among the ectodermal markers: cement gland and neural markers are expressed together at low inducer levels in some experiments, while cement gland and epidermal fates coexist at intermediate concentrations. These data suggest that BMP4 can regulate the choice among three cell types, neural, cement gland and epidermis, in a concentration-dependent manner. Cement gland is formed at concentrations of inducer intermediate between those required to induce large amounts of epidermis and those low enough to permit neural specification, reflecting the position of this ectodermal organ in the embryo when determination occurs.

It is theoretically possible that the various responses of dissociated ectoderm to BMP4 protein could involve distinct sets of responding cells. That is, different doses of BMP could

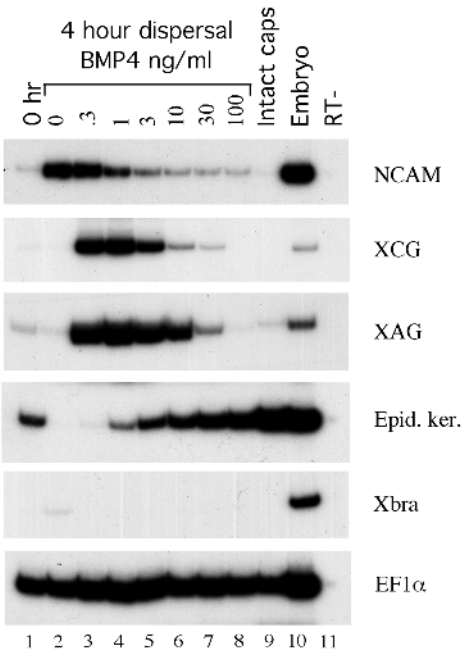

Fig. 1. Concentration-dependent response of dissociated cells to BMP4. Late blastula animal caps were dissociated in Ca^{2+}/Mg^{2+}-free solution, cultured for 4 hours in the presence of various concentrations of BMP4 protein and then reaggregated. RNA was extracted for RT-PCR at the late neurula stage (17-18). Neural (NCAM), cement gland (*XCG*, *XAG*) and epidermal (keratin) markers are expressed in distinct but overlapping ranges of BMP concentration. Only NCAM is strongly expressed in the absence of added factor (lane 2), while cells reaggregated immediately after dispersion (lane 1) as well as intact animal caps (lane 9) form epidermis. The mesodermal marker *Xbra* is not induced by BMP4 at the concentrations used here. The ubiquitous message EF1α is used as a control for RNA recovery and reverse transcription, while cDNA and mock-reversed transcribed RNA from sibling unmanipulated embryos serve as positive and negative controls (lanes 10, 11).

selectively activate differentiation of ectodermal subpopulations according to a prepattern in the animal cap. The most likely prepattern would be along the dorsal-ventral axis, both because this corresponds to the eventual subdivision of the ectoderm and because it has been shown that dorsal and ventral regions of the animal cap respond differently to both neural induction by the organizer and mesoderm induction by activin (Sharpe et al., 1987; London et al., 1988; Sokol and Melton, 1991; Bolce et al., 1992). To address whether such a prepattern could account for the multiple responses to exogenous BMP, we repeated the dose-response experiment using cells from embryos ventralized by UV-irradiation in the first cell cycle (Grant and Wacaster, 1972). As Fig. 2 demonstrates, the response of ectoderm from these embryos is very similar to that of ectoderm from sibling controls, displaying the same transition from neural to cement gland to epidermal marker gene expression with increasing dose. While the ventralized ectoderm seems to form cement gland more readily than the cells from control caps, the epidermal keratin responses are almost identical. The UV-irradiated embryos used in this experiment were highly ventralized, as demonstrated both by the morphology of sibling embryos grown to stage 39, which showed an average DAI of 0.3, and by the almost complete absence of dorsal markers at late neurula stages (see Fig. 2, lane 6) and at gastrula stages (data not shown). Thus ventralized cells show the same range of responses to BMP dose as wild-type ectoderm, demonstrating that the complexity of the response does not depend on dorsoventral prepattern.

In these experiments, BMP4-exposed cells were reaggregated and cultured until late neurula stages, allowing secondary inductive interactions among the reaggregated cells to modify the response to the primary inducer and in principle permitting the determination of cell types not directly induced by BMP4. This has been shown to occur in the wake of activin induction of mesoderm in dispersed cells (Green et al., 1994; Wilson and Melton, 1994). To clarify which aspects of the BMP4 dose response that we observe can be attributed directly to inducer concentration, we repeated these experiments without reaggregating the exposed cells. RNA was extracted from the cell populations at the end of gastrulation (stage 12.5) in order to examine responses as early as possible. In most respects, these results closely resemble the pattern of marker expression obtained with reaggregation (Fig. 3). Although NCAM expression in the absence of BMP is weak, perhaps revealing a role for cell-cell interaction after initial neuralization, *XAG* is strongly induced at low and epidermal keratin at high inducer levels. Thus the specification of cement gland and epidermis are *direct* cell-autonomous responses to different concentrations of BMP4.

In intact animal cap explants as well as in the ventral ectoderm of the gastrula embryo, *endogenous* BMP protein, secreted by the ectodermal cells themselves, is apparently present at sufficient concentration to induce and maintain epidermal fate. Inhibition of BMP signaling, by dissociation or molecular antagonists, results in formation of neural tissue instead (reviewed in Weinstein and Hemmati-Brivanlou, 1997). Our finding that low levels of BMP4 can specify cement gland suggests that partial

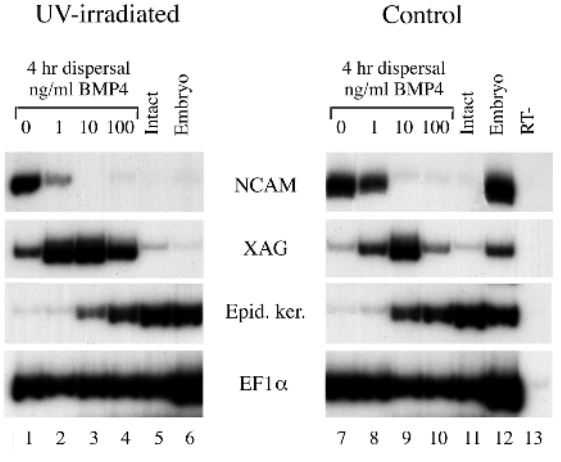

Fig. 2. BMP4 response of ectoderm from ventralized embryos. Animal cap cells from embryos ventralized by UV radiation in the first cell cycle were dissociated and exposed to various doses of BMP4 protein, in parallel with cells from sibling control embryos. Controls and markers are as in Fig. 1. The cells from ventralized embryos show the same general dose-dependent response to BMP4 as the control cells. The neural marker NCAM is almost entirely absent from the UV-irradiated embryos (compare lanes 6 and 12), demonstrating that they were strongly ventralized.

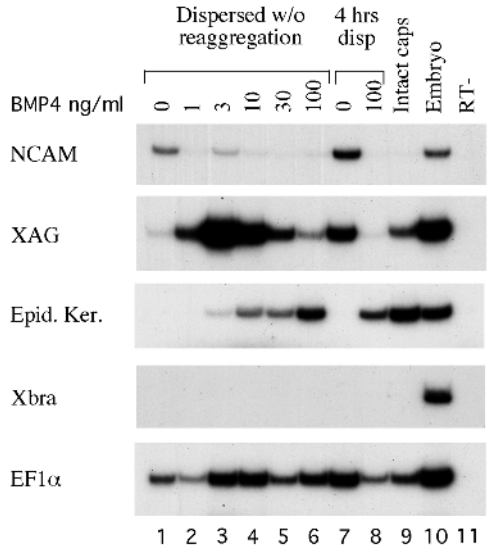

Fig. 3. Dose-response without reaggregation. Dissociated cells were exposed to BMP4 at a range of concentrations and maintained in dispersed culture until the end of gastrulation (stage 12.5), when RNA was extracted (lanes 1-6). As controls, two additional samples from the same pool, cultured in 0 and 100 ng/ml BMP4, were reaggregated after 4 hours as in Fig. 1 (lanes 7,8). The main features of the BMP4 response – induction of epidermis at high concentrations, cement gland at low concentrations – do not require reaggregation of exposed cells.

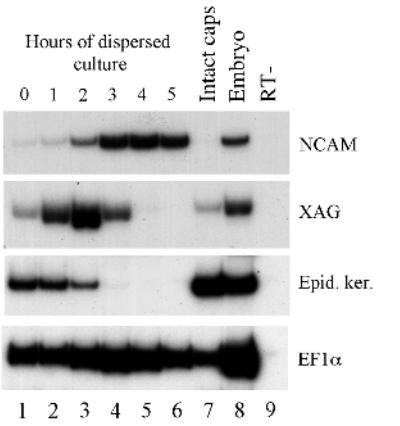

Fig. 4. Ectodermal marker expression after long and short dispersion. Dissociated late blastula ectoderm was maintained in dispersed culture for various periods before reaggregation. All reaggregates were cultured to the end of neurulation. Short dissociations (1-2 hours) promote primarily expression of the cement gland marker *XAG*, while longer dissociation neuralizes the ectoderm, as manifested by the general neural marker NCAM. Epidermal keratin is expressed in intact caps, in immediately reaggregated populations and after short dissociation.

inhibition of endogenous signaling should also promote formation of this cell type. To test this possibility, we first explored the effect of duration of dissociation. If long dissociation interrupts BMP signaling completely enough to neuralize the ectoderm, shorter dissociation might inhibit partially, especially if both duration and intensity of signaling are important. Populations of animal cap cells were dissociated at the start of gastrulation and reaggregated after various periods of dispersed culture. RNA was extracted at late neurula stages for analysis of molecular markers by RT-PCR and the results are shown in Fig. 4. As expected, cells reaggregated immediately after dissociation (0 hours) form primarily epidermis, as do intact control caps. After 3 hours of dissociation, epidermal keratin expression is definitively suppressed, while the general neural marker NCAM is strongly induced. Ectoderm subjected to 1 or 2 hours of dispersion, however, is induced to form cement gland, strongly expressing the cement-gland-specific marker *XAG* after reaggregation and culture. *XCG* and *XA* are also highly expressed by these cells (data not shown). Thus short dissociation promotes adoption of an ectodermal fate lying between the neural plate and the epidermis in normal development.

To ask in another way whether partial inhibition of endogenous BMP signaling can lead to formation of cement gland, we used the dominant-negative type I BMP receptor tBR (Graff et al., 1994; Suzuki et al., 1994). This construct has already been shown to induce both neural tissue and cement gland in animal caps (Suzuki et al., 1995; Xu et al., 1995; Sasai et al., 1995), but the relationship of the two ectodermal fates to inhibitor dose has not been carefully examined. 2-cell-stage embryos were injected in each blastomere near the animal pole with various quantities of tBR RNA; animal caps were cut at late blastula stages and cultured to stage 18. As shown in Fig. 5, *XAG* is induced at even the lowest dose of tBR used, 16 pg, as well as at higher doses. Expression begins to decline at 500 pg. Although traces of NCAM can be seen at lower doses, significant expression begins at about 250 pg and continues to increase with tBR dose. Epidermal keratin is found in all caps, although expression declines markedly at high doses of truncated receptor. The overlap of cell fates seen in this assay presumably reflects the inevitably uneven distribution of injected RNA as well as variation in individual cell response. Thus graded inhibition of endogenous BMP signaling in intact ectoderm can recapitulate in reverse order the series of cell types induced by BMP4 protein in the dissociated cell assay.

In the embryo, BMP signaling is apparently regulated by soluble antagonists secreted by the organizer region of the mesoderm, including noggin, follistatin and chordin. These factors are thought to induce neural fate by inhibiting BMP signaling at the protein level: direct binding to BMP4 has been demonstrated for noggin and chordin (Piccolo et al., 1996; Yamashita et al., 1995; Zimmerman et al., 1996). Thus these organizer signals could also specify cement gland or other neural plate boundary cell types at the limits of their domain of action by partially blocking BMP signaling. To test this hypothesis, we expressed noggin at a range of concentrations in animal cap explants, again by injection of RNA at the 2-cell stage. Fig. 6 shows that *XAG* is again strongly induced at intermediate, subneuralizing concentrations of noggin. In addition, the cement gland marker declines sharply at the highest dose of noggin, while NCAM continues to rise. Thus inhibition of endogenous BMP4 signaling by a secreted organizer factor can account for cement gland specification as well as neural induction in a concentration-dependent manner.

We have shown that quantitative differences in the intensity of BMP4 signaling result in qualitatively different transcriptional responses in ectodermal cells, as expected of a morphogen. To ask at what stage in the transduction of the extracellular signal

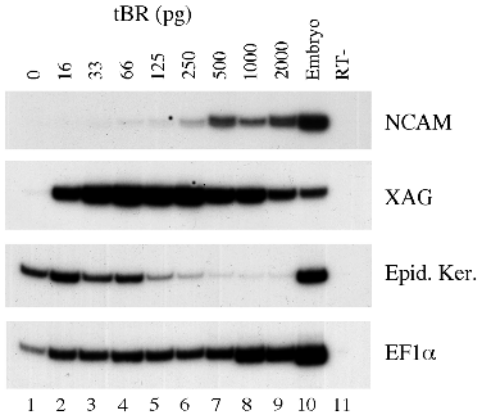

Fig. 5. Dose-response to dominant-negative BMP receptor. Increasing amounts of RNA encoding the truncated BMP type I receptor tBR were injected at the 2-cell stage; animal caps were cut at late blastula and cultured as intact explants until the end of neurulation. The cement gland marker *XAG* is induced at the lowest doses of tBR and declines at the highest doses. NCAM appears only at high doses. Epidermal keratin, the only marker expressed in uninjected caps, declines at the high doses.

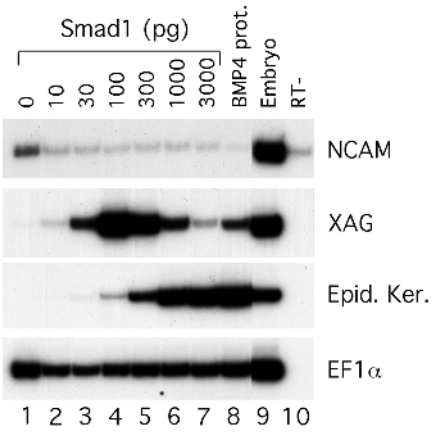

Fig. 7. Dose response to Smad1. Animal cap ectoderm from embryos previously injected with Smad 1 RNA was dissociated for 4 hours, reaggregated and cultured until late neurula stages. The dose response to Smad1 mimics the response to BMP4 protein: low doses induce the cement gland marker *XAG* and suppress NCAM expression in dissociated cells, while high doses activate epidermal keratin and down-regulate *XAG*.

this transformation occurs, we examined marker gene expression after dissociation in cells expressing various doses of Smad1, a signal transducer shown to be able to evoke BMP responses in *Xenopus* and in cell culture (Graff et al., 1996; Hoodless et al., 1996; Liu et al., 1996; Thomsen, 1996). Fig. 7 illustrates that increasing Smad1 dose faithfully reproduces the effect of increasing BMP protein concentration: low doses induce the cement gland marker *XAG* and suppress neural markers, while high doses induce epidermal keratin and suppress *XAG*. Moreover, the ratio of BMP and Smad1 doses that produce a given response remains nearly constant, implying a linear relationship between ligand concentration and transducer activity over a large range.

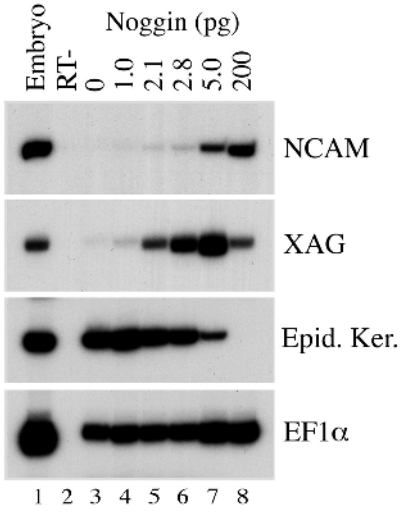

Fig. 6. Dose response to noggin. Ectodermal markers were examined in intact animal caps from embryos injected with various quantities of noggin RNA at the 2-cell stage. RNA was extracted at stage 18. As with tBR, *XAG* is expressed at lower doses than are required to induce NCAM and declines at the highest dose used here.

DISCUSSION

Using dispersed *Xenopus* animal cap cells, we have demonstrated that BMP4 can evoke more than one response in competent gastrula ectoderm, inducing cement gland at lower concentrations, epidermis at high concentrations. When no BMP4 is added, the dispersed cells form neural tissue. Moreover, these distinct responses to different BMP4 concentrations are direct and cell-autonomous, since neither requires reaggregation of the treated cells. Thus BMP4 acting in the *Xenopus* ectoderm satisfies the most fundamental criterion of a classical morphogen, concentration-dependent induction of more than one cell type. Moreover, the same sequence of cell fates – epidermis, cement gland and neural – can be produced by progressively inhibiting *endogenous* BMP signaling in intact animal cap explants, using either a truncated, dominant-negative BMP receptor or the secreted organizer factor noggin, which has been shown to bind BMP4 (Zimmerman et al., 1996). In each case, high doses of the BMP antagonist induce NCAM, while lower doses turn on cement gland markers. We suggest that at these doses BMP signaling is only partially inhibited. Finally, we show that this pattern of marker gene expression can be reproduced in another way, by varying the duration of dissociation in the absence of added BMP4. Short dissociation leads to expression of cement gland markers, while long dissociation neuralizes. Prolonged dissociation presumably neuralizes by inactivating endogenous BMP signaling through dilution (Wilson and Hemmati-Brivanlou, 1995). By the same token, ectoderm dispersed for shorter periods should experience a less complete or, perhaps more plausibly, shorter interruption of BMP signaling, allowing these results to be reconciled with the dose response to tBR or noggin. The hint that the duration of morphogen signaling might be as important as its intensity accords well with the observation that, in the case of activin, another inducer from the same family, longer exposure can substitute for higher concentration (Green et al., 1990; P. A. W., unpublished data).

In fact, the concentration-dependent induction of multiple cell

type markers in dissociated animal cap cells by BMP4 is reminiscent in many ways of the response of the same cell population to activin (Green and Smith, 1990; Green et al., 1992). One notable difference is that the expression domains of ectodermal markers induced by BMP4 overlap, while the mesodermal markers expressed in response to activin are separated by clear thresholds. However, this feature of the activin response has been shown to result from secondary interactions taking place after the induced cells are reaggregated (Wilson and Melton, 1994; Green et al., 1994); when these interactions are prevented, the thresholds do not develop and the induced markers overlap. In fact, the initial dose responses of the mesodermal markers *Xbra* and *goosecoid* to activin (Wilson and Melton, 1994; Green et al., 1994) are strikingly similar to the patterns of *XAG* and epidermal keratin induction by BMP4 reported here. Thus the absence of sharp thresholds in the ectodermal BMP4 response may be typical of at least the initial response to morphogens. Since the various ectodermal cell types are eventually well segregated in vivo, mechanisms must exist to sharpen initially coarse boundaries, perhaps involving short-range cell movement (sorting out), as well as respecification by secondary communication. Our data suggest that this task is accomplished in somewhat different ways in the ectoderm and the mesoderm, since in one case the heterogeneity of the initial responses of dispersed cells is eventually eliminated after reaggregation, while in the other it persists.

The inhomogeneity of ectodermal cell responses to BMP4 and activin apparently does not derive from dorsal-ventral prepattern within the animal cap since, in both cases, cells from highly ventralized UV-treated embryos behave very similarly to cells from control embryos and, at least with BMP4, the overlap of marker gene expression domains is undiminished (Fig. 2; Green et al., 1994). Thus we prefer to attribute this overlap to stochastic rather than spatially organized variation among the responding cells. Moreover, as has been argued in the case of activin, the observation that BMP4 can evoke the full range of cell fates in ventralized cell populations implies that pre-existing dorsal-ventral differences cannot account for the complexity of the response. Since, in intact caps, these regional differences are well documented, they may depend on factors in the extracellular matrix, possibly including BMPs themselves, that are lost when cells are dispersed. We note that, while the responses of dispersed cells from UV-treated and control embryos to activin are essentially identical, the response to BMP4 seems to be shifted to some degree in ventralized embryos. This may reflect the fact that the BMP experiments are performed at a somewhat later stage, when ventralizing factors have had more time to exert their influence.

Our data imply that ectodermal cells of the early gastrula choose among at least three fates – epidermis, cement gland and neural tissue – according to the strength and perhaps duration of local BMP signaling. Experimentally, we have shown that the level of effective BMP action can be manipulated by addition of BMP4 protein to dissociated cells, by inhibiting to various degrees the initially high levels of endogenous signaling, or by stimulating the signal transduction pathway. In the embryo, although several mechanisms presumably regulate BMPs, antagonists secreted by the organizer, including noggin, chordin and follistatin, probably play the central role, since signals from this region of the embryo have long been known to establish the neural plate (Spemann and Mangold, 1924). We propose that the action of these factors, perhaps in conjunction

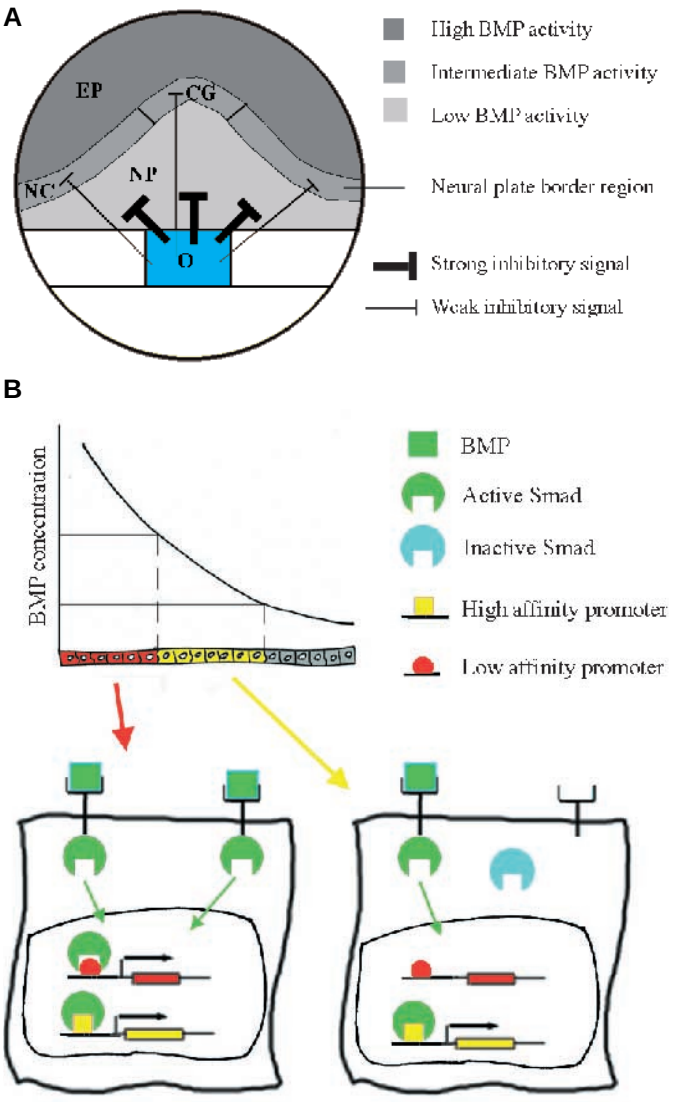

Fig. 8. Model of graded BMP action in the gastrula ectoderm. (A) A schematic fate map of the early gastrula shows the approximate positions of the future neural plate, border region and epidermis, viewed from the dorsal side. The cement gland forms in the border region mid-dorsally (Sive and Bradley, 1996), while the neural crest arises more laterally (Mayor et al., 1995). For simplicity, the ectodermal placodes, which form at various positions in the border region, have been left out. See Hausen and Riebesell (1991), p. 47, for details. We propose that diffusible antagonists produced in the organizer region of the mesoderm, including noggin, chordin and follistatin, result in a graded distribution of BMP signaling in the neighboring ectoderm. EP, epidermis; NP, neural plate; O, organizer; CG, cement gland; NC, neural crest. (B) Differences in local BMP concentration among regions of the ectoderm are reflected in differences in nuclear Smad activity. Low nuclear Smad concentration (right cell) leads to transcription of genes involved in cement gland specification (yellow), through formation of Smad1-containing complexes on high affinity promoter elements. Where BMP signaling is high (left cell), nuclear Smad1 concentration is also high and lower affinity promoters (red) are also activated, leading to epidermal specification. Smad proteins could bind directly to promoter elements; more probably, Smads interact with DNA-binding factors (Chen et al., 1996). Where BMP activity is too low to activate either cement gland- or epidermis-specific genes, neural tissue forms.

with transcriptional or translational regulation, results in a graded distribution of free BMP in the gastrula ectoderm, with lowest levels in the future neural plate on the dorsal side, an intermediate region at its periphery and highest concentrations in the prospective epidermis far from the influence of the organizer (Fig. 8A). In agreement with this hypothesis, the cement gland lies at the anterior edge of the neural plate, where neural inducing factors are too weak to allow neural tissue to form, but may be strong enough to partially inhibit local BMP signaling. Note that in this model the formation of the morphogen gradient does not depend on diffusion from a local source, but on graded inhibition of locally produced protein.

The neural plate border region gives rise not only to the cement gland, but to the neural crest and various ectodermal placodes (pituitary, olfactory, lens and others), raising the possibility that partial BMP inhibition might play a role in the specification of these cell types as well. In our experiments, however, the neural crest marker Xslug (Mayor et al., 1995) is not expressed at any concentration of BMP4, nor in response to tBR or noggin injection (data not shown). We conclude that additional patterning processes are required to induce the neural crest (and inhibit cement gland formation) and advance two hypotheses. First, the caudalizing signals that pattern the early neural plate (reviewed in Doniach, 1995) might operate within the border region as well, causing neural crest to differentiate instead of cement gland away from the anterior edge. Alternatively, secondary interactions with neighboring epidermis or neural plate may regulate which cell types form in which part of the border. Much evidence already supports the importance of these tissue interactions, both in enhancing or inhibiting cement gland determination (Bradley et al., 1996; Drysdale and Elinson, 1993) and in specification of the neural crest (Dickinson et al., 1995; Moury and Jacobson, 1990). (Paradoxically, this neural crest-inducing signal from the epidermis may also be mediated by a BMP (Liem et al., 1995).) In *Xenopus*, however, some neural crest markers are expressed very early, by gastrula stage 11 (Essex et al., 1993), arguing that at least an initial step in specification of this tissue occurs at the same time as neural induction and thus before any neural-epidermal interaction. Thus we propose that a direct response to intermediate levels of BMP signaling, resulting from partial inhibition by organizer signals, may be the first step in the determination of several cell types, including cement gland, neural crest and the ectodermal placodes. This early establishment of the border region would correspond to the initial inducing signal in a recent model of neural crest induction (Mancilla and Mayor, 1996).

The concentration-dependent induction of multiple cell fates poses a special challenge in signal transduction, since variation in the intensity of signaling must be converted into qualitatively distinct cellular responses. This transformation, in which a single pathway branches into several, could in principle occur at any point from receptor to promoter. We have shown that the induction of cement gland and epidermal markers by increasing BMP4 concentration is faithfully reproduced by increasing doses of the BMP signal transducer Smad1, which has been shown to move to the nucleus and activate transcription (Graff et al., 1996; Hoodless et al., 1996; Liu et al., 1996). Our laboratory has recently obtained similar results with a related molecule, Smad5 (Suzuki et al., 1997). Moreover, another member of this transducer family, Smad2, has been shown to mimic the concentration-dependent induction of mesodermal cell types by activin (Graff et al., 1996). These findings suggests that at least for this family of ligands, reading of the morphogen level into discrete responses probably occurs in the nucleus, perhaps on the responding promoters (Fig. 8B). If this proves the case, a surprisingly simple relationship will have emerged between extracellular morphogens acting through cell surface receptors and transcription factors such as dorsal and bicoid, gradients of which pattern the syncytial stage *Drosophila* embryo (Lawrence, 1992). We recognize, however, that these results must be interpreted with caution, since they rely on overexpression of a signal transduction molecule, which would normally require activation to exert its effects. Moreover, the demonstration that different doses of Smad1 can evoke distinct responses of course does not rule out other mechanisms, including for example separate high and low affinity BMP receptors.

Our finding that BMP4 can directly induce more than one ectodermal cell type in a concentration-dependent manner accords closely not only with the behavior of activin in the same system (Green et al., 1992; Green and Smith, 1990; see above), but with the action of the BMP homologue DPP in *Drosophila*. In this organism, recent work on patterning of the wing disc demonstrated by quite different means that DPP can diffuse from its source and activate distinct genes at low and high concentrations (Nellen et al., 1996). DPP is also important in early dorsal-ventral patterning in the fly, where it has also been proposed to act in a gradient to induce more than one cell type (Ferguson and Anderson, 1992; Wharton et al., 1993). However, the relationship between inducer concentration and cell response has not been examined directly in *Drosophila* and, in fact, the action of DPP in the wing disc has been the subject of conflicting interpretations (Lecuit et al., 1996). Thus several independent bodies of work now argue that BMPs or their relatives can induce more than one cell type in the same cell population and it is now possible to speculate that this may prove a general feature of cell response to this family of growth factors. The demonstration of multiple concentration-dependent responses represents a crucial step toward proving the existence of morphogens in embryos.

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