Single cell analysis of mesoderm formation in the *Xenopus* embryo

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Summary

We have examined the developmental specification of individual cells in the *Xenopus* blastula using a new *in vitro* culture system. Regional differences are apparent at the mid-blastula stage when animal hemisphere cells form only ectodermal cell types, while many clones from below the pigment boundary contain mesodermal cell types. A number of clones give rise to more than one differentiated cell type indicating that the initial steps of mesoderm induction are potentially reversible.

Animal hemisphere cells can be induced to form mesoderm by fibroblast growth factor (FGF). Different cell types predominate at different FGF concentrations and the neighbours in this sequence are also the pairs of cell types most usually associated in mixed clones derived from the marginal zone. We propose that the specification of individual cells depends upon both the concentration of inducing factor and on stochastic intracellular events.

Key words: *Xenopus* blastula, mesoderm induction, fibroblast growth factor, clonal analysis.

Introduction

It is often assumed that *individual* cells in developing embryos acquire commitments to form particular terminally differentiated cell types. However, most experiments on which these assumptions are based have been carried out on relatively large multicellular grafts or explants and, even where it has been possible to observe the behaviour of single cells in abnormal positions, the environment as a whole has been a multicellular one (Gardner and Rossant, 1979; Snape *et al.* 1987; Technau, 1987). In the present paper, we have used a new method for the *in vitro* culture of isolated *Xenopus* blastula cells to investigate the acquisition of commitment to form mesoderm. In *Xenopus*, mesoderm is believed to be formed as a result of inductive interactions between vegetal, presumptive endodermal cells and responsive marginal cells and there is mounting evidence that the molecules responsible for induction may be growth factors of the FGF and TGF-β families (reviewed in Gurdon, 1987; Smith, 1989; Dawid *et al.* 1990; Whitman and Melton, 1989). However, the immediate response to induction and the processes by which each cell becomes committed to a particular differentiation pathway are not well understood, although recent studies of the effects of different mesoderm-inducing factors (MIFs) indicate that there may be at least two levels of response possible (Rosa, 1989; Green *et al.* 1990) depending on the type and concentration of inducer.

Two sets of experiments concerning mesoderm formation are described here. Firstly, single cells taken from various regions of mid-blastula stage embryos were cultured in order to determine their states of specification and secondly, the potential of single animal pole cells to form mesoderm was investigated by treating cells with a range of concentrations of FGF. These experiments were designed to examine several questions regarding specification, which we define as the type of commitment measured by culture in isolation. Specification, unlike determination, need not be irreversible. (1) Is the specification of single cells acquired by induction and/or random internal processes? (2) Are all cells from one region similarly specified? (3) Is the specification of single cells the same as that of the tissue region from which they came?

The results showed that by the mid-blastula stage some marginal zone cells have become mesodermally specified, but that many cells fated to become mesoderm in normal development remain uninduced. In general cells from the same region do not all behave in the same way and do not necessarily behave in a similar way to tissue explants. This suggests that cell contacts or extracellular materials contribute to cellular specification *in vivo*. FGF exhibited concentration-dependent effects with respect to the pathway of differentiation followed, but the results indicated that random internal processes also occur.

Materials and methods

Cell culture

Animal pole cells

Cultures were carried out as described in Godsave and Slack (1989). Briefly, single cells from the inner surface of the animal cap were collected in calcium- and magnesium-free medium and one cell was transferred immediately into a well of a Terasaki plate. Culture wells were preincubated with $2\,\mu l$ of a solution containing $100\,\mu g\,ml^{-1}$ fibronectin (Gibco: bovine plasma >95% pure) and $100\,\mu g\,ml^{-1}$ laminin (Gibco: murine >95% pure) for 1–2h. Excess solution was then removed and the wells allowed to dry. The culture medium was $2\,mg\,ml^{-1}$ gamma globulins (BDH: Cohn fraction II) in half-strength Normal Amphibian Medium and aFGF was added to this at the stated concentrations. Cultures were fixed after 48–68h of incubation at 25°C.

Acidic FGF was prepared as described by Slack et al. (1988) by the methods of Esch et al. (1985) and Lobb et al. (1986). Acidic FGF was used for these experiments since at the time they were done, it was available in the largest quantity. More recent experiments with recombinant Xenopus bFGF have yielded very similar results.

Marginal zone cells

The location of the future dorsal side of the embryo was marked at the 4-cell stage when dorsal blastomeres can be seen to be more lightly pigmented in the animal hemisphere than ventral blastomeres. Marking was carried out by holding pieces of dried 5% agarose containing 5% Nile Blue dye against the cleavage furrow between the two dorsal blastomeres for approximately 30 secs. For each batch of embryos, the accuracy of the marking was checked at gastrula or neurula stages in control embryos and was very reliable.

Supraequatorial cells were taken from stage 8 embryos from a region that extended from the pigment boundary up into the animal hemisphere for a distance of 4 cells or about $200\,\mu m$. The piece comprised a quarter of the circumference of the marginal zone and was taken either from the dorsal or the ventral side. Only inner surface, relatively unpigmented cells were cultured. The subequatorial marginal zone cells were taken from stage 8 embryos from the areas shown in Fig. 1. The ring of marginal tissue was dissected into 4 pieces: dorsal, ventral and 2 lateral pieces. Inner cells were collected and cultured in the same way as for animal hemisphere cells.

Cell identification

Cultures were generally fixed with 3:1 methanol:acetic acid and stained with antibodies as described in Godsave and Slack (1989), except that non-specific antibody binding was usually blocked using 1% milk powder and 0.1% Tween 20 in PBS. The antibodies used were: CH14, a rabbit anti-epidermal serum raised against a peptide representing the C-terminal 14 amino

acids of cytokeratin XK70 (Winkles et al. 1985); RT97, a nerve-specific anti-neurofilament monoclonal antibody (Anderton et al. 1982); 12–101, an anti-muscle monoclonal antibody (Kintner and Brockes, 1985); MZ15, an anti-notochord monoclonal antibody recognising keratan sulphate (Smith and Watt, 1985); and anti-IFA, an anti-intermediate filament antibody (Pruss et al. 1981) which was used to stain lateral plate-type cells non-specifically. Antibodies were used in all cases where cell identity was not clear from the morphology.

Following antibody staining, plates were usually poststained in Ehrlichs haematoxylin for 20 min at room temperature, destained for 1 min in 0.5% HCl in 70% ethanol and stopped in 1% NH_3 in 70% ethanol. Stained wells were mounted in glycerol jelly (5% gelatin in glycerol).

In order to try and identify those cell types that were not positively characterised by antibody staining, small pieces of tissue from various regions of later stage embryos were cultured using the conditions described for single cell cultures. The tissue was dissected from embryos in agar-coated Petri dishes containing Normal Amphibian Medium, using electrolytically sharpened tungsten needles. They were cultured for approximately 16h at 25°C to allow cells to spread over the substrate. Cultures were fixed and stained to facilitate photography.
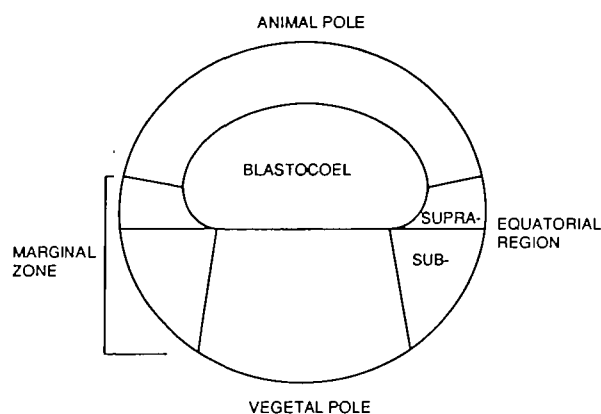

Fig. 1. Regions of the midblastula used for cell cultures. Fig. 1 represents a cross-section through a blastula-stage embryo. The dorsal and ventral sides were distinguished by using Nile blue to mark between the two more lightly pigmented dorsal blastomeres at the 4 cell stage. The equator is marked by the pigment boundary between the animal and vegetal hemispheres. Explants were cut from the regions shown. For the supraequatorial marginal zone pieces, explants extended about four cells or 200 µm into the animal hemisphere and were about a quarter of the circumference of the embryo wide. They were taken either from the dorsal or the ventral side of the embryo. For subequatorial regions, the ring of tissue surrounding the core of vegetal presumptive endoderm was cut into four pieces to give dorsal, ventral and two lateral pieces. Single cells were collected from the inner surface of each explant in calcium- and magnesium-free medium and cultured as described for animal pole (AP) cells previously (Godsave and Slack, 1989).

Results

Cell types formed in culture

Single cells from various regions of the midblastula stage embryo are able to divide in a chemically defined non-nutritive medium, the cells utilising their own yolk granules for development during the culture period of 2–3 days. Almost all of the isolated cells divided to form clones and about 40–70 % (depending on their origin) produced differentiating progeny. The cell types formed are listed here and in Table 1 and are shown in Fig. 2. *Epidermis* forms aggregates or sheets containing ciliated cells and many of the cells stain with the rabbit antibody, CH14, directed against the epidermis-specific cytokeratin XK70. *Neurons* develop long neurites often with growth cones and stain with the monoclonal antibody, RT97, directed against the 200K neurofilament protein. *Mesothelium* consists of sheets of cells, some of which may be ciliated. The cells are smaller than and clearly distinct from epidermal cells. They fail to stain with the antibodies mentioned here but possess endogenous alkaline phosphatase activity and are easily recognisable as a separate cell type. *Large flat cells* also have a distinct appearance. Both mesothelium and large flat cells may be forms of lateral plate mesoderm, since similar cells often appear in cultures of lateral plate from neurulae (data not shown). *Muscle* consists of large cells, which are often elongated and sometimes striated. These all stain with the anti-muscle monoclonal antibody, 12–101. *Notochord* cells are highly vacuolated and are stained by the monoclonal antibody, MZ15, directed against keratan sulphate. (For more details of the antibodies see Methods). In Table 1, we have also included *mesenchyme*, but this is a less clearcut category, because we have occasionally observed cells with a mesenchymal morphology stained with antibodies for other cell types, so mesenchyme is only scored if a significant number of scattered cells are present, rather than a few cells at the periphery of a clone of another type.

Specification states of cells from different regions of the mid-blastula

The results, presented in Table 1, show that there are

Table 1. *Specification of animal and marginal cells from stage 8 blastulae*

			Single cell type						Mixed colonies — Neural+:											
	No.	Diff.	Epi	Neur	Mes	Mst	LFC	Mus	Epi	Mes	Mst	Mus	Mes Mst	Mes LFC	Mes Mst Mus	Mes Mst	Mes Mst LFC	Mst Mus	LFC Mus	Mus Not
(A) Standard Medium																				
Animal Pole Cells																				
AP	62	52	1 (2)	48 (92)					3 (6)											
Supraequatorial Cells																				
DMZ	53	32	3 (9)	28 (88)	1 (3)															
VMZ	59	43	10 (23)	28 (65)		1 (2)			1 (2)	2 (5)	1 (2)									
Subequatorial Cells																				
DMZ	151	61	4 (7)	41 (67)	2 (3)	1 (2)		6 (10)	3 (5)		3 (5)		1 (2)							
LMZ	139	71	3 (4)	37 (52)	3 (4)	2 (3)	5 (7)	7 (10)	4 (6)	2 (3)	3 (4)	1 (1)	1 (1)				1 (1)		1 (1)	1 (1)
VMZ	151	63	1 (2)	18 (29)	4 (6)	1 (2)	6 (10)	13 (21)	4 (6)	1 (2)	4 (6)				2 (3)	1 (2)	1 (2)	3 (5)	2 (3)	2 (3)
(B) Modified Media Animal pole cells																				
Medium	No.	Diff.																		
BSA,L,F	53	21	1 (5)	19 (90)					1 (5)											
Glo,L	30	16	1 (6)	15 (94)																
Glo,F	30	17		17 (100)																

Details are shown of the cell type, or combination of cell types, formed by each differentiating clone from the regions indicated. Abbreviations: No, number of cells cultured; Diff, number of clones in which cells differentiated; Epi, epidermis; Neur, neural; Mes, mesenchyme; Mst, mesothelium; LFC, large flat cells; Mus, muscle; Not, notochord; AP, cells from around the animal pole; DMZ, LMZ and VMZ are dorsal, lateral and ventral marginal zone cells respectively. Numbers in parentheses are the percentages of differentiating clones forming each cell type or combination of cell types.

The modified media used in part B were identical to the standard medium (Part A) except for the protein additives. Standard medium contained gamma globulins, laminin and fibronectin. The modified media contained $2\,mg\,ml^{-1}$ of either gamma globulins (glo) or bovine serum albumin (BSA) in the culture solution and the substrate consisted of laminin (L) and/or fibronectin (F).
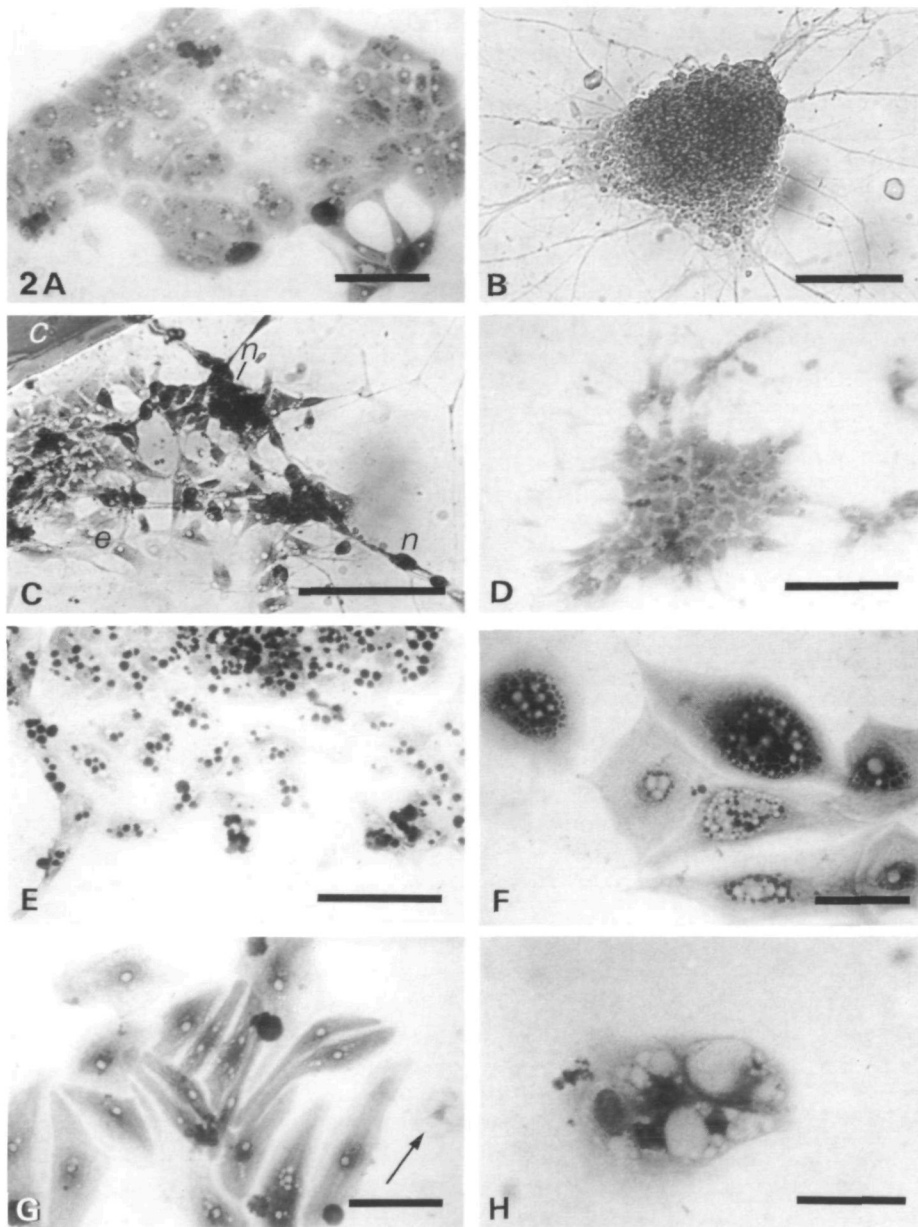
Fig. 2. Photomicrographs of cell cultures in 15 microlitre Terasaki plate wells. Cultures were fixed after 48–64 h at 25°C and stained as described in the Methods. (A) AP epidermis stained with anti-epidermal keratin antibody. (B) Subequatorial MZ cell clone which has differentiated into neurons, stained with RT97 anti-neurofilament monoclonal antibody. (C) Mesothelium and neural tissue co-differentiating from an FGF-treated, single AP cell. Neurons (n), but not mesothelium (e) are stained with anti-neurofilament antibody, RT97. (D) Mesothelium from an FGF-treated, single AP cell. The cytoplasm has been stained with anti-IFA monoclonal antibody which stains all types of intermediate filaments. (E) Large flat cells derived from a subequatorial MZ cell clone. The thin cytoplasm has been stained with anti-IFA antibody. Yolk platelets are darkly stained by haematoxylin. (F) Muscle from a subequatorial MZ cell clone stained with 12–101 anti-muscle antibody. (G) Muscle and notochord from an FGF-treated, animal pole cell clone. Muscle cells have been stained by 12- 101, while notochord (arrow) is unstained. (H) Notochord from an FGF-treated, animal pole cell clone stained with MZ15 anti-notochord antibody. For Fig. 2A an explant of AP tissue taken from a stage 8 embryo was dissected into small pieces when control embryos reached the midgastrula stage and cultured in wells prepared as for single cell cultures. Bars=100 μm.

considerable differences in the cell types formed by clones from each region of the embryo. Animal pole clones only gave rise to ectodermal cell types (usually neural or sometimes epidermal) as did most clones from the supraequatorial marginal zone. However, about 40 % of clones from the subequatorial marginal zone contained cells conventionally regarded as mesodermal (muscle, mesothelium, large flat cells and mesenchyme). Within this region, there appears to be a difference between dorsal and ventral marginal zone cells, with a higher proportion of clones from the ventral side containing the mesodermal types (Fig. 3).

An important feature of the results from subequatorial cells is the relatively high frequency of mixed clones (19 %), including many cases where ectodermal and mesodermal cell types were found in the same clone. Even if mesenchyme is not counted as a discrete cell type, 10 % of clones were mixed.

Since many cells fated to become epidermis gave rise to neurons in culture, several other media were also tested (Table 1B). In case the protein additives contained contaminating inducers, gamma globulins were replaced by RIA grade bovine serum albumin (Sigma) in some experiments and, in others, either fibronectin or laminin alone were used as substrates. However, in each case high levels of neuralization still occurred.

Induction of marginal cells

Since there appeared to be a dorsal-to-ventral difference between the specification states of subequatorial cells, a number of cells from the dorsal subequatorial marginal zone region were treated with FGF to see if they were, in fact, responsive to mesoderm-inducing factors. Only 21 % of untreated dorsal cells gave rise to mesoderm (Table 1), but all the cells treated with
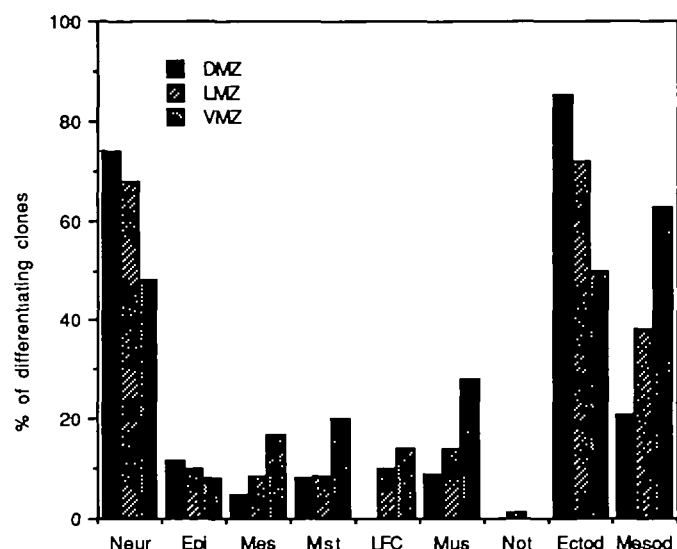
Fig. 3. Regional differences in specification of subequatorial marginal zone cells. Bars show the percentage of differentiating clones from each subequatorial marginal zone region which contained any cells of each type. Clones in which more than one cell type differentiated will be represented in several bars. Abbreviations are as for Table 1. The 'Ectod' section includes all clones in which any epidermis or neural tissue differentiated and 'Mesod' includes all clones in which any other cell type formed.

$10\ ng\ ml^{-1}$ FGF differentiated into mesoderm (Table 2, last row).

Treatment of animal pole cells with FGF

Our observation of mixed clones recalls the mixed clones encountered in comparable experiments on haemopoietic and neuroglial stem cells from late embryos or adults (Suda *et al.* 1983; Temple, 1989). These have been interpreted as showing that the commitments to follow different pathways of differentiation arise by some stochastic mechanism internal to the cell itself. In the case of the early amphibian embryo, there is abundant evidence of the importance of inducing factors in the selection of developmental pathways and, it has sometimes been assumed although without much evidence, that a population of competent cells would all respond in the same way when exposed to the same inducing agent. Our results suggest that there is indeed a random internal component to the decisions and we therefore suggest that the *probabilities* of each decision can be biased by treatment with the inducing factors.

To test this, we have carried out a further series of experiments to observe the effects of different concentrations of an inducing factor, acidic fibroblast growth factor (aFGF), on isolated animal pole (AP) cells. The results are shown in Table 2 and Fig. 4. As shown previously (Table 1 and Godsave and Slack, 1989), in the absence of FGF, animal cells only formed the ectodermal cell types, epidermis and neurons. How-

Table 2. *Differentiation of FGF-treated cells: dose dependent differentiation of animal pole cells and induction of cells from the dorsal subequatorial marginal zone region*

			Single cell type							Mixed colonies								
										Neural+:								
$ng\ ml^{-1}$ FGF	No.	Diff	Epi	Neur	Mes	Mst	LFC	Mus	Not	Epi	Mes	Mst	Mes Mst	Mst Mus	Mes Mst	Mst Mus	Mst Mes Mus	Mus Not
Animal Pole Cells																		
0	62	52	1 (2)	48 (92)						3 (6)								
0.15	45	32		28 (72)							1 (3)	2 (5)	1 (3)					
0.3	73	55		18 (33)		2 (4)					4 (7)	20 (36)	11 (20)					
1	73	50		13 (26)		2 (4)				1 (2)	3 (6)	19 (33)	11 (22)		1 (2)			
2	90	54	1 (2)	4 (7)	2 (4)	9 (17)		4 (7)		1 (2)	1 (2)	19 (35)	11 (20)	1 (2)	1 (2)			
5	65	48			1 (2)	10 (21)		10 (21)				16 (33)	4 (8)	2 (4)	3 (6)	2 (4)		
10	89	49				8 (16)		30 (61)	1 (2)			2 (4)	1 (2)	1 (2)	5 (10)			1 (2)
30	70	55				13 (24)		21 (38)				7 (13)	5 (9)	1 (2)	5 (9)		1 (2)	2 (4)
60	81	55			1 (2)	16 (29)		29 (53)				5 (9)		1 (2)	2 (4)			1 (2)
Dorsal Marginal Zone Cells																		
10 (%)	95	41			2 (5)		2 (5)	32 (78)	4 (10)					1 (2)				

The cell type or combination of cell types formed by each differentiating clone is shown. Abbreviations are as for Table 1. Numbers in parentheses are the percentages of differentiating clones forming each cell type or combination of cell types.
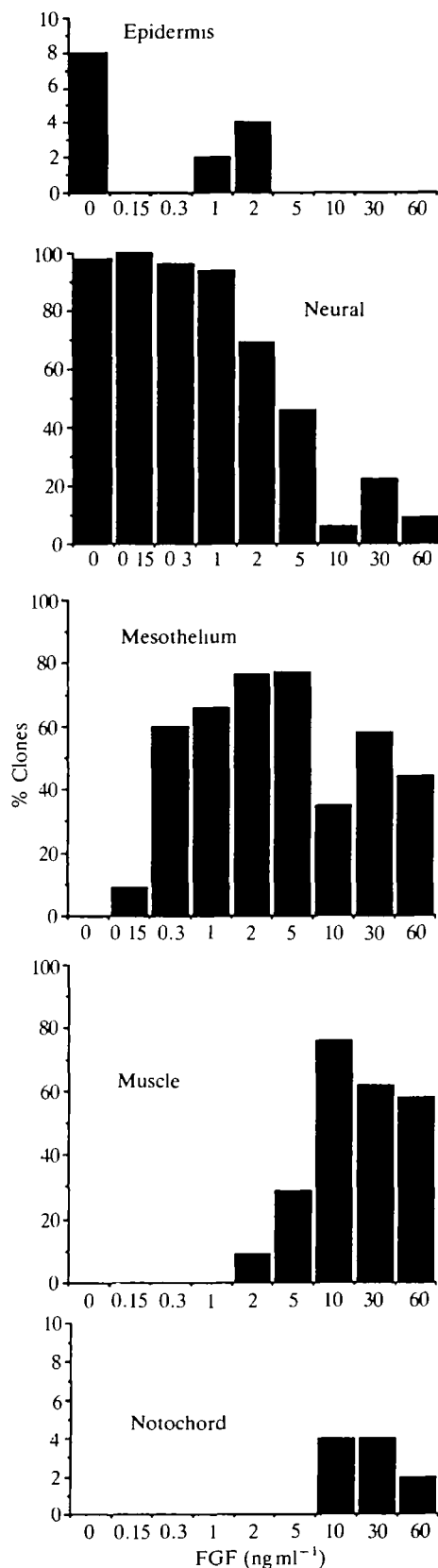
Fig. 4. Concentration dependence of AP cell induction. Bars indicate the percentage of differentiating clones containing each of the cell types shown at each FGF concentration used. Clones in which several cell types formed appear in more than one chart.

ever, when FGF is added, single AP cells can be induced to form mesodermal cell types. In the presence of increasing concentrations of FGF, the frequency of epidermal cells and neurons decreased and of mesothelium and then muscle and notochord increased. The half-maximal formation of mesothelium occurred at a concentration more than 10 times lower than the half-maximal formation of muscle, showing clearly that FGF produces different responses at different concentrations, one of the defining characteristics of a morphogen (Slack, 1987). Mesenchyme is not shown in Fig. 4, but its distribution was similar to mesothelium, with somewhat more of a reduction at the higher concentrations. Clearly individual cells do not respond identically to a given concentration of FGF, but the probability of each cell type forming is dependent upon FGF dose.

The types of cell formed by animal pole region cells in response to FGF are almost the same as those obtained from the marginal zone (Table 1), the only difference being the absence of the large flat cells. Once again, numerous mixed clones are obtained, and these are usually composed of two cell types that are neighbours in the overall sequence, e.g. neural+mesothelium or mesothelium+muscle, but not neural+muscle. We feel that it is significant that the most common sorts of mixed clone arising from marginal zone cells are also of these types. This suggests that mesoderm induction *in vivo* generates the same range of transient cell states as does treatment with FGF *in vitro* and is support for the idea that a form of FGF is a natural mesoderm inducing factor (Slack *et al.* 1987; Kimelman and Kirschner, 1987; Kimelman *et al.* 1988; Slack and Isaacs, 1989).

Discussion

The differences in specification between cells from different regions (Table 1) can conveniently be assessed by comparison with the normal fate map for the early *Xenopus* embryo (Keller, 1975, 1976; Dale and Slack, 1987 Moody, 1987). The fate map shows what will become of each region in normal development, but does not give information about the intrinsic commitment of cells. In at least one respect, the specification data shown here are similar to the fate map, that is that marginal zone but not animal pole cells give rise to mesoderm. However, in two important respects they differ. First, there is a high proportion of neural development, most noticeably from regions that do not normally contribute to the neural plate, particularly the ventral marginal zone. In multicellular explant cultures from the animal pole region or the ventral marginal zone, we never find neural development, even in the same medium as used here, although autoneuralization is common in explant cultures from urodelean amphibian species such as the axolotl (e.g. Holtfreter, 1944; Slack, 1984). We have also observed that, in cultures containing different numbers of animal pole region cells, epidermal differentiation predominates when there are more than about 30 cells in the well (data not

shown). One explanation for this would be that *Xenopus* blastula cells normally produce some inhibitor of neuralization which is diluted out in the single cell cultures. It is also possible that the culture medium induces neuralization of single cells. We have observed neural differentiation in several different media (Table 1) though in each case some protein was present. However, Grunz and Tacke (1989) have recently shown that *Xenopus* blastula or early gastrula animal pole cells kept dissociated for 5 h in protein-free media will frequently give rise to neural structures after reaggregation, while no neuralization occurs if the cells are reaggregated immediately (but also see Symes *et al.* 1988). Dissociation can also cause neuralization in animal pole cells from *Pleurodeles waltl* gastrulae (Saint-Jeannet *et al.* 1990).

The second major difference from the fate map is that, in the cultures, virtually no mesoderm is formed by cells taken from the supraequatorial part of the marginal zone while these cells make a substantial contribution *in vivo*. Since mesoderm is probably formed in response to inducing factors secreted from vegetal cells, this discrepancy may be explained by saying that, at the mid-blastula stage, the inducing signals, or the responses to them, have only reached about half way up the prospective mesodermal region.

In single cell cultures, many marginal zone cells from below the pigment boundary also remain uninduced, but if they are treated with a high concentration of FGF, all become induced (Table 2, last row), showing that they are competent to respond and that the signal must be limiting *in vivo*. The data also suggest that more cells from the ventral than the dorsal subequatorial marginal zone are specified to form mesoderm by the midblastula stage. A greater proportion of ventral than dorsal tissue also appears to be fated to form mesoderm (Dale and Slack, 1987). The major mesodermal tissue fated to form from the dorsal marginal zone is the notochord, very little of which forms in single cell cultures. Perhaps induction in this region is still at an early stage in the mid-blastula.

At the beginning, three questions about specification were posed. We can now suggest the following answers. (1) The mean behaviour of a cell population seems to be controlled by extracellular (inducing) signals, but superimposed on this there is a random element in the response of individual cells. The frequent occurrence of mixed clones shows that the initial specifications can be very finely balanced such that minor perturbations send different daughter cells down different developmental pathways. (2) The specification states of individual cells taken from one region are not necessarily the same though some of the regions used are quite large. Some heterogeneity of behaviour is shown by cells taken from all regions, but particularly the marginal zone. This also suggests a random element in the response of individual cells. (3) The specification of isolated cells is not necessarily the same as that of the tissue mass from which they came, i.e. isolated cells may behave differently to tissue explants. In particular, many single cells from the animal hemisphere give rise to neurons in

culture and many clones from the ventral marginal zone contain neurons or muscle, which is not the case with tissue explants. This may arise from inhibitory effects in the tissue mass and we plan to investigate this further. These results also suggest the need to use the term 'specification' with care.

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