

Mesoderm induction in *Xenopus laevis*: a quantitative study using a cell lineage label and tissue-specific antibodies

L. DALE, J. C. SMITH* AND J. M. W. SLACK

Imperial Cancer Research Fund, Burtonhole Lane, Mill Hill, London NW7 1AD, U.K.

SUMMARY

We have compared the development of the animal pole (AP) region of early *Xenopus* embryos in normal development, in isolation, and in combination with explants of tissue from the vegetal pole (VP) region. For the grafts and the combinations the animal pole tissue was lineage labelled with FLDx in order to ascertain the provenance of the structures formed.

The normal fate of the AP region was determined by orthotopic grafts at stages 7½ (early blastula), 8 (mid blastula) and 10 (early gastrula). At later stages most of the labelled cells were found in ectodermal tissues such as epidermis, head mesenchyme and neural tube (the last from stages 7½ and 8 only). However, in stage-7½ and stage-8 grafts some of the labelled cells were also found in the myotomes and lateral mesoderm.

In isolated explants the AP region of all three stages differentiated only as epidermis assessed both histologically and by immunofluorescence using an antibody to epidermal keratin.

The fate of labelled cells in AP–VP combinations was quite different and confirms the reality of mesoderm induction. In combinations made at stages 7½ and 8 the proportion of AP-derived mesoderm is substantially greater than the proportion of labelled mesoderm in the equivalent fate mapping experiments. This shows that the formation of mesoderm in such combinations is the result of an instructive rather than a permissive interaction.

The formation of mesodermal tissues in stage-7½ combinations was confirmed by using a panel of antibodies which react with particular tissues in normal tailbud-stage embryos: anti-keratan sulphate for the notochord, anti-myosin for the muscle and anti-keratin for epidermis and notochord.

Combinations made at stage 10 gave no positive cases and reciprocal heterochronic combinations between stages 7½ and 10 showed that this is the result of a loss of competence by the stage-10 AP tissue. Whereas stage-7½ AP tissue combined with stage-10 VP tissue gave many positive cases, the reciprocal experiment gave only a few.

We have also tested the regional specificity of the induction. Stage-7½ vegetal pole explants were divided into dorsal and ventral regions and then combined, separately, with stage-7½ animal poles. The dorsovegetal tissue induces ‘dorsal-type’ mesoderm (notochord and large muscle masses) while ventrovegetal tissue induces ‘ventral-type’ mesoderm (blood, mesothelium and a little muscle).

We conclude that mesoderm formation in combinations is an instructive event and propose a double gradient model to explain the complex character of the response.

* *Present address:* Laboratory of Embryogenesis, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

Key words: *Xenopus laevis*, mesoderm induction, lineage labelling, regional markers, specification, gradients, keratin, myosin, keratan sulphate.

INTRODUCTION

The basic body plan of amphibians and other vertebrates is thought to arise as the result of a sequence of inductive interactions between different regions of the embryo (reviewed Nakamura & Toivonen, 1978; Slack, 1983). The first of these is *mesoderm induction* in which it is believed that an equatorial mesodermal rudiment is induced from the animal hemisphere under the influence of a signal from the vegetal hemisphere (Nieuwkoop, 1969, 1973). In the absence of this signal the entire animal hemisphere would become ectoderm. The first experiments on mesoderm induction were performed on the axolotl and similar results were later obtained on *Xenopus laevis* (Nakamura, Takasaki & Ishihara, 1970; Sudarwanti & Nieuwkoop, 1971).

Mesoderm induction is thought to underly the 'rescue' of axial structures in u.v.-irradiated embryos by the orthotopic implantation of unirradiated blastomeres from the dorsovegetal region of the 64-cell stage. These cells cause the formation of axial structures in the host embryos without themselves contributing to them (Gimlich & Gerhart, 1984).

Mesoderm induction is also the only known induction in early development for which a reasonably convincing 'morphogen' has been isolated. The mesoderm-inducing, or vegetalizing, factor is a protein of molecular weight 30 000 isolated from chick embryos. When applied in the form of a pellet to amphibian blastula ectoderm it evokes the differentiation of a range of tissues similar to that induced by vegetal pole tissue (Tiedemann & Tiedemann, 1959; Born *et al.* 1972; Schwartz, Tiedemann & Tiedemann, 1981).

Experiments on induction are often criticized on the grounds that the results may have arisen by selection of precommitted cells rather than by direction of cells along a different developmental pathway from that which they would otherwise have followed. Since methods for cell lineage tracing and cell type identification have greatly improved since Nieuwkoop's experiments, we have reinvestigated the phenomenon of mesoderm induction in the hope of defining clearly its principal biological features, and hence informing future biochemical work.

The differences between the present work and that published previously are fourfold. Firstly, we have fate mapped the target tissue at several stages prior to gastrulation in order to find whether any prospective mesoderm can be found in the region around the animal pole. Secondly, we have used a lineage label, fluorescein-lysine-dextran (FLDx), both for the fate mapping experiments and for the induction experiments in order clearly to distinguish the origins of cells. This label is greatly superior to the tritiated thymidine used previously since it is not diluted on cell division. Thirdly, in order to score the experimental cases we have counted the proportion of the total volume of the responding tissue occupied by each tissue type. Since amphibian embryo tissues show no net growth during early development this allows an accurate assessment of whether the results could have arisen by selection. Finally, we have not relied solely on histology to identify the

tissues in induction experiments but have used a panel of antibodies, each of which is specific for a particular tissue type at the tailbud stage.

Our conclusions are that mesoderm induction is a genuine instructive event and not merely a permissive phenomenon allowing selective differentiation of pre-committed mesodermal cells. However the nature of the primary response remains obscure since different mesodermal tissues are induced by vegetal pole tissue from different stages and from different regions. Also we do not consider it possible at present to decide whether mesoderm induction occurs during normal development, or only in the abnormal tissue configurations created by the experimenter.

MATERIALS AND METHODS

Embryos of *Xenopus laevis* were obtained by artificial fertilization as described by Smith & Slack (1983). They were chemically dejellied with 2 % cysteine hydrochloride (pH 7.8–8.1), thoroughly washed, and transferred to a Petri dish coated with 1 % Noble Agar (Difco) containing 5 % normal amphibian medium (NAM: Slack, 1984). The embryos were staged according to Nieuwkoop & Faber (1967).

Lineage label

The lineage tracer fluorescein-lysine-dextran (FLDx) was prepared as described by Gimlich & Gerhart (1984). Injections were performed using a Burleigh inchworm to drive a 10 μ l pressure-tight syringe connected by a liquid-filled system to the micropipette. Embryos were injected before first cleavage at the vegetal pole with 20 nl of FLDx at 100 mg ml⁻¹ in water. During and after injection embryos were kept in 5 % NAM with 5 % W/V Ficoll (type 400: Pharmacia) which prevents leakage of cytoplasm (Newport & Kirschner, 1982).

Operations

All operations were performed in full-strength NAM using ground forceps, hair loops and electrolytically sharpened tungsten needles. The types of operation performed are shown in Fig. 1. During isolation of the vegetal pole tissue care was taken to ensure that only prospective endoderm was included in the explant. For experiments in which dorsoventral polarity was relevant, it was ascertained at the 8-cell stage when dorsal blastomeres are smaller and lighter than ventral blastomeres (Nieuwkoop & Faber, 1967). The dorsal equatorial zone was then marked with a small crystal of Nile Red (Kirschner & Hara, 1980).

Orthotopic grafts were gradually returned to 5 % NAM and maintained at 22°C for 24 h (st. 25–27) before fixation. Only embryos that had developed normally were examined histologically. Combinations and explants were cultured in full strength NAM at 22°C for either 48 h (controls reach st. 35–38) or 66 h (controls reach st. 40) before fixation.

Histology

Specimens required for routine histological examination were fixed in 4 % paraformaldehyde in 70 % PBSA overnight at 4°C, dehydrated in an ethanol series, embedded in paraffin wax (m.p. 56°C) and sectioned at 10 μ m. Rehydrated sections were either stained with 1 % methyl green for 1 min or with 0.5 μ g ml⁻¹ DAPI (4,6-diamidino-2-phenyl indole: Boehringer) for 1 min. After rinsing in tap water, sections were dehydrated through ethanols, cleared in xylene and mounted in DPX.

Specimens required for immunohistochemistry were fixed in 2 % TCA overnight at 4°C. They were washed in 70 % PBSA and then embedded via acetone in polyethylene glycol 400 distearate (Koch) plus 1 % cetyl alcohol, at 37°C (Dreyer, Wang, Wedlich & Hausen, 1983).

Sections were cut at 6 μm , brought to 70 % PBSA through an acetone series and analysed by indirect immunofluorescence.

Antibodies

Three antibodies were used in this study, 1) a rabbit anti-keratin raised against total keratin from human stratum corneum (Wu & Rheinwald, 1981). This antibody interacts with the epidermis and notochord sheath at all the stages tested (stages 25, 35 and 40) although the notochord sheath is only weakly stained at the earliest stages. 2) MHC2, a rabbit antibody raised in this laboratory against adult *Xenopus laevis* myosin heavy chain and characterized by Western blot. This antibody interacts specifically with muscle from stage 35 onwards, it does not interact with any tissue at earlier stages. 3) MZ15, a monoclonal antibody raised against pig chondrocytes and characterized as recognizing keratan sulphate by radioimmunoassay with different proteoglycan fragments (Zanetti, Ratcliff & Watt, 1985). This antibody interacts specifically with chondrocytes and particularly with the notochord sheath at early stages, and from stage 27 onwards it also interacts with the ventral portion of the ear vesicle lumen (Smith & Watt, 1985). Staining with this antibody is enhanced by pretreating sections with chondroitinase

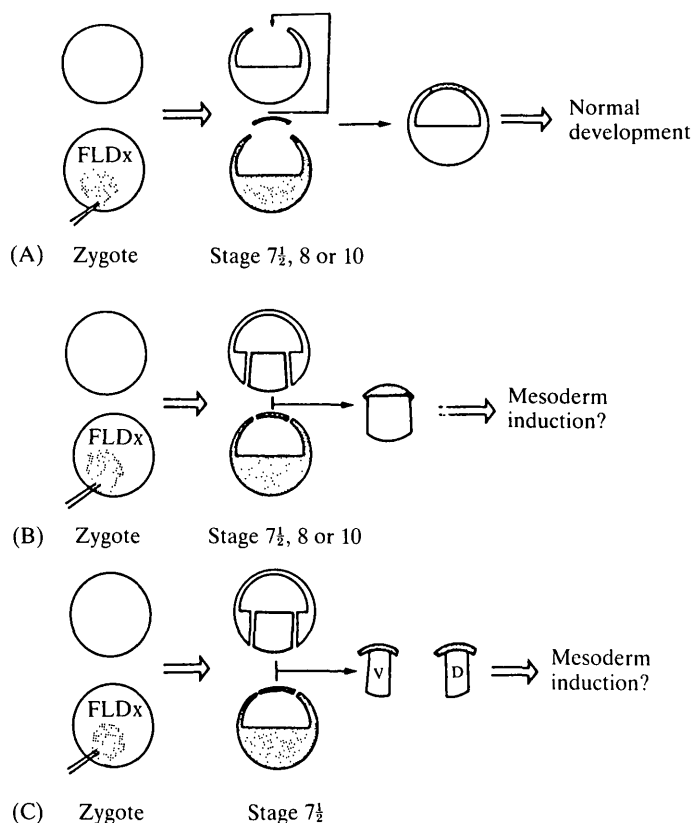


Fig. 1. Experimental design. (A) Orthotopic grafts of animal pole tissue from FLDx-labelled donors into unlabelled recipients. Both donors and recipients were at the same developmental stage. (B) Combination of animal pole tissue from FLDx-labelled embryos with vegetal pole tissue from unlabelled embryos. In most cases combinations were between explants from the same developmental stage. Heterochronic combinations combined explants from different developmental stages (7 1/2 and 10). (C) Combination of FLDx-labelled stage-7 1/2 animal poles and either the dorsal or ventral portion of unlabelled stage-7 1/2 vegetal poles.

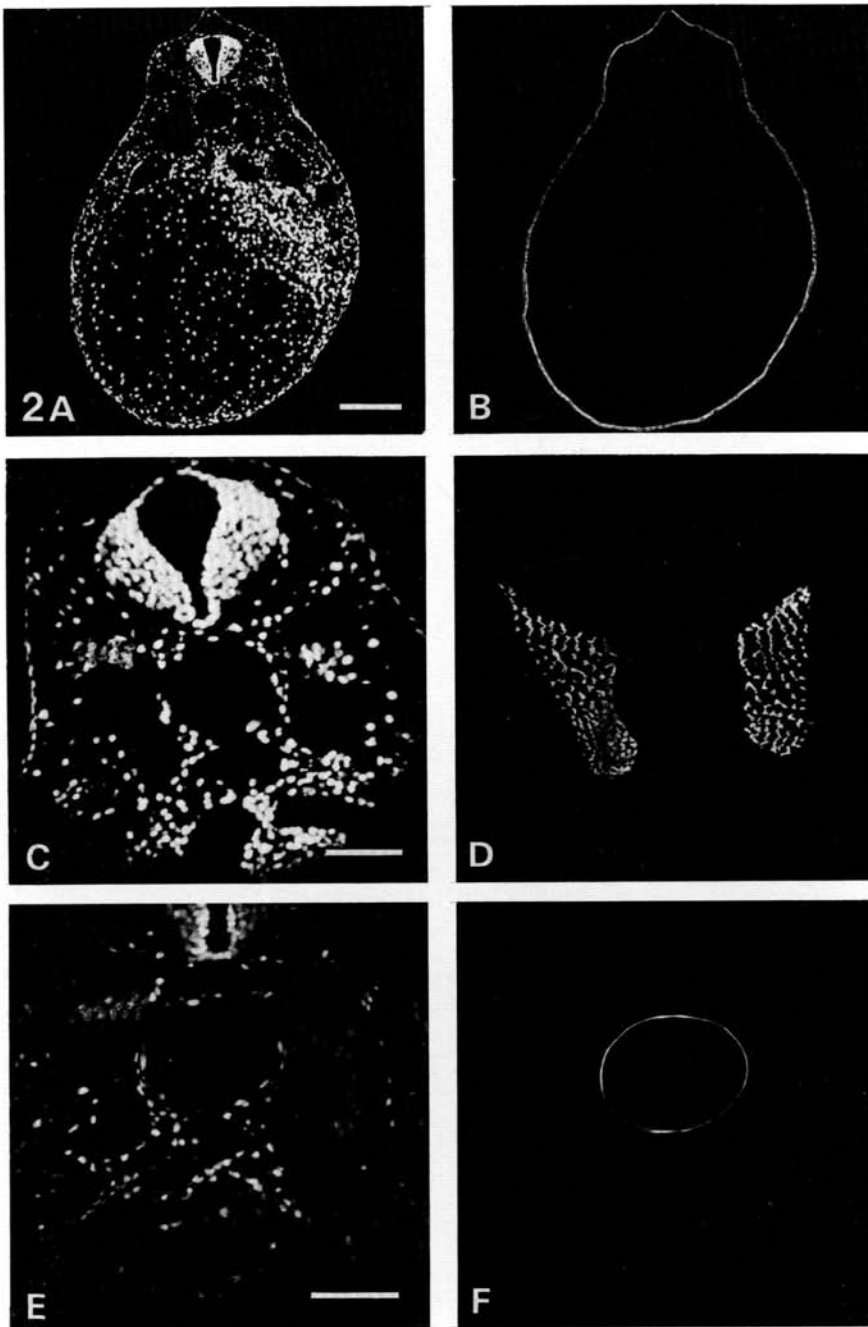
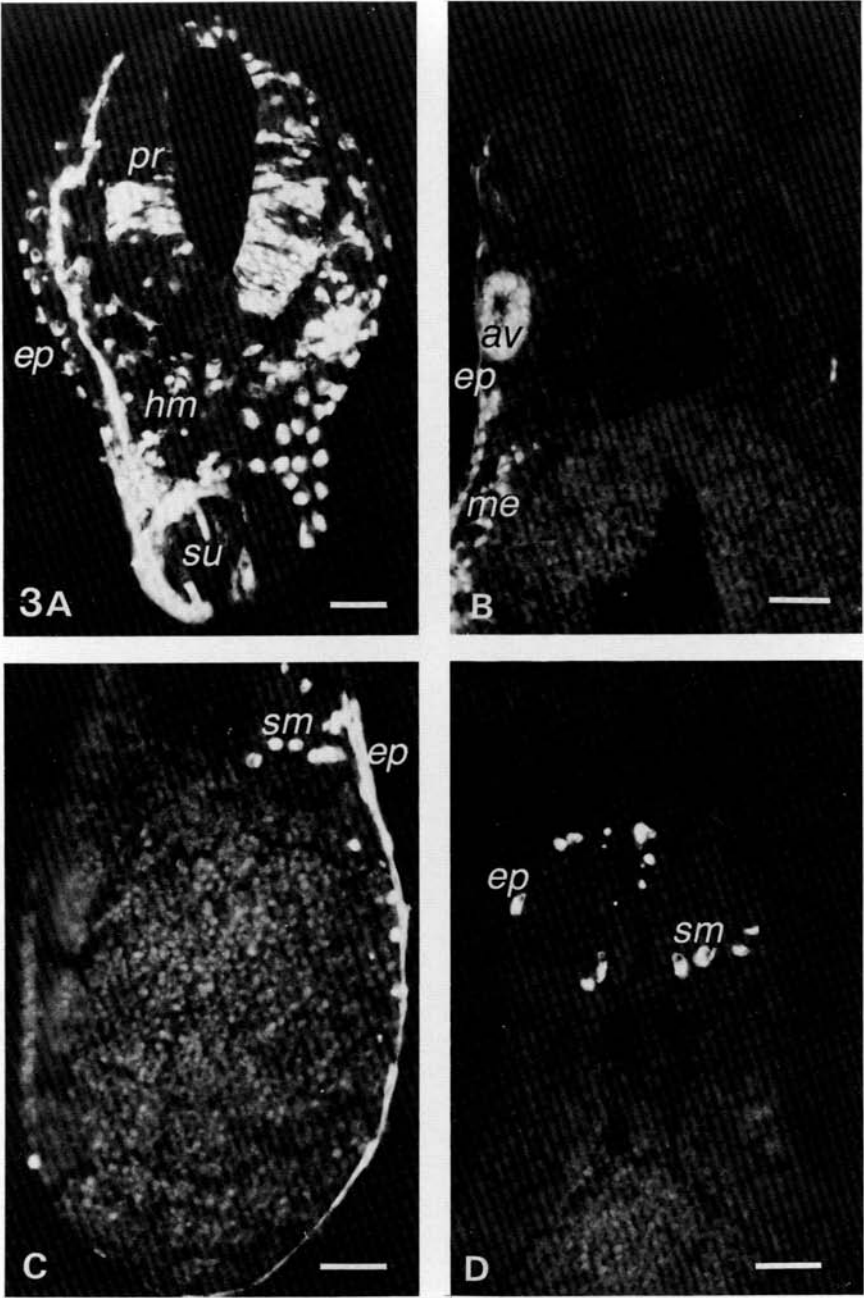


Fig. 2. Tissue specificity of antibodies in 3-day-old (stage-40) *Xenopus* embryos. A, C and E are photographs of DAPI fluorescence, B, D and F are photographs of immunofluorescence. (A, B) antikeratin, labels epidermis and notochord (faintly). Scale bar = 200 μm . (C, D) MHC2, labels muscle. Scale bar = 100 μm . (E, F) MZ15, labels notochord. Scale bar = 100 μm .



(Smith & Watt, 1985). The regional specificity of these antibodies on sections of normal stage-40 embryos is shown in Fig. 2.

Immunofluorescence

Before staining, sections to be incubated with MZ15 were pretreated with $1\ \mu\text{l ml}^{-1}$ of chondroitinase (Sigma) for 1 h. All sections were then 'blocked' with 4 % BSA and either 10 % goat serum (Miles) or 10 % rabbit serum (Miles) in 70 % PBSA. Sections were incubated for 30 min with either 1 % antikeratin serum or 1 % MHC2 serum, or for 60 min with 0.5 % MZ15 (ascitic fluid). They were then washed in 70 % PBSA for 3×15 min followed by 30 min incubation with either 2.5 % FITC-conjugated goat anti-rabbit IgG (Miles), or 2.5 % FITC-conjugated rabbit anti-mouse IgG (Miles). All sections were then washed in 70 % PBSA (1×15 min) and stained for 1 min with $0.5\ \mu\text{g ml}^{-1}$ DAPI. Finally they were thoroughly washed in 70 % PBSA (3×15 min) and mounted in a gelvatol-based medium containing n-propyl gallate to retard quenching (Giloh & Sedat, 1982).

Scoring of specimens

All specimens were scored using a Zeiss photomicroscope with appropriate filter sets for fluorescein, rhodamine and DAPI. Specimens were analysed every fifth section to determine the types of tissue differentiated, emphasis being placed on those tissue types differentiating from the lineage labelled (i.e. former animal pole) region. They were also analysed every tenth section to determine the absolute and relative volumes of each tissue type differentiated from the animal pole region. This was calculated by dividing the field of view (mag. $\times 250$) into squares of side $15\ \mu\text{m}$ using an eyepiece graticule and counting those squares in which at least 50 % of the area contained FLDx. The readings were summed for each of the labelled tissues, providing a measure for the volumes of the various tissue types that were labelled. Results were analysed statistically using 't' test, and the Wilcoxon two sample rank test. The 'P-values' quoted in the text refer to the t test but all significant results were significant by both tests.

RESULTS

Normal fate of the animal pole (AP) region

The animal pole region referred to here and subsequently is a disc of tissue from the centre of the pigmented hemisphere subtending a solid angle of about 60° . The normal fate of this region was determined by orthotopic grafts between FLDx-labelled donors and unlabelled hosts (Fig. 1A). Operations were performed at stages $7\frac{1}{2}$ (early blastula), 8 (mid blastula) and 10 (early gastrula). Since the AP region gets thinner as development progresses from stage $7\frac{1}{2}$ to stage 10, the volume of tissue fate mapped gets smaller. At stages 8 and 10 healing of the graft and host was generally very good and most embryos developed normally. This was not the case at stage $7\frac{1}{2}$: healing was often very poor and many embryos developed abnormally. Only those embryos that showed a normal external morphology and upon examination a normal internal anatomy were included in the results.

Fig. 3. Normal fate of the FLDx-labelled stage- $7\frac{1}{2}$ animal pole region following orthotopic grafts into unlabelled stage- $7\frac{1}{2}$ recipients. These pictures show the distribution of label in parts of tailbud-stage embryos. (A) Head section, labelled cells found in prosencephalon (*pr*), head mesenchyme (*hm*), sucker (*su*) and epidermis (*ep*). (B) Head section, labelled cells found in auditory vesicle (*av*), epidermis (*ep*) and mesoderm (*me*). (C) Trunk section, labelled cells found in epidermis (*ep*) and somitic mesoderm (*sm*). (D) Trunk section, a few labelled cells found in the somitic mesoderm (*sm*) and epidermis (*ep*). Scale bars = $50\ \mu\text{m}$.

Table 1. *Normal fate of animal pole region*

Tissue type	Stage		
	7½	8	10
Epidermis	66 ± 5	84 ± 3	94 ± 2
Neural	10 ± 3	7 ± 3	0
Head mesenchyme	9 ± 3	6 ± 1	5 ± 2
Trunk neural crest	1 ± 0	0	0
Total ectoderm	86 ± 3	97 ± 1	100
Notochord	1 ± 1	0	0
Somite	9 ± 3	2 ± 1	0
Lateral plate	3 ± 1	1 ± 0	0
Total mesoderm	14 ± 3	3 ± 1	0
Yolk mass	0	0	0
Number of cases	12	13	16

Figures show the percentages ± the standard error of each tissue type in the labelled tissue.

In this and subsequent tables all figures are rounded to the nearest integer. Deviations from additivity arise only from rounding errors.

When embryos derived from all three types of graft were examined at later stages (stage 25–27) labelled cells were mainly found in the ventrolateral epidermis and epidermal derivatives such as the lens and otic vesicle (Fig. 3 and Table 1). At stages 7½ and 8 a significant proportion of the label was found in neuroectodermal tissues, particularly anterior regions of the brain (prosencephalon and optic cup) and head mesenchyme, but the spinal cord and trunk neural crest also contained a few labelled cells. In the mesoderm, labelled cells were mainly found in the posterior trunk and tail somites and a few cells were also found in the lateral plate and occasionally, at stage 7½, in the notochord. Although some of these cells may be of neural crest origin, it is clear that the AP region does make a contribution to the mesoderm, although this contribution is stage dependent. Thus the highest proportion of labelled mesoderm was found following grafts at stage 7½ (14 %) and was significantly less at stages 8 and 10 (3 % and 0 % respectively, Table 1). Similarly the proportion of label in the neurectoderm was progressively reduced as grafts were performed at later stages. We can therefore conclude that both the prospective epidermis/mesoderm and prospective epidermis/neural tube boundaries move towards the vegetal pole as the embryos progress through the later blastula stages.

Differentiation of animal pole, vegetal pole (VP) and intermediate zone (IZ) isolates

Explants from the AP, VP and IZ regions of stage-7½, -8 and -10 embryos were cultured for two days to test their differentiative capacity in isolation. In this experiment intermediate zone refers to that region remaining following the isolation of the AP and VP regions being therefore somewhat larger than the

marginal zone of the gastrula. All three regions differentiated in a stage-independent manner.

Isolates of the AP region (33 cases) typically differentiate into a mass of morphologically atypical epidermal cells surrounded by a layer of irregularly shaped epidermis. The AP region consists of two cell layers with different cell affinities, a superficial layer which probably differentiates into the irregularly shaped epidermis and a deep layer which probably differentiates into the central mass of epidermal cells (Asashima & Grunz, 1983). No cases of endodermal, mesodermal or neuroepithelial differentiation were observed. That isolates of the stage-7½ AP region differentiate only into epidermis was demonstrated by the use of the panel of regionally specific antibodies described above. All the cells stained with the anti-keratin but none with the anti-myosin or anti-keratan sulphate.

Those isolates of the VP region (20 cases) that did not completely disintegrate upon culture formed only spherical masses of large yolky cells. No differentiated tissues of ecto- or mesodermal type were observed. Stage-7½ VP isolates were not stained by any of the three antibodies.

Isolates of the intermediate region (35 cases) differentiated into the whole range of ecto- and mesodermal tissues including notochord, muscle, neuroepithelium and epidermis (Fig. 4). Few differentiated endodermal structures were observed although there were often undifferentiated regions which some authors might count as endoderm. As expected, following immunofluorescence, stage-7½ IZ isolates were stained in the appropriate regions by all three antibodies (Fig. 4). In each case the histological appearance of the positive tissues resembles that of the corresponding tissues in intact embryos (Fig. 2) and this makes us confident that these tissues can usually be correctly identified by histological criteria alone, although the antibodies can give extra information in some borderline situations.

Combinations of AP and VP explants

The AP region of embryos uniformly labelled with FLDx were isolated at stages 7½, 8 and 10 and combined with the unlabelled VP region from embryos of the same stage (Fig. 1B). The two pieces adhered to each other very rapidly and remained in close contact throughout the culture period. The reciprocal experiment, FLDx- or RLDx-(rhodamine-lysine-dextran) labelled VP regions recombined with unlabelled AP regions, proved not to be possible. After two days of culture no labelled VP blastomeres were found in the combination and no mesodermal structures were formed by the unlabelled AP region. Why the labelled VP blastomeres should die is unclear since blastomeres labelled *in situ* survive and contribute to the prospective endoderm (data not shown; also see Gimlich & Gerhart, 1984).

The tissue types formed in these experiments were stage dependent, only those constructed at stages 7½ and 8 formed mesodermal tissues. Positive cases could be divided into two groups, one of which we refer to as 'dorsal-type' differentiation and the other as 'ventral-type'. This distinction is based on the behaviour, in isolation, of marginal zone tissue from early gastrulae (Slack & Forman, 1980).

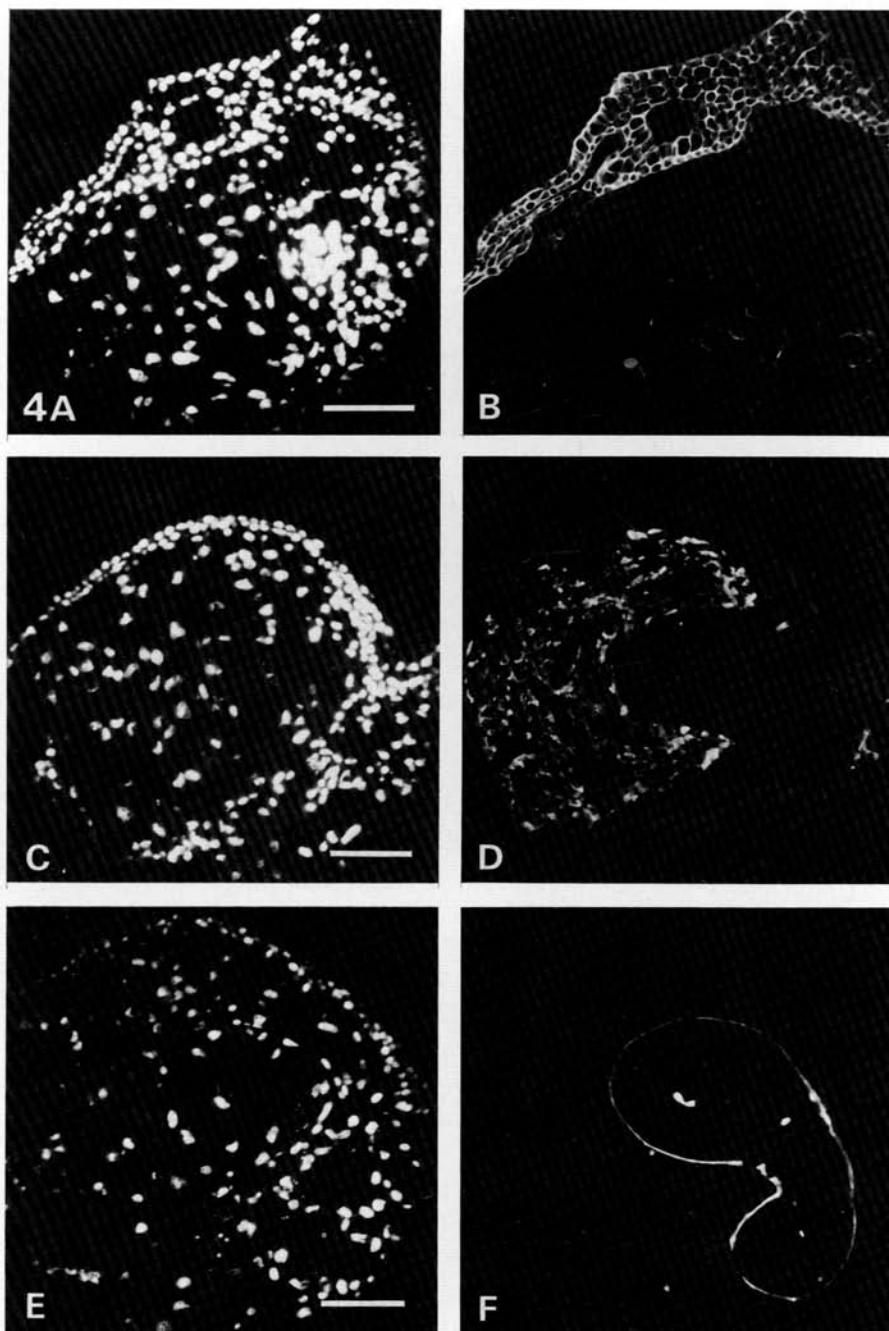


Fig. 4. Tissue specificity of antibodies in 3-day-old intermediate zone explants. A,C and E are photographs of DAPI fluorescence, B,D and F are photographs of immunofluorescence. (A,B) Anti-keratin, labels epidermis and notochord. (C,D) MHC2, labels muscle. (E,F) MZ15, labels notochord. Scale bars = 100 μ m.

Thus dorsal-type differentiation consists mainly of muscle and notochord while ventral-type differentiation consists of erythrocytes, mesothelium and mesenchyme (see Fig. 5).

All cases of combinations made at stage 7½ contained muscle, about half contained notochord (7/16) and a small number of cases contained erythrocytes (3/16), mesothelium (2/16) and pronephros (3/16). Although there was some variability between combinations, on average 90 % by volume of identifiable mesoderm was muscle, 4 % notochord and 1–2 % erythrocytes, mesothelium and pronephros (Table 2). It is clear that the majority of cases (13/16) produced dorsal-type mesoderm, while only three cases produced ventral-type mesoderm. All the identifiable mesoderm was labelled with FLDx and therefore clearly derived from the AP region. Occasionally a small number of unlabelled cells were found intermingled with labelled muscle, however they could not histologically be clearly identified as muscle. The remaining labelled tissue mainly differentiated as irregular-shaped epidermis or was undifferentiated. Very small amounts also differentiated as mesenchyme and neuroepithelium. Most of the yolky part of the combinations was both undifferentiated and unlabelled showing that it is derived from the vegetal component, yet many cases also contain a few labelled but unidentifiable cells in this region as well.

The same experiment performed at stage 8 gave similar results (Table 2) except that mesoderm was almost all present as muscle (>99 %); a small amount of notochord was found in a single combination and no cases produced erythrocytes, mesothelium or pronephros. All the positive cases were therefore of dorsal type. Not all cases produced mesoderm, however. In three cases labelled tissue differentiated entirely as atypical and irregular-shaped epidermis (similar to isolates) while in two others labelled tissue was enclosed by unlabelled, undifferentiated yolky cells and had failed to differentiate. Stage-10 combinations gave only a few

Table 2. *Fate of animal pole tissues in animal-vegetal combinations*

Tissue	Stage		
	7½	8	10
Epidermis	46 ± 5	43 ± 9	98 ± 1
Neural	0	1 ± 1	0
Mesenchyme	1	0	2 ± 1
Total ectoderm	47 ± 5	44 ± 9	100
Notochord	2 ± 1	0	0
Muscle	31 ± 4	41 ± 9	0
Mesothelium	0	0	0
Erythrocytes	1	0	0
Total mesoderm	34 ± 4	41 ± 9	0
Unidentified	21 ± 3	17 ± 7	0
Number of cases	16	14	5

Figures show percentages ± standard error of each tissue type in the labelled tissue.

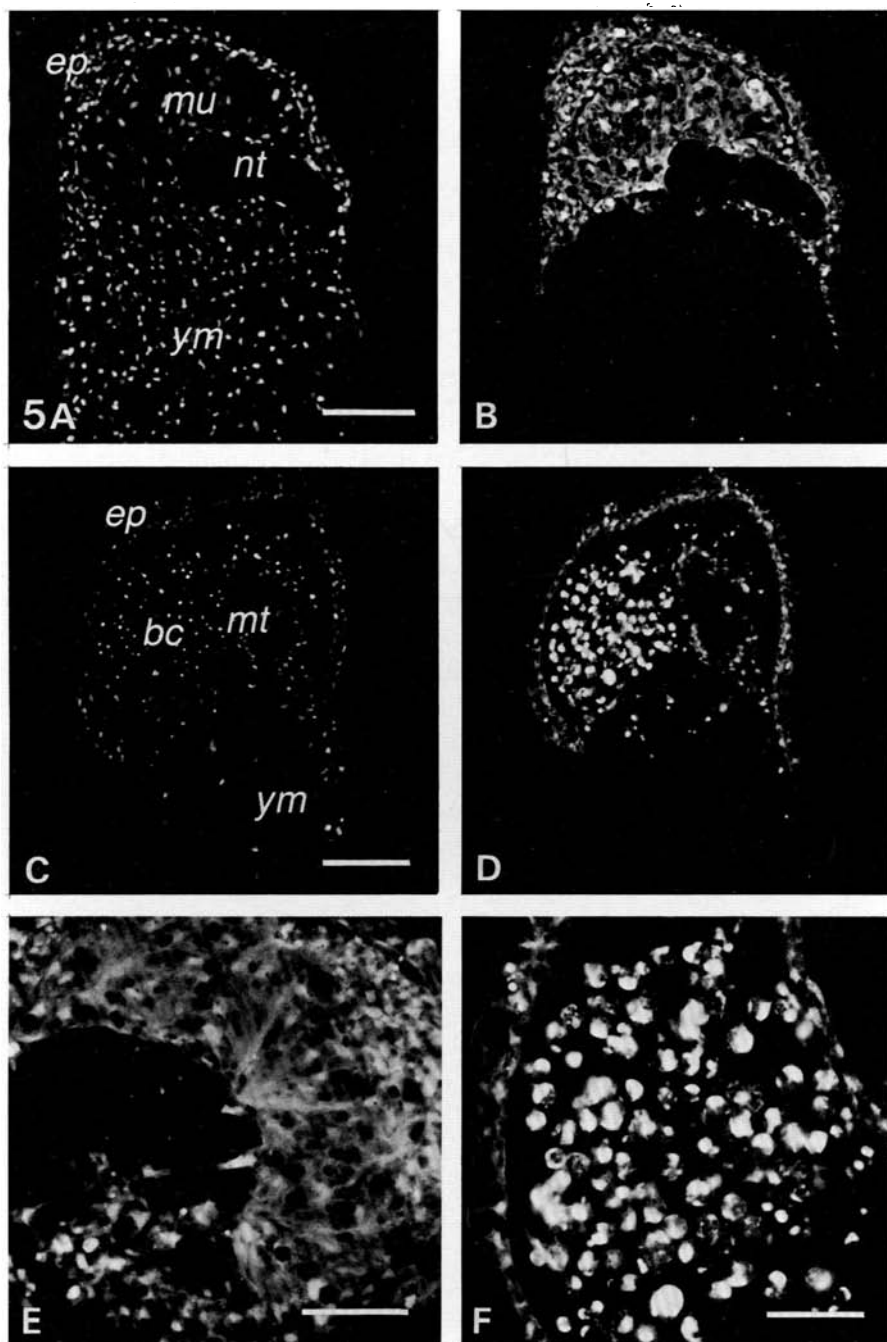


Table 3. *Proportions of animal-pole-derived tissue forming tissue types considered as ectoderm and mesoderm*

	St7½	Stage St8	St10
		<i>Isolates</i>	
Ectoderm	100	100	100
Mesoderm	0	0	0
		<i>Normal development</i>	
Ectoderm	86 ± 3	97 ± 1	100
Mesoderm	14 ± 3	3 ± 1	0
		<i>Animal-vegetal combinations</i>	
Ectoderm	47 ± 5	44 ± 9	100
Mesoderm	34 ± 4	41 ± 9	0

positive cases and these only produced a small amount of mesenchyme. In no cases were labelled and unlabelled cells found intermingled.

Several series of stage-7½ combinations were also prepared for immunofluorescence; in these experiments no lineage label was used since the procedures required for good immunohistochemical results do not fix FLDx or RLDx. Although serial sections were not taken in these cases, those sections that were examined contained all the ecto- and mesodermal tissue types found in earlier combination experiments. The identities of the tissues were fully confirmed by staining with the panel of antibodies. All cases contained epidermis and muscle and about half contained notochord. All of the unidentifiable tissues in these combinations were unstained. The use of these antibodies confirms that mesodermal tissues differentiate in stage-7½ combinations.

A comparison of Tables 1 and 2 is shown as Table 3 and demonstrates a clear and significant difference between the differentiation of the blastula AP region in isolates, orthotopic grafts and AP-VP combinations. So it is clear that the developmental pathway of the AP region of the blastula can be altered by environmental factors. Whereas in isolation it forms only epidermis and in normal development there is only a small contribution to the mesoderm, in combinations a substantial proportion ($P < 0.01$ at stages 7½ and 8) of the AP region differentiates into mesodermal as opposed to ectodermal tissues. Since the total volume is constant these results cannot be explained by selection and the

Fig. 5. Mesoderm induction. (A,B) animal pole-dorsovegetal pole combination. (A) DAPI fluorescence: *ep*, epidermis; *nt*, notochord; *mu*, muscle; *ym*, yolk mass. Scale bar = 100 µm. (B) Fluorescein fluorescence: epidermis, notochord and muscle are all labelled and therefore of animal pole origin. The yolk mass is unlabelled and therefore of vegetal pole origin. (C,D) animal pole-ventrovegetal pole combination. (C) DAPI fluorescence; *ep*, epidermis; *bc*, blood cells; *mt*, mesothelium; *ym*, yolk mass. Scale bar = 100 µm. (D) Fluorescein fluorescence: epidermis, blood cells and mesothelium are all labelled and therefore of animal pole origin, whereas the yolk mass is unlabelled and therefore of vegetal pole origin. (E) Higher power to show labelled muscle and notochord in animal-dorsovegetal combination. Scale bar = 50 µm. (F) Higher power to show labelled blood cells in animal-ventrovegetal combination. Scale bar = 50 µm.

Table 4. *Heterochronic animal-vegetal combinations*

Tissue type	AP(st7½)-VP(st10)	AP(st10)-VP(st7½)
Epidermis	58 ± 5	94 ± 4
Neural	1 ± 1	1 ± 1
Mesenchyme	2 ± 1	0
Total ectoderm	61 ± 5	95 ± 3
Notochord	1 ± 1	1 ± 1
Muscle	15 ± 4	2 ± 2
Mesothelium	3 ± 1	0
Erythrocytes	4 ± 1	0
Total mesoderm	23 ± 4	3 ± 3
Unidentified	16 ± 3	2 ± 1
Number of cases	28	28

Figures show percentages ± standard error of each tissue type in the labelled tissue.

Table 5. *Regional specificity of induction*

Tissue type	Inductor (st7½)	
	Dorsal	Ventral
Epidermis	47 ± 4	61 ± 4
Neural	5 ± 2	0
Mesenchyme	0	1
Total ectoderm	53 ± 4	62 ± 4
Notochord	10 ± 3	0
Muscle	23 ± 3	2 ± 2
Mesothelium	0	5 ± 1
Erythrocytes	0	13 ± 4
Total mesoderm	33 ± 3	20 ± 4
Unidentified	13 ± 2	17 ± 3
Number of cases	27	17

Figures show percentages ± standard error of each tissue type in the labelled tissue *Tissue type*.

formation of mesoderm in the combinations must be the product of an instructive, rather than permissive, action of the VP component upon the AP component. The stage specificity of mesoderm formation suggests either that the competence of the AP component to respond, or of the VP component to instruct, or both, is lost by stage 10. In order to find which of these alternatives was correct we carried out heterochronic combinations.

Heterochronic combination of AP and VP regions

Reciprocal heterochronic combinations were performed with labelled AP and unlabelled VP regions isolated from stage-7½ and stage-10 embryos. In both cases the two regions adhered rapidly and remained in close contact throughout the culture period.

Combinations of stage-10 AP region with stage-7½ VP region usually failed to produce any mesoderm; in 25 out of 28 combinations the entire labelled region differentiated into atypical and irregular-shaped epidermis. A few of these cases also contained a few mesenchyme cells and a small amount of tissue that could not be identified. In two of the remaining cases most of the labelled tissue (91 %) differentiated as epidermis and the remainder differentiated as muscle. The final case contained large amounts of labelled muscle (56 %), notochord (19 %) and neural tissue (25 %), no epidermis was found. The average figures given in Table 4 thus indicate a small number of significant inductions rather than frequent small inductions. By contrast, the reciprocal experiment, stage-7½ AP combined with stage-10 VP, gave many positive cases. 21 out of 28 formed mesoderm, which was always labelled and therefore of AP origin. 11 of the 21 cases behaved as dorsal-type isolates; all containing muscle and seven notochord. Mesoderm accounted for 40 % of the label in these combinations, 37 % being muscle. The remaining label was found in epidermis (37 %), neural tissue (4 %), mesenchyme (1 %) and tissue that could not be identified (19 %). Ventral-type differentiation was found in the remaining 10 cases, with the peculiarity that one contained a large amount of muscle (26 % of labelled tissue). This combination, however, like all the others, contained erythrocytes. Nine of the combinations contained mesothelium and seven mesenchyme. On average, mesoderm accounted for only 19 % of the label in these combinations, the remainder being epidermis (67 %), mesenchyme (2 %) and tissue which could not be identified (18 %). A few isolated labelled cells were found amongst the unlabelled VP component in dorsal- but not ventral-type combinations.

The experiments show that the failure of mesoderm induction in stage-10 combinations is the result of a loss of competence in the AP component. Stage-10 VP regions remain capable of inducing mesoderm formation in a stage-7½ AP region. However, it is also clear that some change has occurred in the VP region which was capable of inducing more mesoderm, and in particular more dorsal-type mesoderm, at stage 7½ than at stage 10.

Regional differences in the stage-7½ VP region

To test for regional differences in the inductive capacity of the blastula VP region, unlabelled stage-7½ VP regions were divided into dorsal and ventral halves. Each explant was then combined with a labelled AP explant from an embryo of the same stage (Fig. 1C). As in previous experiments the two regions adhered rapidly and contact was maintained throughout the culture period.

Division of the VP region did not affect the number of positive cases obtained; for both dorsal and ventral regions over 90 % of the combinations produced some mesoderm. However, both the amount and the type of mesoderm formed were quite different. Combinations including dorsal VP regions produced dorsal-type mesodermal tissues in 25 out of 26 positive cases; they always contained muscle and most (18/26) also contained notochord (Fig. 5). A small amount of pronephros was also found in a few cases. The remaining positive case produced a

small amount of ventral-type mesodermal tissues, erythrocytes and mesothelium. Most of the mesodermal tissues were labelled and therefore of AP origin (Fig. 5), but in three cases a few unlabelled cells were found amongst the labelled muscle and in one case unlabelled cells had clearly differentiated into notochord. Around half of the labelled tissue in these combinations differentiated as ectoderm (47 % epidermis and 5 % neural tissue), a smaller but significant amount by comparison with normal development (33 %, $P < 0.001$) differentiating as mesoderm. The remaining labelled tissue (13 %) could not be identified. As in previous experiments muscle accounted for most of the mesoderm (73 %) but notochord also accounted for a significant proportion (24 %).

Combinations including ventral VP regions produced mesoderm in 16 out of 17 cases and in all these all the mesoderm was labelled with FLDx (Fig. 5). Of the 16 positive cases, 15 differentiated as ventral-type mesoderm (Fig. 5) and one differentiated as dorsal-type mesoderm. Once again much of the labelled tissue (62 %) differentiated as ectoderm, all of it as epidermis. Only 20 % of the label formed mesoderm (not significantly greater than normal development: $P > 0.05$), the remainder being found in tissue that could not be identified (17 %). However, within the mesoderm most of the label was found in erythrocytes (61 %), mesothelium (21 %) and muscle (15 %), only small amounts were found in notochord (2 %) and pronephros (1 %). Most of the muscle and all of the notochord was found in the single case that differentiated as dorsal-type mesoderm.

It is clear that regional differences in the inductive capacity of the blastula VP region do exist. Dorsal regions induce dorsal-type mesoderm while ventral regions induce ventral-type mesoderm. It is not known whether this is the result of qualitatively different inducing capacities or quantitative differences in the same inducing capacity. Indeed since the amount of mesoderm formed in ventral combinations is not significantly higher than that found in stage-7½ orthotopic grafts, it is possible that the ventral VP region does not induce mesoderm at all, but merely provides the permissive environment required for ventral differentiation in an otherwise specified mesodermal rudiment. Dorsal combinations form similar amounts of mesoderm as combinations including the whole VP region. However, the proportion of the two dorsal tissue types are dissimilar, dorsal combinations contain more notochord and less muscle than combinations including the whole VP region. Combinations including dorsal VP regions are therefore stronger inducers of dorsal mesoderm than combinations including the whole VP region.

DISCUSSION

Identification of tissues

An embryologist familiar with his material is able to recognize quite subtle features of regional organization in the normal intact embryo when the degree of cytological differentiation is still very low. This is because the cells are clumped into tissue masses having characteristic shapes and orientations. However when

such judgement is used to assess the character of tissues or structures in small tissue explants there is a danger of the process becoming too subjective to be easily repeated in other laboratories. We believe that ideally all identifications should be made using reagents which are specific for the corresponding tissue or region in the intact embryo. In this work we have used a monoclonal antibody to keratan sulphate (MZ15) to identify notochord, an antibody to myosin heavy chain (MHC2) to identify muscle, and an antibody to stratum corneum keratin to identify epidermis. The use of these reagents has produced no surprises and we are confident that the histological identification of these tissues in explants and combinations is correctly made.

Although no specific reagents were available we have scored neuroepithelium, mesothelium and erythrocytes by the usual histological criteria. We have refrained, however, from attempting to score endodermal structures since these are poorly differentiated in the culture periods used. Although yolk-filled cells are often counted as endodermal by other workers we feel that they could equally well be any early embryo cell type which had failed to differentiate.

Fate map of animal pole region

The experiments involved fate mapping a 60° solid angle of tissue by orthotopic grafting at three stages, 7½, 8 and 10. During this period the animal pole tissue becomes noticeably thinner, hence the volume of tissue mapped is getting smaller, and it seems as though cells must already be moving in a vegetal direction as they will continue to do during gastrulation. These movements have been observed before and are referred to as pregastrulation movements by Nieuwkoop & Faber (1967).

The effect of this is that the fate map expands from the animal pole. Where the same area of tissue is labelled at different stages, fewer structures become populated the later the stage of labelling. The results show that our piece of tissue spans the epidermis – neural tube boundary at stage 7½–8 but not at stage 10 and indeed in Keller's (1976) fate map of stage 10 this boundary is shown at 45° from the animal pole on the dorsal meridian.

With respect to the origins of the mesoderm it is important to notice that in the early blastula (stage 7½) there is a significant contribution of around 14 % of the animal pole tissue. So experiments on mesoderm induction at this stage need to produce significantly more mesoderm than this to avoid the charge of selection. At stage 8 the contribution to mesoderm has become much lower at 3 %, but is still greater than zero.

Since this is a fate mapping experiment the demonstration that 14 % of animal pole cells enter the mesoderm in normal development in no way shows that they are committed to do so at stage 7½. In view of the fact that no mesoderm at all appears in isolates from this region it is much more likely that this 14 % represent the cells which happen to move furthest towards the equator of the blastula and hence enter the province of the mesoderm inducing signal. This result is in fact the

best evidence that at least some mesodermal induction occurs during normal development.

Mesoderm induction in animal-vegetal combinations

At stages $7\frac{1}{2}$ and 8 the proportion of animal pole derived tissue to become mesoderm in the combinations significantly exceeds that which does so in normal development ($P < 0.01$) but this is no longer the case for stage 10 ($P > 0.05$). When the combinations are compared to the animal pole isolates the formation of mesoderm is not just significantly greater but represents a qualitative difference in the development of the tissue. We therefore conclude that mesoderm induction really occurs and that it is an instructive rather than a permissive interaction.

The FLDx label allows us to say with confidence that the overwhelming majority of mesodermal cells in the combinations were derived from the animal pole component. In this regard we agree with the previous conclusions of Nieuwkoop & Ubbels (1972) who used xenoplastic combinations and combinations in which one component was labelled with [^3H]thymidine. There are however often a few unlabelled cells within the muscle masses and so we cannot at present definitely exclude any 'animalization' of the vegetal tissue. Indeed Heasman, Wylie, Hausen & Smith (1984) have recently shown that animalization of single vegetal pole blastomeres from stage-8 *Xenopus* embryos can occur. When labelled *in situ* the progeny of these blastomeres contribute only to the endoderm, yet if transplanted into the blastocoel of a stage-9 embryo they contribute to all three germ layers.

The main difference between stages $7\frac{1}{2}$ and 8 is that nearly all the induced mesoderm is muscle at stage 8 whereas a wider spectrum of tissues, including in particular notochord, is formed at stage $7\frac{1}{2}$. This raises the question of the nature of the primary response which will be considered in greater detail below.

Times of signalling and competence

The lack of induction at stage 10 might, *prima facie*, be due to a loss of the signal or a loss of the capacity to respond, or both. The heterochronic combinations show that it is in fact the competence of the animal pole region which is lost. This result is the only one of the present series which is in disagreement with those previously obtained (Nakamura *et al.* 1970). We cannot account for this difference but there are two reasons why we prefer our result. Firstly if the signal is not turned off it accounts for the persistence of mesoderm-inducing activity in mesoderm-derived tissues of larvae and adults. Secondly it seems a theoretical necessity that the mesodermal competence should be turned off. During gastrulation the dorsal ectoderm comes under the influence of the neural inducing stimulus from the archenteron roof. Since mesoderm induction is homoeogenetic (*i.e.* induced mesoderm is itself a mesoderm inductor (Kurihara & Sasaki, 1981)) neural induction could not occur at all unless the competence of the ectoderm was altered.

A series of combinations between stage-8 AP and stage 8-, 9- and 10-VP explants was also reported by Boterenbrood & Nieuwkoop (1973) using axolotl

embryos. They found that the ventral vegetal region retained its inductive ability but that the dorsal vegetal region lost its activity. This is more similar to our result in that our AP(7½)–VP(10) combinations gave many ventral type inductions while the AP(7½)–VP(7½) or AP(8)–VP(8) did not. We suspect that the dorsal inducing activity is not actually lost but that the active tissue has migrated away from the vegetal pole in the course of the gastrulation movements and thus is not included in the explants from the later stages.

Regional specificity of induction

We have confirmed the earlier reports that dorsovegetal tissue induces 'dorsal-type' mesoderm containing notochord and large muscle masses while ventro-vegetal tissue induces 'ventrolateral-type' mesoderm containing erythrocytes, mesothelium and a little muscle (Boterenbrood & Nieuwkoop, 1973). As mentioned above the heterochronic combinations AP(7½)–VP(10) also gave many ventral-type inductions. In earlier work on the organization of the mesoderm at stage 10 we have shown that when dorsal and ventral mesodermal tissues are joined the ventral component becomes 'dorsalized' and forms large masses of muscle, although not notochord (Slack & Forman, 1980; Smith & Slack, 1983). We have thus considered 'dorsalization' to be a distinct inductive interaction occurring in normal development after mesoderm induction and before neural induction (Slack, Dale & Smith, 1984), and consider that the response to mesoderm induction must embody the precondition for dorsalization. This means that the initial mesodermal rudiment would contain at least a dorsal and a ventral zone, the latter subsequently becoming subdivided under the influence of the former.

In combinations ventral-type mesoderm would then only be expected to differentiate if the dorsal zone had failed to be induced and in the combination of animal pole tissue with ventrovegetal pole tissue this is because the dorsovegetal region has been deliberately removed.

The primary response to mesoderm induction

The evident regional and temporal specificity of mesoderm induction shows that it is a rather complex process which cannot be explained in terms of a simple morphogen gradient (Weyer, Nieuwkoop & Lindenmayer, 1978). The simplest interpretation is probably that there are two signals, evoking dorsal and ventral mesoderm, which are emitted respectively by dorsal and ventral vegetal regions. Whether the evoked states correspond to specifications for the tissues notochord and blood, or to positional codings higher in the developmental hierarchy than histological tissue types, we cannot at present know. It is necessary to assume that the intermediate mesodermal tissue types (muscle, kidney, or their precursor positional codings) arise by subsequent dorsalization of the ventral territory as referred to above.

This interpretation is sufficient to account for the results of experiments in which animal pole tissue is used as the responding tissue. If we wish to argue that

mesoderm induction occurs in normal development and results in the formation of the equatorial marginal zone then there are certain additional problems.

Firstly it is known that at stage 10 the entire prospective mesoderm lies below the embryo surface (Keller, 1975; Smith & Malacinski, 1983). Outside it in the superficial marginal zone lies suprablastoporal endoderm which becomes archenteron roof (midventrally: posterior archenteron floor) in the course of the gastrulation movements. If this is also true at the earlier stages when mesoderm induction is believed to occur then the implication is that deep and superficial tissues respond differently to the signal. This would not be consistent with the experimental finding that outer as well as inner layer cells from the animal pole region are equally mesodermalized by vegetal tissue (Sudarwati & Nieuwkoop, 1971) or by vegetalizing factor (Asashima & Grunz, 1983).

Secondly, there is the problem of the fate map for the internal subdivisions of the mesoderm. The exact mesoderm fate map before stage 10 is not known but if Keller's (1976) results are back-projected along the involution trajectories we obtain a map approximately as in Fig. 6. If this map shows us the location of the specified states arising from mesoderm induction then we must conclude that the dorsal-type inductions (specified notochord+somite) arise from a weaker signal than the ventral-type (specified mesenchyme+blood) since the prospective regions lie further away from the vegetal signalling area. This seems odd since various authors using vegetalizing factor have claimed to be able to shift the responding tissue from ventral to dorsal by *increasing* the dose or time of contact (Noda, Sasaki & Iyeri, 1972; Grunz, 1983). Of course the fate map alone cannot tell us about the state of commitment or specification in the mesoderm at these early stages (Slack, 1983). The specification map may be less complex than the fate map and in the simplest case mesodermal induction may simply specify a mesodermal rudiment with a dorsoventral polarity. Regionalization could then

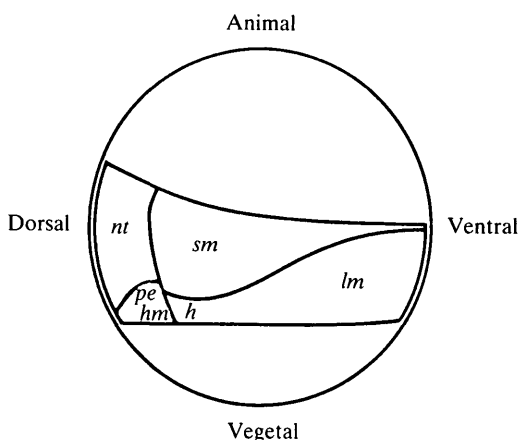


Fig. 6. Fate map of *Xenopus* gastrula mesoderm backprojected onto the blastula. Prospective areas are labelled as follows: *nt*, notochord; *pe*, pharyngeal endoderm; *hm*, head mesoderm; *h*, heart mesoderm; *sm*, somitic mesoderm; *lm*, lateral mesoderm. Based on Keller (1976).

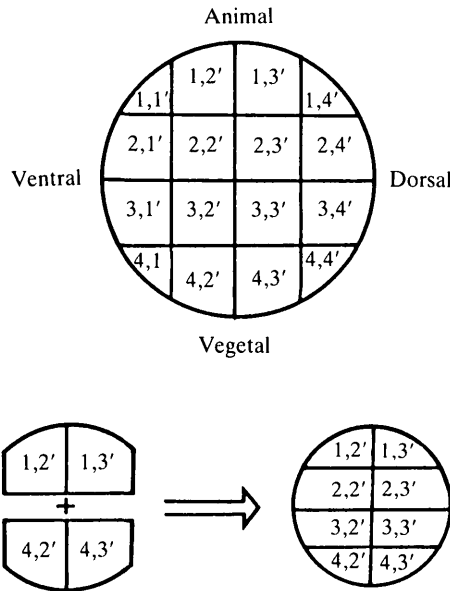


Fig. 7. Formal description of mesoderm induction in the amphibian blastula. Two gradients, one along the animal–vegetal axis the other along the dorso–ventral axis, partition the blastula into territories arbitrarily labelled 1–4 and 1'–4'. Each territory is identified by two codings and left and right sides are mirror images with identical codings. Upon combination of the extreme animal and vegetal territories, the animal–vegetal gradient smooths out discontinuities in the animal–vegetal axis resulting in the reestablishment of intermediate territories. Based on Slack (1983).

be explained by later dorsalization of the ventral rudiment during dorsal convergence.

A third factor which some authors have considered to be a problem for the conception of an obligatory mesoderm induction in normal development is the ability of very early isolates to self differentiate into mesodermal tissues. This has been demonstrated for the marginal zone of blastulae (Nakamura & Matsuzawa, 1967; Nakamura & Takasaki, 1970), for the vegetal four cells of the 8-cell stage (Ruud, 1925; Grunz, 1977; Gurdon *et al.* 1984), and even for fertilized egg fragments containing some equatorial material (Gurdon, Brennan, Fairman & Mohun, 1984). However in all these experiments the isolates contain some animal and some vegetal tissue and so it is quite possible that the induction occurs at a later stage during cultivation.

The complex character of the response suggests that what is being transmitted is not a set of instructions to differentiate into particular tissue types but rather a set of positional codings which are subsequently interpreted as terminal differentiation pathways (Wolpert, 1969). Various authors have proposed double gradient models for the early amphibian embryo (Dalcq & Pasteels, 1937; Yamada, 1950; Toivonen & Saxen, 1955; Slack, 1983) in which one set of

positional codings runs from the vegetal to the animal pole and another from the dorsal to the ventral pole. According to modern terminology, both gradients are capable of smoothing out discontinuities introduced by the experimenter, and do so in such a way that the 'low' end (animal or ventral) becomes promoted to an intermediate value while the 'high' end (vegetal or dorsal) remains as it was. Isolation experiments however suggest that neither gradient is capable of restoring the poles if these are removed (for DV separations see Ruud, 1925; Kageura & Yamana, 1983; Cooke & Webber, 1985). Looked at from this point of view 'mesoderm induction' is the name we give to the regulative behaviour of the vegetal-animal gradient and 'dorsalization' is the name we give to the regulative behaviour of the dorsoventral gradient (Fig. 7). The organizer is the dorsovegetal region of tissue which shares the high positional codings of both gradients and can therefore induce any other combination of codings under the appropriate circumstances.

The issue of whether or not mesoderm induction occurs in normal development then becomes the same as asking how much regional specification has occurred by the end of the postfertilization cytoplasmic movements. If a complete set of AV codings (or the equivalent continuous gradient of a substance) is already in place, then mesoderm induction does not normally occur, whereas if only the extreme values (or the equivalent sources and sinks for a morphogen) are in place then it does normally occur.

It seems unlikely that any further biological experiments will be capable of answering this question and that we shall have to look to biochemistry for significant new developments. Since we already know of a protein which can cause mesoderm induction it seems appropriate to ask what is the stage and regional representation of the endogenous vegetalizing factor. This is now under active investigation and we hope to report some results in a subsequent paper.

REFERENCES

- ASASHIMA, M. & GRUNZ, M. (1983). Effects of inducers on inner and outer gastrula ectoderm layers of *Xenopus laevis*. *Differentiation* **23**, 206–212.
- BORN, J., GEITHE, H. P., TIEDEMANN, H., TIEDEMANN, H. & KOCHER-BECKER, U. (1972). Isolation of a vegetalizing inducing factor. *Z. Physiol. Chem.* **353**, 1075–1084.
- BOTERENBROOD, E. C. & NIEUWKOOP, P. D. (1973). The formation of the mesoderm in urodelean amphibians. V. Its regional induction by the endoderm. *Wilhelm Roux Arch. EntwMech. Org.* **173**, 319–332.
- COOKE, J. & WEBBER, J. A. (1985). Dynamics of the control of body pattern in the development of *Xenopus laevis*. I. Timing and pattern in the development of dorsoanterior and of posterior blastomere pairs, isolated at the 4 cell stage. *J. Embryol. exp. Morph.* **88**, 85–112.
- DALCQ, A. & PASTEELS, J. (1937). Une conception nouvelle des bases physiologiques de la morphogénèse. *Archs Biol.* **48**, 669–710.
- DREYER, C., WANG, W. H., WEDLICH, D. & HAUSEN, P. (1983). Oocyte nuclear proteins in the development of *Xenopus*. In *Current Problems in Germ Cell Differentiation* (ed. A. McLaren & C. C. Wylie), pp. 329–352. Cambridge, England: Cambridge University Press.
- GILOH, H. & SEDAT, J. W. (1982). Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by n-propyl gallate. *Science* **217**, 1252–1255.
- GIMLICH, R. L. & GERHART, J. C. (1984). Early cellular interactions promote embryonic axis formation in *Xenopus laevis*. *Devl Biol.* **104**, 117–130.

- GRUNZ, M. (1977). Differentiation of the four animal and the four vegetal blastomeres of the eight-cell-stage of *Triturus alpestris*. *Wilhelm Roux' Arch. devl Biol.* **181**, 267–277.
- GRUNZ, H. (1983). Change in the differentiation pattern of *Xenopus laevis* ectoderm by variation of the incubation time and concentration of vegetalizing factor. *Wilhelm Roux' Arch. devl Biol.* **192**, 130–137.
- GURDON, J. B., BRENNAN, S., FAIRMAN, S. & MOHUN, T. J. (1984). Transcription of muscle-specific actin genes in early *Xenopus* development. Nuclear transplantation and cell dissociation. *Cell* **38**, 691–700.
- HEASMAN, J., WYLIE, C. C., HAUSEN, P. & SMITH, J. C. (1984). Fates and states of determination of single vegetal pole blastomeres of *X. laevis*. *Cell* **37**, 185–194.
- KAGEURA, H. & YAMANA, K. (1983). Pattern regulation in isolated halves and blastomeres of early *Xenopus laevis*. *J. Embryol. exp. Morph.* **74**, 221–234.
- KELLER, R. (1975). Vital dye mapping of the gastrula and neurula of *Xenopus laevis*. I. Prospective areas and morphogenetic movements of the superficial layer. *Devl Biol.* **42**, 222–241.
- KELLER, R. (1976). Vital dye mapping of the gastrula and neurula of *Xenopus laevis*. II. Prospective areas and morphogenetic movements of the deep layer. *Devl Biol.* **51**, 118–137.
- KIRSCHNER, M. W. & HARA, K. (1980). A new method for local vital staining of amphibian embryos using Ficoll and crystals of Nile Red. *Mikroskopie* **36**, 12–15.
- KURIHARA, K. & SASAKI, N. (1981). Transmission of homoigenetic induction in presumptive ectoderm of newt embryo. *Devl Growth Diffn.* **23**, 361–369.
- NAKAMURA, O. & MATSUZAWA, K. (1967). Differentiation capacity of the marginal zone of the morula and blastula of *Triturus pyrrhogaster*. *Embryologia* **9**, 223–237.
- NAKAMURA, O. & TAKASAKI, H. (1970). Further studies on the differentiation capacity of the dorsal marginal zone in the morula of *Triturus pyrrhogaster*. *Proc. Japan Acad.* **46**, 546–551.
- NAKAMURA, O. & TAKASAKI, H. & ISHIHARA, M. (1970). Formation of the organizer from combinations of presumptive ectoderm and endoderm. I. *Proc. Japan Acad.* **47**, 313–318.
- NAKAMURA, O. & TOIVONEN, S. (1978). *Organizer – A Milestone of a Half-century from Spemann*. Amsterdam, Oxford, New York: Elsevier/North-Holland Biomedical Press.
- NEWPORT, J. & KIRSCHNER, M. (1982). A major developmental transition in early *Xenopus* embryos. I. Characterisation and timing of cellular changes at the midblastula stage. *Cell* **30**, 675–686.
- NIEUWKOOP, P. D. (1969). The formation of mesoderm in Urodelean amphibians. I. Induction by the endoderm. *Wilhelm Roux Arch. EntwMech. Org.* **162**, 341–373.
- NIEUWKOOP, P. D. (1973). The 'organization center' of the amphibian embryo, its origin, spatial organization and morphogenetic action. *Adv. Morphogen.* **10**, 1–39.
- NIEUWKOOP, P. D. & FABER, J. (1967). *Normal Table of Xenopus laevis (Daudin)*. Amsterdam: (North Holland).
- NIEUWKOOP, P. D. & UBBELS, G. A. (1972). The formation of mesoderm in Urodelean amphibians. IV. Quantitative evidence for the purely "ectodermal" origin of the entire mesoderm and of the pharyngeal endoderm. *Wilhelm Roux Arch. devl Biol.* **169**, 185–199.
- NODA, S., SASAKI, N. & IYEIRI, S. (1972). Time dependence of the inductive effect of bone marrow extracts. I. Alterations of the contact between the inductor and the ectoderm. *Zool. Mag. (Jap.)* **81**, 59–62.
- RUUD, G. (1925). Die Entwicklung isolierter Keimfragmente frühester Stadien von *Triton taeniatus*. *Wilhelm Roux Arch. EntwMech. Org.* **105**, 209–293.
- SCHWARTZ, W., TIEDEMANN, H. & TIEDEMANN, H. (1981). High performance gel permeation chromatography of proteins. *Mol. Biol. Rep.* **8**, 7–10.
- SLACK, J. M. W. (1983). *From Egg to Embryo. Determinative Events in Early Development*. Cambridge: Cambridge University Press.
- SLACK, J. M. W. (1984). Regional biosynthetic markers in the early amphibian embryo. *J. Embryol. exp. Morph.* **80**, 289–319.
- SLACK, J. M. W., DALE, L. & SMITH, J. C. (1984). Analysis of embryonic induction using cell lineage markers. *Phil. Trans. Roy. Soc. Lond. B.* **307**, 331–336.
- SLACK, J. M. W. & FORMAN, D. (1980). An interaction between dorsal and ventral regions of the marginal zone in early amphibian embryos. *J. Embryol. exp. Morph.* **56**, 283–299.

- SMITH, J. C. & MALACINSKI, G. M. (1983). The origin of mesoderm in an anuran, *Xenopus laevis*, and a Urodele, *Ambystoma mexicanum*. *Devl Biol.* **98**, 250–254.
- SMITH, J. C. & SLACK, J. M. W. (1983). Dorsalization and neural induction: properties of the organizer in *Xenopus laevis*. *J. Embryol. exp. Morph.* **78**, 299–317.
- SMITH, J. C. & WATT, F. M. (1985). Biochemical specificity of the *Xenopus* notochord. *Differentiation* (in press).
- SUDARWATI, S. & NIEUWKOOP, P. D. (1971). Mesoderm formation in the anuran *Xenopus laevis* (Daudin). *Wilhelm Roux Arch. EntwMech. Org.* **166**, 189–201.
- TIEDEMANN, H. & TIEDEMANN, H. (1959). Versuche zur Gewinnung eines mesodermalen Induktionsstoffes aus Hühnerembryonen. *Hoppe Seylers Zeitschrift für physiologische Chemie.* **314**, 156–176.
- TOIVONEN, S. & SAXEN, L. (1955). The simultaneous inducing action of liver and bone-marrow of the guinea-pig in implantation and explantation experiments with embryos of *Triturus*. *Expl Cell Res. (Suppl.)* **3**, 346–357.
- WEYER, C. J., NIEUWKOOP, P. D. & LINDENMEYER, A. (1978). A diffusion model for mesoderm induction in amphibian embryos. *Acta Biotheor.* **26**, 164–180.
- WOLPERT, L. (1969). Positional information and the spatial pattern of cellular differentiation. *J. theoret. Biol.* **25**, 1–47.
- WU, Y. & RHEINWALD, J. G. (1981). A small (40Kd) keratin filament protein made by some cultured human squamous cell carcinomas. *Cell* **25**, 627–635.
- YAMADA, T. (1950). Regional differentiation of the isolated ectoderm of the *Triturus* gastrula induced through a protein extract. *Embryologia* **1**, 1–20.
- ZANETTI, M., RATCLIFFE, A. & WATT, F. M. (1985). Two subpopulations of differentiated chondrocytes identified with a monoclonal antibody to keratan sulphate. *J. Cell Biol.* **101**, 53–59.

(Accepted 16 April 1985)