

## The specification of heart mesoderm occurs during gastrulation in *Xenopus laevis*

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### Summary

The establishment of heart mesoderm during *Xenopus* development has been examined using an assay for heart differentiation in explants and explant combinations in culture. Previous studies using urodele embryos have shown that the heart mesoderm is induced by the prospective pharyngeal endoderm during neurula and postneurula stages. In this study, we find that the specification of heart mesoderm must begin well before the end of gastrulation in *Xenopus* embryos. Explants of prospective heart mesoderm isolated from mid- or late neurula stages were capable of heart formation in nearly 100% of cases, indicating that the specification of heart mesoderm is complete by midneurula stages. Moreover, inclusion of pharyngeal endoderm had no statistically

significant effect upon either the frequency of heart formation or the timing of the initiation of heartbeat in explants of prospective heart mesoderm isolated after the end of gastrulation. When the superficial pharyngeal endoderm was removed at the beginning of gastrulation, experimental embryos formed hearts, as did explants of prospective heart mesoderm from such embryos. These results indicate that the inductive interactions responsible for the establishment of heart mesoderm occur prior to the end of gastrulation and do not require the participation of the superficial pharyngeal endoderm.

Key words: heart induction, pharyngeal endoderm, gastrula, *Xenopus*.

### Introduction

The heart mesoderm of vertebrate embryos is thought to arise through an inductive interaction between the pharyngeal endoderm and the paired regions of anterior lateral mesoderm. Evidence for the induction of heart mesoderm by pharyngeal endoderm arises from a variety of experimental embryological studies (e.g. Balinsky, 1939), as well as analyses of a mutation affecting this inductive interaction (Lemanski *et al.* 1979) and observations of human teratology (Hommes, 1957). This work is reviewed in Jacobson & Sater (1988). In brief, studies of heart differentiation in explants of prospective heart mesoderm isolated from urodele embryos at various stages throughout neurulation indicate that heart induction occurs during early to midneurula stages (Jacobson & Duncan, 1968). Removal of the entire endoderm from urodele embryos at various stages during neurula and postneurula development prevents heart formation in a large percentage of cases (Balinsky, 1939; Nieuwkoop, 1947; Chuang & Tseng, 1957; Jacobson & Duncan, 1968). These findings suggest that inductive interactions critical to heart formation continue well into postneurula development.

Further evidence for inductive interactions during

postneurula stages is provided by the work of Lemanski *et al.* (1979) on the *cardiac lethal* mutation in axolotls. In embryos homozygous for this mutation, heart development is arrested at early stages of heart morphogenesis, and hearts fail to beat (Humphreys, 1972). Explant recombinations between mutant and wild-type tissues demonstrate that the phenotypic lesion resulting from this mutation resides within the endoderm; mutant heart mesoderm is able to develop normally when combined with wild-type endoderm (Lemanski *et al.* 1979). Presumably, the mutation disrupts the production of signals by the endoderm that are required for normal heart development.

Some of the previous studies (e.g. Ekman, 1921; Lemanski *et al.* 1979) fail to distinguish between interactions of heart mesoderm with pharyngeal endoderm that are essential for the initial acquisition of heart-forming potency, and interactions between these tissues that merely support the induced mesoderm along its course of differentiation. In recent years, at least two levels of developmental commitment have been defined (Slack, 1983). The specification of a tissue represents acquisition of a level of developmental commitment that is sufficient for the formation of a given organ when the tissue is cultured in isolation. A greater level of

developmental commitment is represented by the ability of a tissue to undergo organ formation when transplanted to a new site on the embryo, since under these conditions, the tissue is exposed to novel environmental influences; Slack (1983) refers to the acquisition of this latter level of developmental commitment as the determination of the tissue. Further interactions occur after determination that support complete differentiation, and these interactions are called 'formative influences' (Holtfreter & Hamburger, 1955; Mangold, 1957; Jacobson, 1961).

In this study, we assay heart formation in explants; therefore, we are studying heart specification. The appearance of specification is generally considered one of the early steps along the progression of developmental commitment that is necessary for organ formation *in vivo*. As such, it should reflect primarily the tissue interactions responsible for the acquisition of heart-forming potency, rather than more subsidiary tissue interactions, which merely support subsequent differentiative events.

This study examines the role of superficial pharyngeal endoderm in the specification of heart mesoderm in embryos of *Xenopus laevis*. The results indicate that the specification of heart mesoderm in *Xenopus* embryos is complete shortly after the beginning of neurulation, much earlier than has been observed in urodeles. Thus, the induction of heart mesoderm occurs during gastrulation. In addition, after the beginning of gastrulation, interactions with the superficial pharyngeal endoderm are not essential for the specification of heart mesoderm.

## Materials and methods

### Embryos

*Xenopus laevis* embryos were obtained from natural matings. Adult *Xenopus* were induced to mate by injection of chorionic gonadotropin (HCG) (Sigma Chemicals, St Louis, MO), according to the following schedule: males received two injections of 150 international units (i.u.) HCG in sterile 9% NaCl 32 h and 8 h before they were placed in the mating tank; females received one injection of 500 i.u. when they were placed in the mating tank, preceded by one injection of 250 i.u. 6 to 8 h earlier. Pairs of adults were allowed to mate overnight. The following morning, embryos were collected and dejellied by incubation for 5 min in 2.5% cysteine HCl, pH 7.8. Embryos were then washed extensively in 10% Holtfreter's solution (Jacobson, 1967) and cultured in 10% Holtfreter's solution at 17°C.

### Preparation and culture of explants

Embryos were transferred to sterile Niu-Twitty solution, an inorganic saline medium (Jacobson, 1967), and their vitelline layers were removed with electrolytically sharpened watchmaker's forceps. Embryos were transferred for microsurgery to fresh sterile Niu-Twitty solution in a Petri dish containing a layer of 2% agar. Explants containing mesoderm, endoderm, and ectodermal epithelium were removed with electrolytically sharpened tungsten needles (Jacobson, 1967).

When experiments called for the separation of mesoderm and endoderm, embryos were dissected in the presence of

0.01% trypsin (Sigma Type IX) in Niu-Twitty solution. This treatment permits the separation of tissue layers while preserving cohesiveness within each cell layer (Slack, 1984). Explants isolated in the presence of trypsin were subsequently incubated in 0.02% soybean trypsin inhibitor (SBTI; Sigma Type II-S) in Niu-Twitty solution for 2–4 min.

Explants were cultured in hanging drops of Niu-Twitty solution plus 50 i.u. ml<sup>-1</sup> penicillin and 50 µg ml<sup>-1</sup> streptomycin (pen/strep) at 17°C. Several small drops of culture medium were placed on coverslips that had previously been boiled for several minutes in distilled water, air-dried, and baked at approximately 160°C for two hours. Explants were then transferred by mouth pipette into the drops. Each coverslip was then attached to an upside-down flat-bottomed well slide by a bead of vaseline drawn out around the outside of the well. In this configuration, the drops stand upright in an airtight chamber, and the embryonic tissues are allowed to heal without being exposed to the air–water interface at the surface of the drop. After 36–48 h, the cultures were inverted to permit observation of the culture in the now-hanging drop through the coverslip. Embryos were initially viewed with a dissecting microscope; after the fourth or fifth day, when explants were sufficiently translucent to permit observation under transillumination, explants were observed with a compound microscope.

### Microsurgical operations and culture of gastrula embryos

Microsurgical operations on gastrula embryos were performed using eyebrow hair knives (Hardin & Keller, 1988). Operations were performed in Niu-Twitty solution on a bed of 2% agar. Extirpation of the bottle cells and suprablastoporal endoderm from gastrula embryos could be accomplished without the use of trypsin. The bottle cells and suprablastoporal endoderm to be removed were separated from the surrounding tissue with an eyebrow hair knife. Thus loosened, the layer of bottle cells and suprablastoporal endoderm was grasped at one corner with watchmaker's forceps and gently teased from the embryo with an eyebrow hair knife. Following the operations, embryos were allowed to heal for at least 30 min at room temperature and then transferred to Petri dishes containing Niu-Twitty solution plus pen/strep. Embryos were subsequently cultured at 17°C.

### Histology

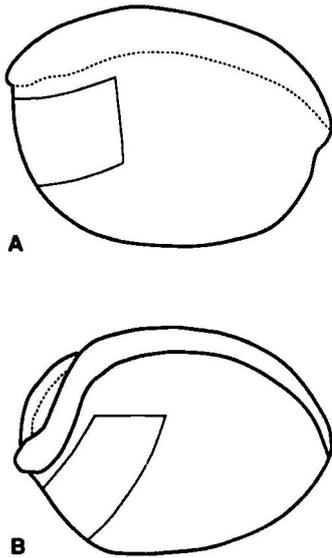
Embryos were fixed in Kahle's solution (Jones, 1966) for at least 24 h at 4°C. Embryos were then dehydrated through an ethanol–butanol series, embedded in Para-plast (Scientific Products), and sectioned at 8 µm. Sections were collected on albumin-coated slides and dewaxed through a xylene–ethanol series. They were then stained with 0.2% neutral red and counterstained with 0.1% Janus green (Jones, 1966).

## Results

### Effects of pharyngeal endoderm on heart formation in mesodermal explants

Explants of presumptive heart mesoderm with and without the underlying pharyngeal endoderm were removed at various stages during neurulation and cultured within vesicles of ectodermal epithelium using the hanging drop culture system described above. The region of tissue included in these explants is shown in Fig. 1.

Explants were viewed initially on the third day of

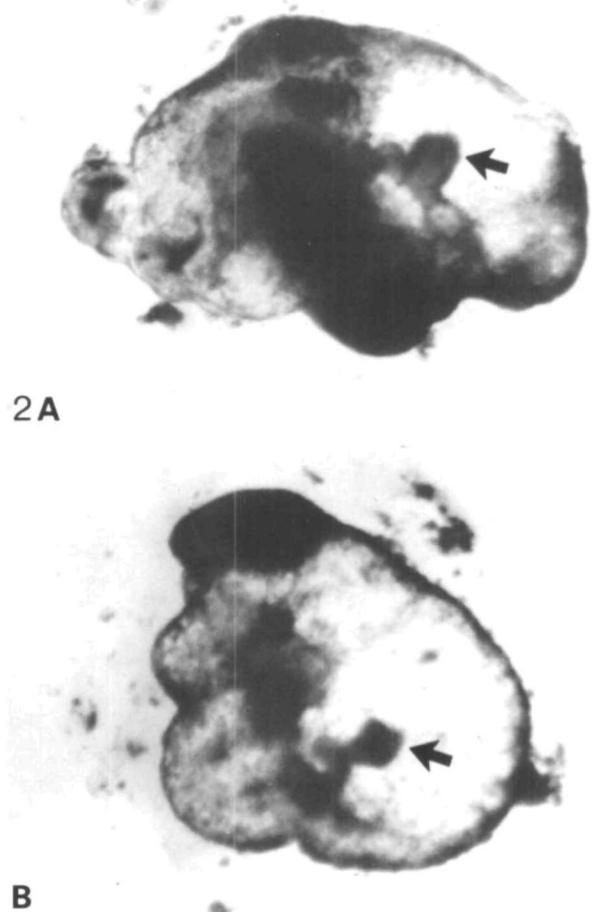


**Fig. 1.** Explants of heart mesoderm during neurulation. Anterior is at the left. (A) Tissue explanted from embryos at stages 12.5 to 13. Explants include anterior lateral tissue up to anterior midline. (B) Tissue explanted from embryos at stages 14 to 19. Explants include anterior lateroventral tissue up to ventral midline. Explants shown in (A) and (B) include the same region of tissue. The difference between (A) and (B) reflects the morphogenetic movements occurring throughout the embryo during neurulation, especially the ventrally-directed movement of the prospective heart mesoderm at the anterior lateral edge of the mesodermal mantle. All explants included mesoderm and epidermis in the presence or absence of the underlying endoderm.

culture, and daily or every other day thereafter. By the third day, explants had healed into a ciliated vesicle. Dissociated cells were often present, and explants containing endoderm occasionally showed exposed regions of endoderm. Over subsequent days, the vesicular explants became inflated and translucent, and eventually, as more of the yolk was metabolized, transparent. Explant cultures were observed over a period of at least two weeks, although explants could persist for up to four or five weeks.

Beating hearts were visible in inflated areas within the explant as cylindrical tissue undergoing the regular peristaltic contractions characteristic of myocardium. In better-developed hearts, major regions of the heart were delineated by the 'looping and bending' morphology of the heart tube. Typical explants of mesoderm alone and in combination with endoderm are shown in Fig. 2.

Explants of mesoderm plus pharyngeal endoderm generally form larger hearts that frequently display the characteristic 'looping and bending' morphology; occasionally, valves are visible in these hearts. In contrast, hearts formed by mesodermal explants in the absence of endoderm are smaller and thinner, often without the large inflated lumen. Sometimes explants of mesoderm alone contain multiple foci of beating tissue (Fig. 3). Usually, these foci include one larger, visibly differen-



**Fig. 2.** Explants of heart-forming regions from stage 19 embryos after seven days in culture. Explants are enclosed within epithelial vesicles. (A) Mesoderm + endoderm. (B) Mesoderm only. Both explants have formed beating hearts (indicated by arrows).

tiated region easily recognizable as a heart, accompanied by one or more small tissue clumps showing no obvious morphological differentiation and exhibiting a characteristic 'heartbeat' pattern of contractility. In some cases, multiple foci would beat synchronously; in others, the foci would beat independently of one another. The smaller foci were visible only through the compound microscope.

Frequently, several other organs or structures differentiated in these explant cultures. Eyes surrounded by pigmented retinal epithelia, otocysts, and melanocytes were often observed in explants removed from late gastrula to midneurula embryos. Nearly all explants removed from neurulae included mesenchyme and mesentery, as well as a cement gland, which forms immediately anterior to the heart in intact embryos. Occasionally, pronephric tubules were also observed in these cultures.

The frequency of beating heart formation in cultures of mesoderm with or without the underlying endoderm is shown in Fig. 4. All explants developed within vesicles of ectodermal epithelium. Both sets of explants display an increase in the frequency of beating heart

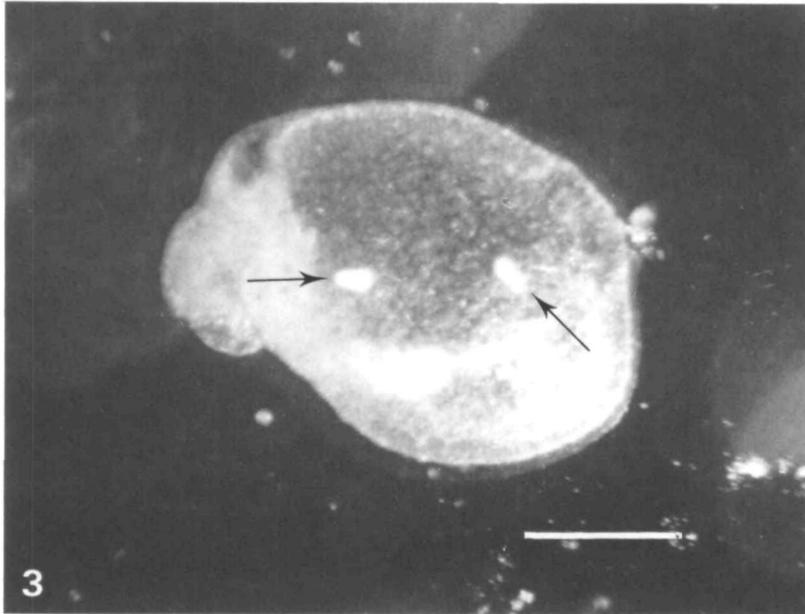


Fig. 3. Explant of mesoderm from a stage 13 embryo after 5 days in culture. Arrows point to foci of beating tissue; bar, 0.4 mm.

formation when removed from successively older stages between the end of gastrulation at stage 12.5 and early neurula stage 14. Mesodermal explants removed from embryos older than stage 14 undergo beating heart formation in nearly 100% of cases, regardless of whether or not pharyngeal endoderm is included in these explants. This result indicates that the specification of heart mesoderm is complete by stage 14.

Explants of mesoderm from stage 12.5 embryos form beating hearts in over 40% of cases when cultured in the absence of endoderm. In contrast, explants containing both mesoderm and endoderm from stage 12.5 embryos form beating hearts in approximately 70% of cases. The difference in the frequency of heart formation in mesodermal explants with and without endo-

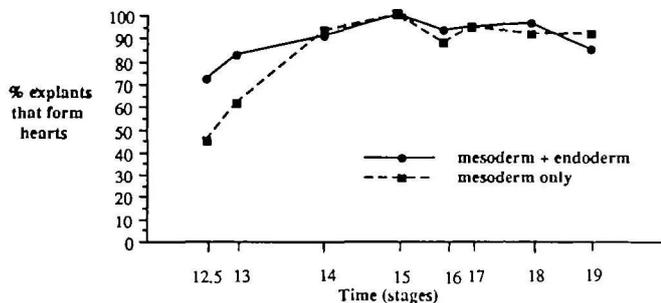


Fig. 4. The frequency of heart formation in mesodermal explants in the presence and absence of endoderm. Explants were removed at stages ranging from the end of gastrulation to the end of neurulation. A chi-squared test was used to determine that differences in the frequency of heart formation in the presence and absence of endoderm are statistically insignificant at all stages examined. Sample sizes for explants of mesoderm + endoderm: stage 12.5,  $n = 18$ ; stage 13,  $n = 23$ ; stage 14,  $n = 32$ ; stage 15,  $n = 17$ ; stage 16,  $n = 27$ ; stage 17,  $n = 21$ ; stage 18,  $n = 23$ ; stage 19,  $n = 20$ . Sample sizes for explants of mesoderm: stage 12.5,  $n = 22$ ; stage 13,  $n = 37$ ; stage 14,  $n = 29$ ; stage 15,  $n = 17$ ; stage 16,  $n = 25$ ; stage 17,  $n = 20$ ; stage 18,  $n = 39$ ; stage 19,  $n = 24$ .

derm is not statistically significant ( $P > 0.1$ ), as shown by the chi-squared test using Yates' correction for continuity. Inclusion of endoderm has no statistically significant effect on the amount of time required to initiate heartbeat at any of the stages tested (data not shown).

These results demonstrate that the specification of heart mesoderm is well under way by the end of gastrulation. It is not possible to examine the specification of the anterior lateral mesoderm prior to stage 12.5, since this is the earliest stage at which all three germ layers are distinct (Nieuwkoop & Faber, 1967). Thus, the anterior lateral mesoderm cannot be isolated using microsurgery.

#### *Removal of superficial pharyngeal endoderm does not prevent heart formation*

The high frequency of heart formation in mesodermal explants from neurula stages cultured in the absence of endoderm suggests that the induction of heart mesoderm must occur prior to the end of gastrulation. At the beginning of gastrulation at stage 10, there are two regions of prospective dorsoanterior endoderm: the superficial pharyngeal endoderm, which includes the dorsal and dorsolateral bottle cells (Keller, 1976, 1981), and a region of deep dorsal endoderm that lies internal to the mesodermal deep zone. The fate of this deep endoderm is unknown; it may contribute to the pharyngeal and branchial endoderm, and/or it may come to rest at the anterior edge of the large endodermal mass that constitutes the archenteron floor posterior to the branchial region. The deep endoderm is in contact with the head and heart mesoderm prior to the onset of gastrulation, and it is not possible to separate these tissues using microsurgery. The superficial pharyngeal endoderm, however, is an epithelial sheet and can easily be removed from early gastrula embryos.

It is not known whether these two regions of dorso-

**Table 1.** The effects of the removal of bottle cells and suprablastoporal endoderm on heart formation *in vivo*

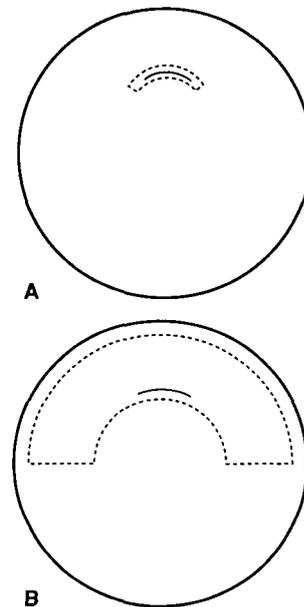
Tissue removed	Stage at operation	No. of embryos with hearts	Total no. of embryos
Dorsal bottle cells	10–10·25	4	4
Dorsal and dorsolateral bottle cells	10–10·25	6	6
Dorsolateral bottle cells	10–10·25	2	2
Ventral bottle cells	11	6	6
Dorsal and dorsolateral bottle cells + suprablastoporal endoderm	10–10·25	21	22

anterior endoderm have equivalent inducing capabilities. Either or both may participate in the induction of heart mesoderm, although the role of the superficial pharyngeal endoderm, which first comes into contact with the prospective heart mesoderm during gastrulation, may differ from that of the deep endoderm, which is in continuous contact with the prospective heart mesoderm. To determine whether interactions with the superficial endoderm are necessary for the induction of heart formation during gastrulation, the superficial pharyngeal endoderm was removed at the beginning of gastrulation.

The dorsal bottle cells, whose formation represents the first external sign of gastrulation, give rise to the superficial pharyngeal endoderm; they have been shown to form the lining of the anterior archenteron, extending as far posterior as the liver diverticulum (Keller, 1981). A diagram showing their removal is shown in Fig. 5. Bottle cells are joined very tightly at their apical ends; thus, once they have been released from their normally-shaped neighbors, the bottle cells can be removed as a cohesive cell mass.

Dorsal bottle cells were removed from stage 10–10·25 embryos. Other series of operations included removal of dorsolateral bottle cells, removal of bottle cells from the entire dorsal half of the embryo at stage 10–10·25, and removal of ventral bottle cells from stage 11 embryos. A summary of the results is presented in Table 1. Removal of dorsal and dorsolateral bottle cells resulted in embryos with diminished anterior structures at late neurula and postneurula stages. Embryos from which the ventral bottle cells had been removed showed no apparent defects at late neurula or postneurula stages. When observed at stage 40, all embryos displayed beating hearts.

This result suggests that the superficial pharyngeal endoderm is not required after the onset of gastrulation for heart formation *in vivo*. It is possible, however, that in the absence of dorsal bottle cells, the endoderm lining the posterior archenteron roof migrates forward to replace the dorsal bottle cells. In such an event, this more posterior endoderm might substitute for the derivatives of the dorsal bottle cells in interactions with the prospective heart mesoderm. Prior to gastrulation, the endoderm destined to line the posterior archenteron roof forms an external layer immediately above the dorsal bottle cells; this suprablastoporal endoderm involutes around the dorsal lip of the blastopore to form the roof and walls of the archenteron posterior to the

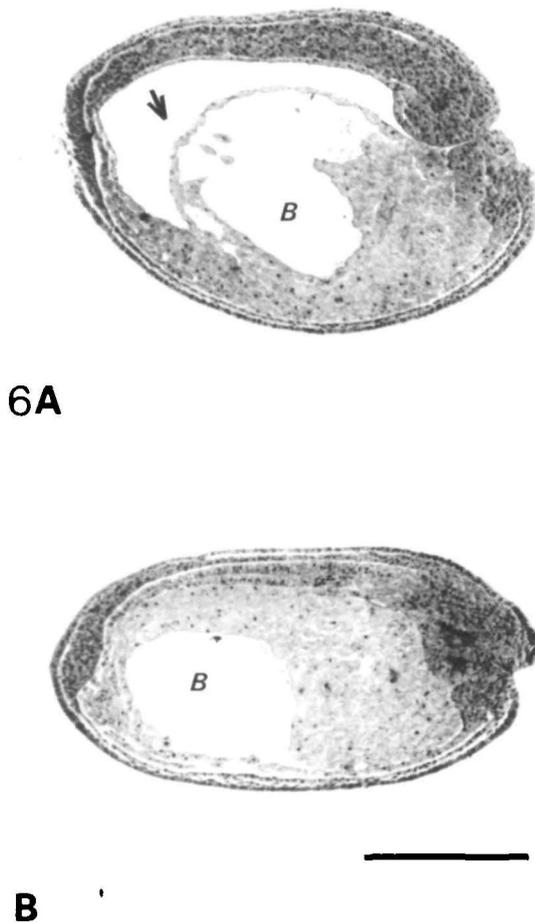


**Fig. 5.** Extirpation of superficial pharyngeal endoderm at the beginning of gastrulation. Broken lines delimit excised tissue. (A) Extirpation of dorsal bottle cells. (B) Extirpation of bottle cells and suprablastoporal endoderm from the entire dorsal half of the embryo.

liver diverticulum (Keller, 1976, 1981). Both the suprablastoporal endoderm and the dorsal bottle cells are easily distinguished by their smaller size and relatively non-yolky appearance from the large, yolky endoderm cells that constitute the archenteron floor.

The dorsal bottle cells and the suprablastoporal endoderm were removed from early gastrula (stage 10–10·25) embryos, as shown in Fig. 5, to determine whether cells destined to give rise to the posterior archenteron can replace the dorsal bottle cells. With one exception, all embryos subjected to this operation eventually formed beating hearts, although in 4 of 21 cases, the appearance of a beating heart was delayed by one day compared to intact controls. The single embryo in which a beating heart was not visible exhibited highly abnormal development: the heart region was opaque and appeared to be occluded with cellular debris. A summary of the results obtained from removal of the dorsal bottle cells and the suprablastoporal endoderm is presented in Table 1.

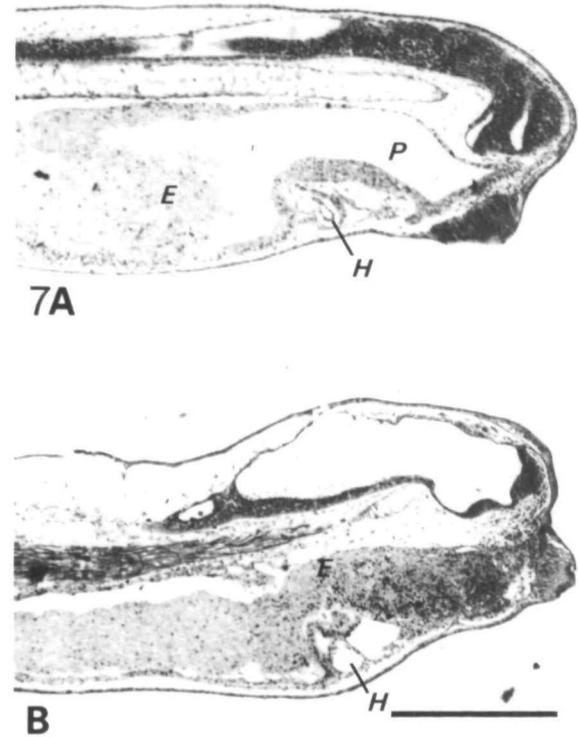
Midsagittal sections of early neurula (stage 14) embryos lacking dorsal bottle cells and the suprablastoporal endoderm, and intact early neurula embryos are



**Fig. 6.** Midsagittal view of intact embryos and embryos lacking derivatives of the dorsal bottle cells and suprablastoporal endoderm at stage 14. The blastopore is to the right. (A) Intact embryo. The archenteron has expanded (indicated by arrow). (B) Operated embryo. The archenteron is not visible; B, blastocoel; bar, 0.5 mm.

shown in Fig. 6. Embryos subjected to the operation show no sign of an archenteron, whereas the archenteron is clearly visible in the control embryos. Expansion of the anterior archenteron, apparent in the control embryos, has not occurred in embryos subjected to the operation. The expansion of the anterior archenteron, which is initiated at stage 12 and is complete by stage 13.5 (Nieuwkoop & Faber, 1967), is due to changes in cell shape by the dorsal and dorsolateral bottle cells (Keller, 1981; Hardin & Keller, 1988). In addition, the anterior ventral region of the operated embryo is somewhat diminished in comparison with that of the control embryo.

Fig. 7 shows midsagittal sections of experimental and intact embryos at stage 35/36. The hearts are clearly visible in the midsagittal sections of both embryos. However, the organization of pharyngeal tissue, dorsal and slightly anterior to the heart, is very different in these embryos. Pharyngeal pouches can be observed in



**Fig. 7.** Midsagittal sections of operated and intact embryos at stage 35/36. (A) Intact embryo. (B) Operated embryo; bar, 0.8 mm. (C) Anterior region of intact embryo. The pharyngeal cavity, P, is visible; bar, 0.5 mm. (D) Anterior region of operated embryo. The pharyngeal cavity is occluded by endoderm, E, from more posterior regions.

the control embryo, while the experimental embryo lacks pharyngeal pouches. Instead, the experimental embryo exhibits a large endodermal mass extending along the floor of the pharyngeal region back into the trunk of the embryo, with no histological evidence of differentiation along its length.

Embryos from which the superficial pharyngeal endoderm has been removed lack another derivative of pharyngeal endoderm, the thyroid anlage. In intact embryos, the thyroid anlage appears as a ventrally and caudally directed protrusion from the pharyngeal floor at the level of the first pharyngeal pouch. No such structure is visible in embryos lacking the superficial pharyngeal endoderm. Furthermore, the head regions of experimental embryos are considerably narrower than those of the control embryos. This difference results from the fact that removal of the superficial pharyngeal endoderm prevents the expansion of the anterior archenteron, as noted earlier. More posterior regions of the embryos are virtually identical.

Again, these results indicate that, after the beginning of gastrulation, the superficial pharyngeal endoderm is not necessary for the induction of heart mesoderm. Alternatively, heart formation in embryos from which the dorsal bottle cells and the superficial blastoporal

endoderm have been removed may also be due to regulation within the endoderm following gastrulation. In other words, endodermal cells that are normally posterior to the pharyngeal region may move anteriorly following gastrulation and induce heart formation when they come into contact with the prospective heart mesoderm in the anterior ventrolateral region.

This possibility was tested by removing the dorsal/dorsolateral bottle cells and suprablastoporal endoderm at the beginning of gastrulation (stage 10 to 10.25). Embryos subjected to this operation were then allowed to complete gastrulation to the equivalent of stages 12.5 to 13. Explants of the prospective heart mesoderm and the overlying epidermis were then removed and placed in hanging drop cultures, as described earlier. Explants were also prepared that included the underlying endoderm, as well as the prospective heart mesoderm and the epidermis. The frequency of heart formation in mesodermal explants from embryos lacking dorsal bottle cells and suprablastoporal endoderm was compared with the frequency of heart formation in mesodermal explants from intact embryos at the same stage.

A summary of the results presented in Table 2 shows no statistically significant difference in the frequency of heart formation between explants from these two operations. When mesodermal explants are prepared from embryos at stage 12.5 that lack the bottle cells and suprablastoporal endoderm, the frequency of heart formation is 47%, compared with 45% in explants from intact embryos. At stage 13, the frequency of heart formation in the absence of endoderm is 69% for explants from embryos lacking bottle cells and suprablastoporal endoderm, and 62% for explants from intact embryos. Explants of mesoderm with endoderm from embryos lacking bottle cells and suprablastoporal endoderm formed beating hearts in 87% of cases, while similar explants from intact embryos formed beating hearts in 82% of cases. These results indicate that removal of the superficial pharyngeal endoderm does not measurably affect the specification of heart mesoderm by the end of gastrulation.

## Discussion

The principal findings of this work are twofold. First, processes governing the specification of the heart meso-

derm in *Xenopus* embryos are under way during gastrulation. Second, the superficial pharyngeal endoderm is not required for the specification of heart mesoderm during gastrulation.

The specification of heart mesoderm occurs earlier in *Xenopus* development than it does during the development of any of the urodeles examined in previous studies. In *Xenopus* embryos, specification of the heart mesoderm is complete in 100% of cases by early neurula stages. In contrast, the specification of heart mesoderm in the urodele *Taricha torosa* is complete in only 10% of cases by early neurula stages (Jacobson & Duncan, 1968). Several studies of heart development in urodeles have demonstrated that heart formation *in vivo* requires the presence of endoderm during neurula and postneurula stages (Balinsky, 1939; Chuang & Tseng, 1957; Nieuwkoop, 1947; Jacobson, 1960; Jacobson & Duncan, 1968), suggesting that induction of heart formation by pharyngeal endoderm continues well past the end of neurulation. An alternative explanation for these latter observations is that continued induction is necessary to overcome the effects of interactions with the neural plate and neural folds that suppress heart formation (Jacobson & Duncan, 1968); these suppressive interactions are avoided by the use of explant cultures to assay developmental commitment.

This difference in the timing of heart specification between *Xenopus* and several urodeles may reflect a basic difference between anuran and urodele development. Perhaps in *Xenopus* embryos, the prospective heart mesoderm is in more extensive contact at an earlier stage in comparison with urodele embryos. Unfortunately, studies that would permit a comparison with other anuran species are not available.

On the other hand, it may reflect a shift in the timing of regional specification events during *Xenopus* development, compared with the development of other amphibians. At a given temperature, *Xenopus* embryos develop far more rapidly than do embryos of the urodeles, e.g. *Taricha*, *Ambystoma*, and *Triton*, in which the establishment of heart mesoderm has been examined. In rapidly-developing embryos, regional specification and the acquisition of developmental commitment may occur at younger stages than they do in slower-developing species. For example, commitment to characteristically dorsal developmental pathways apparently occurs relatively early during the course of *Xenopus* development. In embryos exhibiting a com-

**Table 2.** The effects of the removal of bottle cells and suprablastoporal endoderm on the specification of heart mesoderm

	No. of explants that form hearts	Total no. of explants	% of explants that form hearts	% explants from intact embryos that form hearts
<i>Mesoderm only</i>				
Explant at st. 12.5	9	19	47%	45%
Explant at st. 13	22	32	69%	62%
<i>Mesoderm + endoderm</i>				
Explant at st. 12.5	N/D			
Explant at st. 13	13	15	87%	83%

mon, naturally-occurring variant of the normal cleavage pattern, determination of dorsal mesoderm occurs during midcleavage stages (Gimlich, 1986). Dorsoventral differences in the degree of commitment to ectodermal differentiative pathways have been shown to arise during cleavage stages (London *et al.* 1988) or early gastrula stages (Sharpe *et al.* 1987).

Our results also indicate that the presence of pharyngeal endoderm in explants of heart mesoderm from late gastrula and early neurula embryos does not significantly affect the timing of heart formation in these cultures. This finding differs markedly from those of Jacobson & Duncan (1968), who report that in explants of heart mesoderm from midneurula-stage *Taricha torosa* embryos, the most pronounced effect of pharyngeal endoderm on heart formation is an increase in the rate of heart formation, as assayed by the initiation of heartbeat. The difference between the present findings and theirs may simply reflect the determination of heart mesoderm at a relatively early stage in *Xenopus* embryos. Alternatively, this inability to detect a decrease in the amount of time required for the initiation of heartbeat may stem from the relatively rapid rate of *Xenopus* development. These cultures were monitored at intervals of one day; small decreases in the amount of time required for the initiation of heartbeat might be apparent only through more frequent observations.

Following gastrulation, pharyngeal endoderm does appear to play an important role in supporting heart morphogenesis, however. Mesodermal explants lacking endoderm occasionally showed multiple foci of beating tissue. These multiple foci were never observed in mesodermal explants that also included endoderm. While such multiple foci of beating tissue could conceivably arise as an artifact of the trypsin treatment or the microsurgical procedures used to separate the presumptive heart mesoderm from the pharyngeal endoderm, this seems unlikely. Multiple beating foci were never observed in explants of presumptive heart mesoderm in combination with pharyngeal endoderm, even though these explants were also removed in the presence of trypsin (albeit the heart mesoderm in these explants was not exposed directly to trypsin). In addition, explants of anterior lateral mesoderm isolated from postneurula embryos often formed hearts, but did not exhibit multiple beating foci despite the fact that they were isolated in the same manner (Sater, unpublished observations).

Hearts that formed in the absence of pharyngeal endoderm were smaller and not as well-developed as hearts that developed in the presence of pharyngeal endoderm. Clearly, the pharyngeal endoderm contributes to heart morphogenesis in some way. In early gastrulae, the prospective heart mesodermal regions appear as a loose crawling population of cells in the dorsolateral regions of the deep zone (Gerhart & Keller, 1986). This noncohesive tissue organization remains unchanged at least throughout the anteriorward migration of the mesodermal mantle during gastrulation, and probably even longer. Heart formation *in vivo* involves the ventralward migration of the paired

dorsolateral heart mesodermal regions which then fuse at the ventral midline. Thus, the substratum upon which the prospective heart mesodermal cells move during gastrulation and neurulation may direct or support this mesodermal migration. The pharyngeal endoderm may be involved in organizing this substratum.

The results presented here support the contention that events responsible for the specification of heart mesoderm occur prior to the end of gastrulation. Interactions between the presumptive heart mesoderm and the pharyngeal endoderm during later stages do not have a significant quantitative effect upon either the frequency of heart formation or the amount of time required for heart formation. However, pharyngeal endoderm may enhance heart morphogenesis during subsequent stages by assisting the migration of the heart mesoderm.

#### *Superficial pharyngeal endoderm is not required for heart induction*

Unfortunately, the experimental design used to investigate heart specification during neurulation cannot be used to determine which tissue interactions during gastrulation are necessary for heart formation, because the prospective heart mesoderm cannot be isolated from neighboring tissues prior to the end of gastrulation (Nieuwkoop & Faber, 1967). It is possible, however, to remove the superficial pharyngeal endoderm at the beginning of gastrulation and determine whether this operation affects heart formation or the specification of heart mesoderm in any way. As discussed earlier, the prospective heart mesoderm is presumably in contact with the deep dorsal endoderm from blastula stages onward. However, the first contact between the prospective heart mesoderm and the superficial pharyngeal endoderm is made at the onset of gastrulation, when morphogenetic movements bring the bottle cells at the tip of the archenteron in apposition to the prospective heart mesoderm. Since this period coincides with the stages during which the specification of heart mesoderm presumably occurs, it is possible that interactions between the prospective heart mesoderm and the superficial pharyngeal endoderm are critical to the acquisition of heart-forming potency.

To test this possibility, the bottle cells and the superficial blastoporal endoderm from the dorsal half of the embryo were removed from embryos at the onset of gastrulation. Embryos subjected to this operation were able to form beating hearts, suggesting that interactions between the prospective heart mesoderm and the superficial pharyngeal endoderm are not essential for the acquisition of heart-forming potency. An alternative explanation for this result is that more posterior regions of the endoderm have undergone regulation to replace the superficial pharyngeal endoderm *via* anteriorward migration. In this case, heart mesoderm could be induced following the regulative replacement of the superficial pharyngeal endoderm during subsequent development. Support for this possibility may be found

in Fullilove's (1970) map of the regions of the endoderm of *Taricha torosa* neurulae that are capable of heart induction; her work indicates that endodermal regions posterior to the pharyngeal endoderm itself have some capability for heart induction, although it is considerably less than the heart-inducing capability of the pharyngeal endoderm itself.

Two studies were performed to determine whether heart formation in embryos lacking the superficial pharyngeal endoderm is the result of interaction with endoderm that has undergone regulative replacement of the superficial pharyngeal endoderm. First, explants of heart mesoderm were made from embryos lacking the superficial pharyngeal endoderm when the embryos had reached stages 12.5 to 13. The second study involved the histological examination of pharyngeal structures in midtailbud embryos following removal of the superficial pharyngeal endoderm.

Mesodermal explants prepared from embryos lacking the superficial pharyngeal endoderm underwent heart formation at approximately the same frequency observed in comparable explants from intact embryos. This finding indicates that the removal of superficial pharyngeal endoderm does not prevent the specification of heart mesoderm. It is highly unlikely that regulative replacement of the superficial pharyngeal endoderm by more posterior endoderm could occur before the beginning of neurulation, since the superficial pharyngeal endoderm undergoes involution at the beginning of gastrulation, well before the involution of more posterior endodermal regions at later stages of gastrulation (Keller, 1975).

To determine whether regulative replacement of the superficial pharyngeal endoderm occurs during subsequent stages of development, embryos lacking the superficial pharyngeal endoderm were subjected to histological examination at early neurula and midtailbud stages. The most conspicuous defects in these embryos at midtailbud stages are the absence of pharyngeal pouches and the narrow pharyngeal region. These embryos also lack at least one derivative of the pharyngeal region, the thyroid anlage. The lack of pharyngeal pouches and their associated structures indicates that regulative replacement of the superficial pharyngeal endoderm has not occurred by these stages. Since the heart has begun to beat by midtailbud stages, any regulative replacement of the superficial pharyngeal endoderm at later stages cannot be involved in heart formation. The narrow pharyngeal region probably arises from the absence of dorsal bottle cells. Bottle cells change shape during the latter half of gastrulation, becoming considerably shorter and widening in the plane of the archenteron epithelium. The resulting increase in the surface area of the epithelium lining the anterior archenteron drives the expansion of the anterior archenteron itself, forming a large cavity that will eventually become the pharynx and branchial region (Keller, 1981; Hardin & Keller, 1988). The removal of the dorsal bottle cells at the onset of gastrulation precludes the expansion of the anterior archenteron during late gastrula and early neurula

stages and prevents the pharyngeal region from reaching its full mediolateral extent during tailbud stages.

These results demonstrate that interactions between the prospective heart mesoderm and the superficial pharyngeal endoderm are not essential for the specification of heart mesoderm. In addition, the results indicate that more posterior regions of the endoderm do not undergo regulative replacement of the superficial pharyngeal endoderm. In view of these results, the deep dorsal endoderm is perhaps more likely to contribute to the specification of heart mesoderm than is the superficial pharyngeal endoderm, although the latter may provide some nonessential contribution as well. A subsequent paper will address the tissue interactions that contribute to the establishment of heart mesoderm during gastrulation.

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