NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

CONTROL OF HEART DEVELOPMENT IN THE MEXICAN AXOLOTL
(*Ambystoma mexicanum*)

by

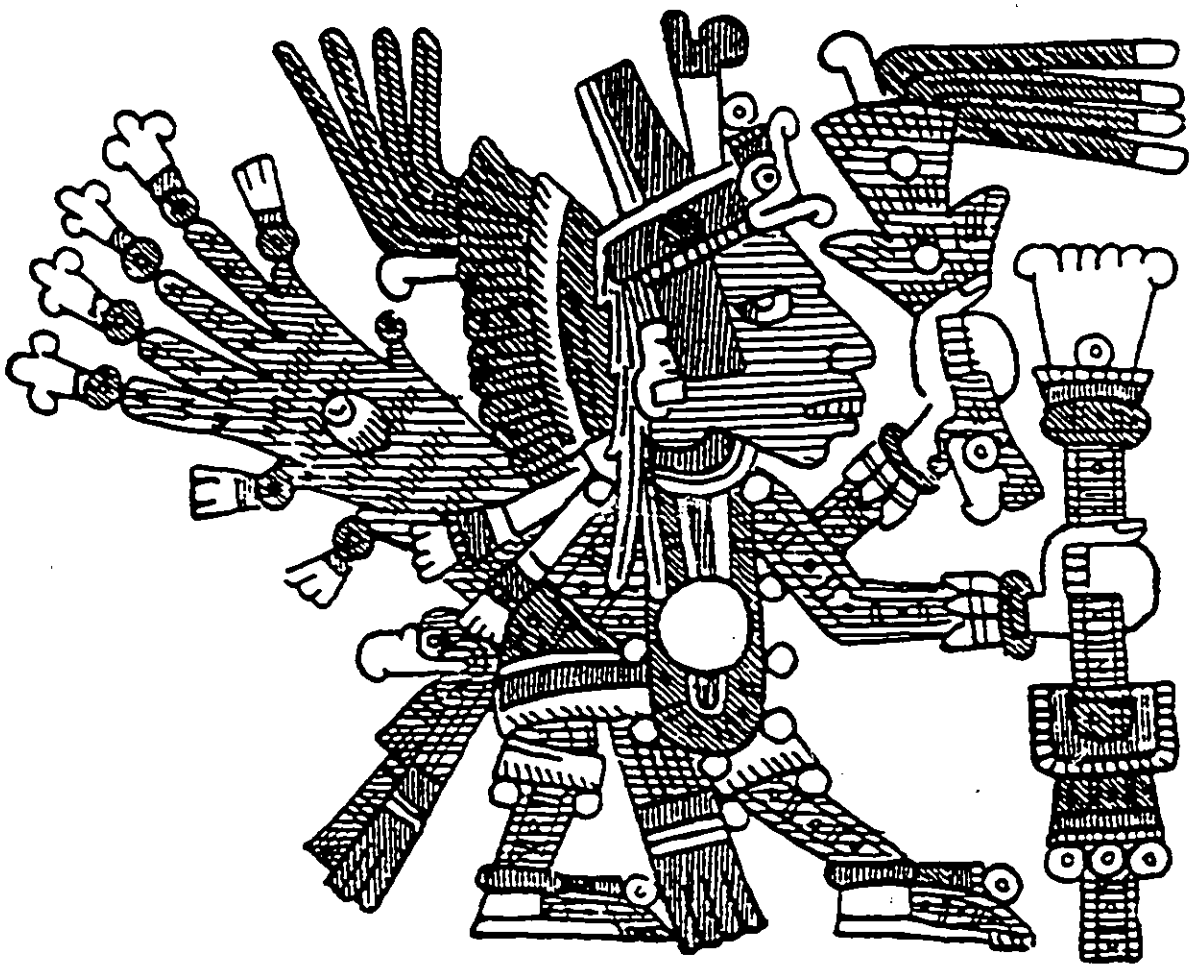
STEVEN C. SMITH

Thesis submitted to
the School of Graduate Studies and Research
in partial fulfillment of the requirements for the
Ph.D. degree in Biology.

University of Ottawa/Université d'Ottawa

ISBN 0-315-62337-3

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA



Frontispiece: Xolotl (from Smith, 1989), for whom the axolotl is named; Aztec god of games and, one hopes, patron of stumbling development biologists.

ACKNOWLEDGEMENTS

A thesis cannot be completed without the help of many people. Therefore, I offer my thanks to all of these people (whether mentioned or not) for their patience and excellence.

Special thanks go to my research supervisor, Dr. John B. Armstrong, who has repeatedly demonstrated that exacting scientific excellence can be achieved without sacrificing humanity, decency, compassion, and humour. He has renewed my enthusiasm for research.

I thank my research committee, Drs. Dave Brown, Cathy Morris, and Jim Neelin, for their guidance and encouragement. Though occasionally heated, our discussions always ended amicably and provided me with valuable food for thought.

I also thank the Canadian Heart Foundation for the financial support which made this study possible, and the Ottawa-Carleton Centre for Biological Microscopy for the use of their electron microscopy and darkroom facilities, and for photographic supplies.

To Mr. William S. Fletcher (a.k.a. the Doctor of Love), curator of the University of Ottawa Axolotl Colony, I offer my gratitude for his excellent work and his companionship. His expertise in axolotl husbandry and ability to procure embryos from (sometimes) uncooperative animals made this

study possible. I wish him my best in all his future endeavours.

My thanks also to my fellow graduate student, Dr. Mary H. Whiteley, for sharing the ups and downs of graduate work, the joys of axolotls, and her good humour. It's been a pleasure - good luck in Bethesda.

Thanks to our fourth-year students, Tammy Murray, Alison Doucet, and Marjorie McIsaac, for allowing me to show them a few of the wonders (and some of the trials) of research.

Mes sincère remerciements à M. Jacques Hélie pour avoir fait des objets d'arts de mes dessins rudimentaires, et à M. Paul Brunon pour avoir fait les photos. Vos efforts sont apprécié.

Je remercie aussi Pascale Garber et Yvan Chapdelaine pour avoir corrigé, plusieurs fois, mon français barbare. Grace à eux, j'ai l'air un peu moins ignorant.

My profoundest gratitude goes to my parents, Charles and Joanna, for having raised me to believe that I could do anything I wanted to, for always having encouraged me to do my best, and most of all for their love, good humour, and support, through good times and bad. Thank you says so little; without you, this would not have been possible.

Finally, I wisn to thank my colleague, partner, proofreader, critic, confessor, lover, and friend - my wife, Dr. Ann C. Graveson. Enfin, ensemble, on a réussi!

To Ann

ABSTRACT

The locations and migration of the embryonic primordia which form the heart are well known. However, the processes whereby the heart-forming mesoderm is induced, and the later mechanisms controlling the differentiation and morphogenesis of the heart are only poorly understood for any system.

An important model system for studying heart induction and differentiation is the cardiac-lethal (c) mutant in the axolotl (*Ambystoma mexicanum*). Embryos homozygous for the c gene develop hearts which never begin to beat, become severely deformed due to the lack of circulation, and die shortly after hatching. The mutation was believed to affect the tissue responsible for heart induction, the anterior (pharyngeal) endoderm, rendering it incapable of supplying the appropriate inductive stimuli.

The inductive failure hypothesis is largely based on an assumption. The assumption is that the timing of heart induction is the same in the axolotl as has been reported for another urodele species (*Taricha torosa*). As well, this hypothesis is based on the finding that wild-type heart mesoderm does not form beating hearts when transplanted into c/c embryos at late tailbud stages 28-29. (However, for this evidence to support the inductive failure hypothesis, the

the preceding assumption must be correct.) On the basis of this evidence, it has been suggested that wild-type heart mesoderm does not receive the proper inductive signals in the mutant environment. However, it has also been suggested that the induction occurs much earlier in another species of *Ambystoma* than in *T. torosa*; the timing of the inductive process in the axolotl has never been determined.

Therefore, I have examined the temporal and spatial parameters of heart induction in wild-type axolotl embryos using an *in vitro* assay for the formation of functional myocardial tissue. In this species, the inductive interaction is completed by the end of neurulation (stage 20), earlier than had been previously assumed.

Myocardial cell differentiation is induced by the pharyngeal endoderm, as has been reported for other species. However, the evidence presented in this thesis suggests that the inductive activity is highest in the mid-ventral (future heart site) endoderm, and appears to be distributed in a gradient, since there is less inductive activity in more dorsal areas. This is the first time that evidence for a gradient of heart inducer has been reported.

Attempts to characterize the inductive agent(s) were unsuccessful; no significant inductive activity was present in any of the cell-free preparations examined. This included RNA-containing extracts from inductive endoderm, similar to

those reported to "induce" *c/c* hearts to begin beating *in vitro*.

The finding that heart induction in the axolotl is completed much earlier than previously suspected casts serious doubt on the inductive failure hypothesis for *c/c* embryos. It suggests that the wild-type heart mesoderm transplantations into mutant embryos were performed well after the mesoderm was fully induced. This mesoderm must have been actively inhibited from completing its differentiation in the *c/c* host embryos. This alternative has been previously suggested, but largely ignored in subsequent studies. As well, RNA-containing preparations, which are capable of stimulating heartbeat in fully-formed *c/c* hearts, contain no detectable inductive activity when assayed with uninduced wild-type heart mesoderm.

Therefore, in the second phase of this study I re-examined the nature of the defect in cardiac-lethal mutant embryos. Using *in vitro* combinations of uninduced *c/c* and wild-type heart mesoderm and inductive endoderm, I demonstrated that mutant endoderm produces normal inductive signals, and that the mutant heart mesoderm is incapable of responding normally to the induction. As well, when fully induced (but undifferentiated) mutant and wild-type heart mesoderm are combined *in vitro*, the mutant mesoderm forms beating tissue. This suggests that an "activator",

responsible for controlling myofibrillogenesis, is present in wild-type heart mesoderm, and absent in mutant tissue.

In vivo transplantations of wild-type heart mesoderm into c/c embryos at different stages corroborate the *in vitro* results, and further demonstrate that mutant heart mesoderm produces a specific inhibitor of cardiomyocyte differentiation.

The presence of a specific activator and inhibitor of heart differentiation, both produced by the heart mesoderm itself, provides evidence that the later phases of heart formation (*i.e.* the organization of contractile proteins into functional sarcomeres, and possibly the early morphogenesis of the heart tube) are probably under the control of a two-morphogen reaction-diffusion system. Such systems have been demonstrated to control pattern formation in one invertebrate organism, and have been proposed to control morphogenesis in a variety of other systems. However, this study is the first direct, experimental evidence for a reaction-diffusion mechanism controlling the development of any vertebrate organ system.

RESUME

Alors que les emplacements et la migration des ébauches embryonnaires du coeur sont très bien connus, les processus de l'induction et les mécanismes contrôlant la différenciation et la morphogénèse du coeur ne sont pas bien compris.

Le mutation cardiaque-léthal (*c*) chez l'axolote (*Ambystoma mexicanum*) est un modèle important pour l'étude de l'induction et de la différenciation cardiaque. Bien que les coeurs des embryons homozygotes pour le gène *c* soient formés, ils ne commencent jamais à battre. Ces embryons développent des malformations sévères et ils meurent peu de temps après l'éclosion. On soupçonnait que la mutation affecte l'endoderme pharyngien (le tissu inductif) pour rendre ce dernier incapable d'élaborer des signaux inductifs normaux.

Cette hypothèse est fondée en grande partie sur deux évidences. La première étant la supposition que l'induction se produisait aux mêmes stades de développement chez l'axolote que chez une autre espèce d'urodèle (*Taricha torosa*). La seconde (dépendant absolument de la première supposition) étant le fait que le mésoderme pré-cardiaque de type sauvage ne forme pas de coeur battant quand il est transplanté dans un embryon mutant aux stades 28-29. Avec

ces preuves, on a suggèré que le mésoderme pré-cardiaque de type sauvage ne reçoit pas les signaux inductifs normaux lorsqu'il est dans l'environnement mutant. Mais il a aussi été postulé que l'induction du coeur a lieu plus tôt chez une autre espèce d'*Ambystoma* que chez *T. torosa*. L'aspect temporel de l'induction n'a jamais été déterminé chez l'axolote.

J'ai donc examiné les caractères temporaux et spatiaux de l'induction chez les axolotes de type sauvage en utilisant un système *in vitro* mesurant la formation du tissu myocardiaque fonctionel. Chez l'axolote, l'interaction inductif est complété dès la fin du neurulation (stade 20), c'est à dire plus tôt qu'on ne le supposait.

L'endoderme pharyngien induit la différenciation des cellules myocardiaques, tel que rapporté pour d'autres espèces. Mais les preuves présentées dans cette thèse suggèrent que la concentration de l'activité inductive forme un gradient: les concentrations les plus élevées se retrouvent dans l'endoderme mi-ventral (site futur du coeur), alors que les plus faibles se retrouvent dans les régions plus dorsales. C'est la première fois qu'un tel gradient a été démontré pour l'inducteur cardiaque.

Les tentatives pour caractèriser l'agent(s) inductif(s) n'ont pas réussi; une activité inductive n'a jamais été détectée dans les préparations acellulaires examinées. Parmi

ces préparations inactives, il y avait des extraits contenant de l'ARN de l'endoderme inductif, semblables à ceux qui ont été rapporté comme étant capables "d'induire" les coeurs *c/c* à commencer de battre *in vitro*.

La preuve que l'induction du coeur est complété plus tôt que suspecté chez l'axolote met en doute l'hypothèse que l'induction est empeché dans les embryons *c/c*. Elle suggère plutôt que le mésoderme de type sauvage a été transplanté dans les embryons mutants après que l'induction ait été completée. La différenciation de ce mésoderme a dû être inhibée chez les embryons hôtes mutants. Cette explication alternative a déjà été suggérée, mais elle a été largement ignoré dans les études succédants. De plus, les préparations contenant de l'ARN, capables de stimuler le battement des coeurs *c/c*, ne contiennent aucune activité inductive quand essayée sur le mésoderme pré-cardiaque non-induit de type sauvage.

Dans la deuxième phase de cette étude, j'ai donc réexaminé la nature du défaut chez les embryons mutants. En combinant *in vitro* du mésoderme non-induit et de l'endoderme inductif *c/c* et de type sauvage, j'ai pu démontré définitivement que l'endoderme mutant produit des signaux inductifs normaux, et que le mésoderme mutant est incapable de répondre de façon normale. De plus, le mésoderme mutant se différencie en tissu battant quand il est cultivé *in vitro*

avec du mésoderme de type sauvage induit mais non-différencié. Ceci suggère que le mésoderme de type sauvage contient un "activateur" qui contrôle la myofibrillogénèse, et que l'activateur est absent dans le mésoderme mutant.

Les transplantations *in vivo* du mésoderme de type sauvage dans les embryons *c/c* à des stades différents confirment les résultats obtenus *in vitro*, et ils démontrent que le mésoderme pré-cardiaque produit un inhibiteur de la différenciation des cellules myocardiaques.

La présence d'un activateur et d'un inhibiteur de la différenciation cardiaque, produits par le mésoderme pré-cardiaque lui-même, suggère que les phases plus tardives du développement du coeur (soit l'organisation des protéins contractiles en sarcomères fonctionelles, et peut-être la morphogénèse précoce du tube cardiaque) sont contrôlées par un système de réaction-diffusion composé de deux morphogènes. De tels systèmes sont impliqués dans la formation des patrons d'un organisme invertébré, et ils ont été proposés théoriquement commes mécanismes contrôllant la morphogénèse dans divers autres systèmes. Mais ceci est la première evidence expérimentale directe impliquant ce mécanisme de contrôle dans le développement d'un système d'organe chez les vertébrés.

CONTENTS

CONTENTS (CONT.)

CONTENTS (CONT.)

LIST OF FIGURES

LIST OF FIGURES (CONT.)

LIST OF TABLES

LIST OF TABLES (CONT.)

We dance around in a ring and suppose

But the Secret sits in the middle and knows

From: "The Secret Sits". In: *The Poetry of Robert Frost* (1971; E. Connery, ed.). Holt, Rinehart, & Winston, NY, USA. (With apologies to F.L. Strand, who thought of it first.)

CHAPTER I

INTRODUCTION

One of the major unresolved problems of developmental biology is that of inductions. During the course of early development, interactions occur between responsive tissues and other tissues and organs (the inductors) which influence the developmental fate of the former. However, the inductive processes whereby multipotent embryonic tissues become committed to differentiate into specific adult tissues are only poorly understood for any system.

One organ system which is dependent on inductive interactions for its development is the heart. While some of the inductive interactions necessary for heart formation have been elucidated, particularly in the amphibian, the nature of the inductive process(es) remains obscure.

1.1 Origin of the heart:

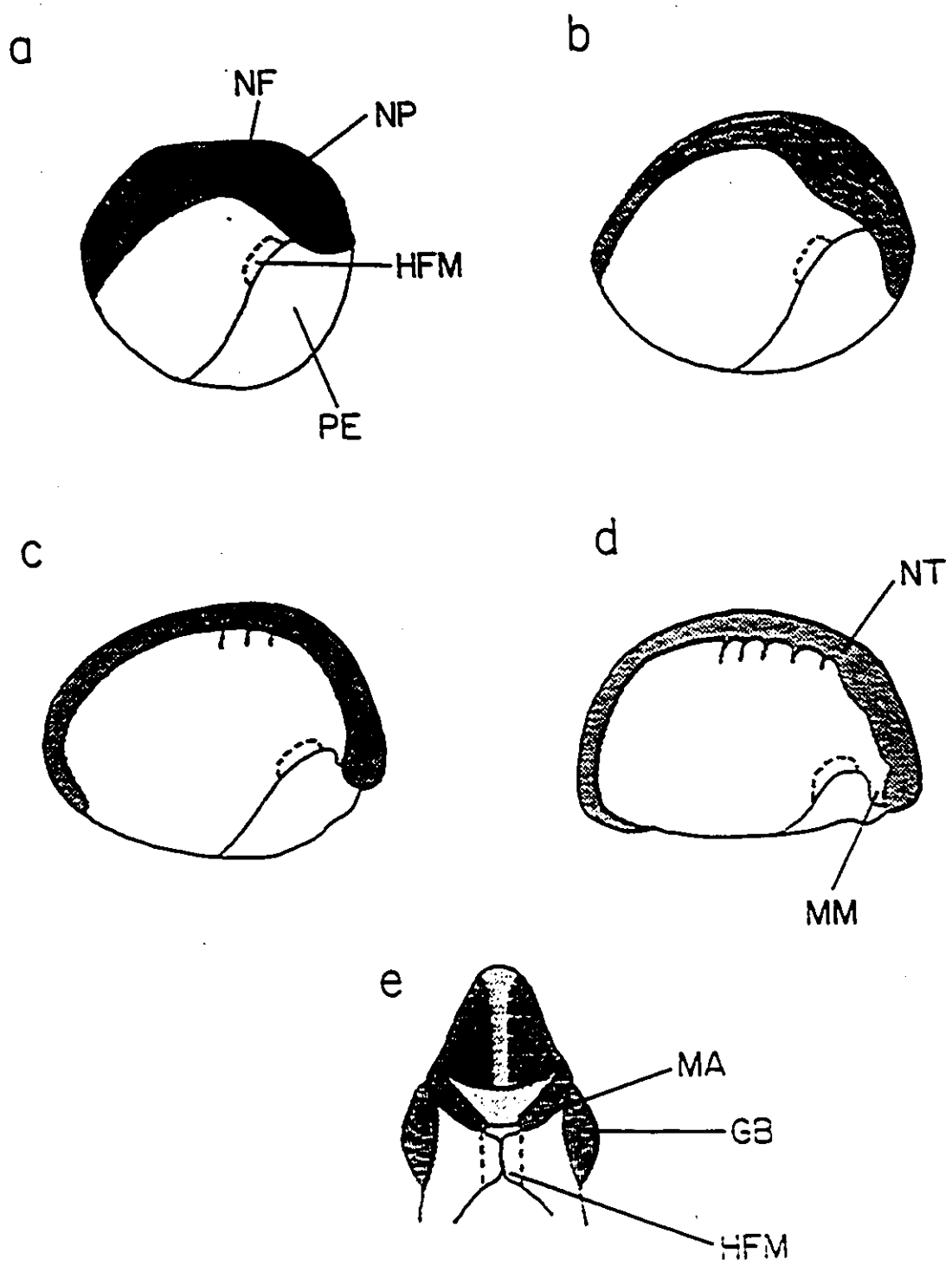
At the turn of the century, the embryonic origin of the heart was unclear. It was disputed whether the heart arose from the endoderm, the mesoderm, or a combination of these tissue layers (see Copenhaver, 1926; Wilens, 1955, for discussion). Early extirpation experiments by Copenhaver (1926) demonstrated conclusively that the amphibian heart

arises from the lateral mesoderm.

The locations and movements of the early heart primordia have been mapped using vital stains (Wilens, 1955). During early neurula stages, the heart consists of paired primordia located at the leading edge of the mesodermal mantle, immediately subjacent to the presumptive inner ear ectoderm and just ventral to the neural folds. During later neurula and tailbud stages, the primordia migrate antero-ventrally until they meet and fuse at the ventral midline of the pharyngeal cavity (Fig. 1-1). The fused primordia form a tube which begins to contract rhythmically, and fold upon itself to form the usual three-chambered amphibian heart. If the two primordia are separated, each is capable of forming a complete heart independently (Fales, 1946).

Recently, it has been shown that the cranial neural crest also contributes to normal heart morphogenesis in chicks. This tissue appears to be important for the formation of normal septation in the outflow region of the heart (Kirby *et al.*, 1983; Kirby and Bockman, 1984; Besson *et al.*, 1986; Phillips *et al.*, 1987). However, neural crest cells do not appear to be necessary for the differentiation of functional myocardial tissue, since mesoderm alone can differentiate into beating cells in the absence of neural crest (Bacon, 1945; Jacobson, 1960; 1961; Jacobson and Duncan, 1968). Thus, the mesoderm alone is responsible for

Figure 1-1: a-d) Diagrams of the right sides of stage 14, 16, 18, and 20 neurulae (respectively), with the ectoderm removed, showing the locations and migration of the heart-forming mesoderm (HFM). The mesoderm (lightly stippled) migrates antero-ventrally down both sides of the embryo during neurulation and early tailbud stages. The two heart primordia (one on each side) are located at the anterior edges of the mesodermal mantle. e) At stage 29 (ventral view) the two primordia meet at the ventral mid-line and begin to fuse. In (a-d), anterior is to the right, dorsal at the top; in (e), anterior is at the top. NF: neural fold; NP: neural plate; PE: pharyngeal endoderm; NT: neural tube; MM: mandibular mesoderm; MA: mandibular arch; GB: gill bulge.


a
NF
NP
HFM
PE
b
c
d
NT
MM
e
MA
GB
HFM

the formation of the myocardium.

1.2 Induction of the heart:

Although the movements of the heart primordia (Wilens, 1955; Hirakow *et al.*, 1987) and the subsequent morphogenesis of the heart (see Manasek and Monroe, 1972; Ojeda and Hurle, 1981; Manasek *et al.*, 1984; Hirakow, 1986) have been adequately described, the inductive processes whereby the mesoderm becomes committed to form beating cardiac tissue have not been as well characterized.

A classic study by Bacon (1945) was the first real attempt to elucidate the mechanisms of heart induction. He concluded that the presumptive heart mesoderm of *Ambystoma maculatum* (*punctatum*) underwent "complete primary organization" and could self-differentiate if isolated from early gastrulae. However, Bacon's (1945) criterion for differentiation was merely the formation of a tissue mass which morphologically resembled the gross structure of a heart, regardless of whether any contractions were observed. Therefore, what Bacon (1945) describes as the ability to self-differentiate might not have included differentiation at all. Indeed, Bacon (1945) recognized that morphogenesis could occur independently of cell differentiation (as indicated by the presence of pulsating cells) and *vice versa* (see also Manasek and Monroe, 1972).

A further problem arises when the nature of Bacon's (1945) explant cultures is considered. He reported that a "secondary organizing activity" was present in the floor of the archenteron (*i.e.* pharyngeal endoderm). This activity was potent enough to induce heart differentiation in some mesoderm not normally destined to become heart. However, the explants from early gastrulae, which Bacon (1945) described as capable of self-differentiation, likely contained presumptive pharyngeal endoderm as well as presumptive heart-forming mesoderm. His corroborative experiments involved implanting mesoderm in the archenteron, where close association between the implant and other tissues (notably pharyngeal endoderm) was possible (see Copenhaver, 1955).

Therefore, Bacon's (1945) study was inconclusive. The pharyngeal endoderm admittedly had some organizing activity, and all of the explant cultures and implants had the opportunity to be in close contact with this tissue. Thus, it is possible that the heart mesoderm was merely induced later by tissues explanted with it or present at the site of implantation, rather than being truly capable of self-differentiation at early (gastrula) stages. This is particularly true since embryos from which the entire endoderm is removed at early neurula stages fail to develop hearts (Balinsky, 1939; Nieuwkoop, 1947; Bacon, personal communication in Copenhaver, 1955; Jacobson, 1960).

The most extensive study of heart induction in amphibians was reported in a series of papers by Antone Jacobson (Jacobson, 1960; 1961; Jacobson and Duncan, 1968). It should be noted that, in all of these studies, the presence of beating tissue was used as the diagnostic, whether or not the morphology of the structure resembled a heart. Therefore, the inductions described are those responsible for the differentiation of functional myocardial tissue and not (necessarily) for the morphogenesis of the heart as an organ.

Using a series of cultures from neurulating *Taricha torosa* embryos, Jacobson (1960) showed that heart mesoderm from early neurulae, explanted alone, began to beat in only a small number of cases. By combining this mesoderm with various other embryonic tissues, he showed that both ectoderm and, especially, anterior (pharyngeal) endoderm increased the proportion of explants with beating tissue. Cultures which contained neural tissue, however, failed to begin beating. Indeed, whole embryos which had their entire endoderm removed never formed hearts, although they could if the neural plate was also removed. Jacobson (1960) concluded that heart formation was stimulated by anterior endoderm but inhibited by anterior neural plate and fold.

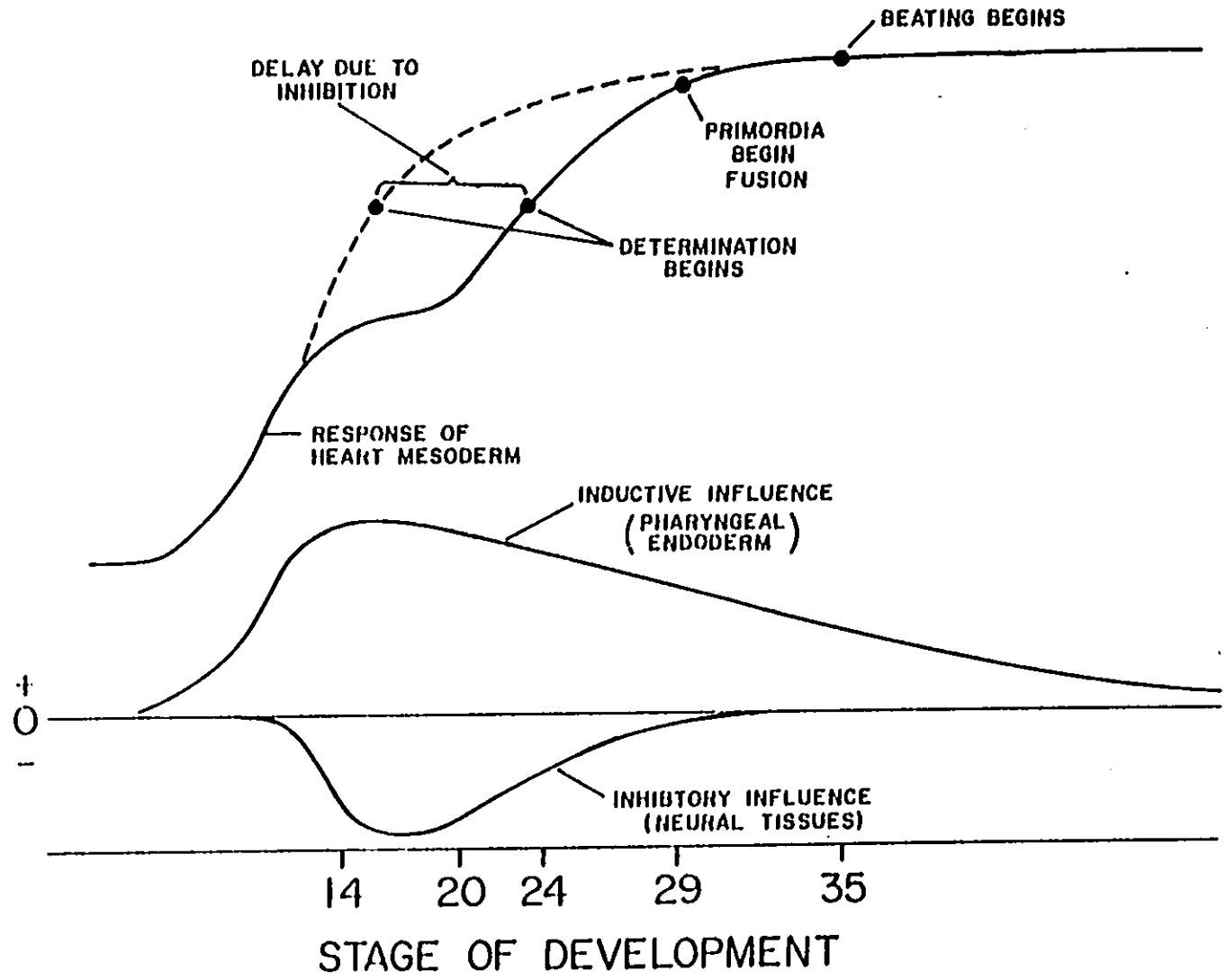
In 1961, Jacobson took his study further, establishing that: i) ectoderm was apparently not necessary for heart

determination, but merely provided a substrate for the migration of the heart-forming mesoderm, and ii) that anterior neural plate, but not neural fold, suppressed heart formation. He concluded that anterior endoderm induced heart differentiation. This induction began during gastrulation, since some beating tissue could form if mesoderm alone was explanted at this early stage. However, the heart-forming mesoderm was insufficiently induced to overcome the inhibitory effects of the anterior neural plate until as late as mid-tailbud stages (stages 24-26).

Jacobson and Duncan (1968) finally used this, and some new, data to propose a model. In this model, heart induction was described as a gradual, cumulative process involving both inductive and inhibitory interactions between the heart-forming mesoderm and other, adjacent, tissues (Fig. 1-2). They concluded that the major inductive stimulus was supplied by the anterior (pharyngeal) endoderm, the presence of which increased both the frequency and rate of heart differentiation. As well, they describe the presence of a "general stimulatory factor" in all ectoderm, which increased the proportion of cultures which began to beat (the frequency), but not the rate at which they differentiated.

Finally, Jacobson and Duncan (1968) described the presence of an inhibitory influence in the anterior neural plate and fold which could repress heart differentiation.

Figure 1-2: Graphic interpretation of the process of heart induction (after Jacobson and Duncan, 1968). The inductive influence of the pharyngeal endoderm causes the heart mesoderm to gradually become determined. This is delayed by the inhibitory effect of the neural plate and folds. The inhibitory influence decreases with age due to the ventral migration of the mesoderm, away from the neurectoderm, rather than to a loss of inhibitory activity in the latter (see Jacobson and Duncan, 1968, Fig. 7).


BEATING BEGINS
DELAY DUE TO INHIBITION
PRIMORDIA BEGIN FUSION
DETERMINATION BEGINS
RESPONSE OF HEART MESODERM
INDUCTIVE INFLUENCE (PHARYNGEAL ENDODERM)
+
0
−
INHIBITORY INFLUENCE (NEURAL TISSUES)
14
20
24
29
35
STAGE OF DEVELOPMENT

Curiously, the inhibitor was reported to be ineffective in explant cultures, and its presence could only be demonstrated in whole embryos from which the endoderm had been removed. Based mainly on their extirpation experiments, Jacobson and Duncan (1968) identify stages 24-26 (mid-tailbud) as the point at which the heart-forming mesoderm is "determined" and will form a functional myocardium even in the presence of neural tissues. However, they believe that the time of determination is due largely to the inhibitory influence of the neural tissues, which delay determination; determination of heart tissue occurs during neurulation in the absence of cranial neural plate and fold (Jacobson and Duncan, 1968, Fig. 8, p. 83).

These experiments were performed on *Taricha torosa* embryos. It is important to note that these workers also report that "... heart determination occurs at earlier morphological stages ..." in embryos of *Ambystoma tigrinum* (Jacobson and Duncan, 1968, p. 81, and Fig. 12). This distinction becomes important when interpreting studies of the cardiac-lethal mutant of *Ambystoma mexicanum* (see Section 1.4).

Fullilove (1970) attempted to determine the levels of inductive activity in different areas of the endoderm of *Taricha torosa* embryos. She concluded that inductive activity was broadly based within the anterior (pharyngeal)

endoderm, but was not present in posterior endoderm. Curiously, when Fullilove's (1970) data are translated into heart differentiation coefficients (HDC; used by Jacobson and Duncan, 1968, as a measure of heart differentiation in the same species), only low levels of inductive activity appear to be present in any of the endodermal regions tested. This correlates well with Jacobson and Duncan's (1968) data, which show that uninduced (stages 14-14.5) heart mesoderm cultured with "anterior dorsal endoderm" did not begin to beat (Jacobson and Duncan, 1968, Fig. 13 and Table 2). Only when older (already somewhat induced) mesoderm was used did these areas of endoderm increase the HDC.

If Jacobson's (1960; 1961; Jacobson and Duncan, 1968) endoderm extirpation experiments were correct, and pharyngeal endoderm is the major inductor of heart differentiation, it suggests that the areas of anterior endoderm tested by Fullilove (1970) and Jacobson and Duncan (1968) were not the major source of the inductor. Therefore, the inductive activity of the endoderm may have a more restricted distribution than reported by these authors.

In similar studies, other workers have reported that anterior endoderm is responsible for heart induction in several other species. These include a variety of amphibians (Amano, 1961; Fautrez and Amano, 1961; Bride, 1979; Sater and Jacobson, 1989), and chicks (Orts-Llorca, 1963; Orts-Llorca

and Gil, 1965), and Hommes (1957, as cited in Jacobson and Duncan, 1968) has implied the same for humans.

1.3 Characterization of the inducer:

Having determined the inductors necessary for heart differentiation, the next logical step is to attempt to characterize and isolate the actual factor(s) (inducers) responsible for the induction. The first study of this type was performed by Jacobson and Duncan (1968). These workers tested Sephadex "membrane" fractions of a variety of homogenized embryonic tissues for their ability to induce heart-forming mesoderm in culture. However, their results failed to demonstrate the presence of a specific inducer of heart differentiation. Even cultures treated with extracts from supposedly inhibitory (neural) tissues differentiated almost as well as cultures containing inductive endoderm or fractions thereof. Indeed, they state that:

> "... Any additional tissue or any membrane fraction of embryonic tissue that is combined with the heart mesoderm seems to promote more heart differentiation than obtained with heart rudiments cultured alone ..." (Jacobson and Duncan, 1968, *pp.* 97-98).

This strongly suggests that the effects observed by these workers were non-specific. Perhaps due to this, no further attempts have been made to elucidate the nature of the inductive signal(s) which pass from the pharyngeal endoderm to the heart-forming mesoderm, although Jacobson and Sater (1988) hold out hope that this may be accomplished using the more sophisticated techniques now available.

Concomittantly with this study in amphibians, work by others into heart induction in chicks held great promise for its potential to identify the nature of the inducer. Butros (1965) reported that crude RNA extracts from embryonic chick hearts were able to enhance the formation of beating tissue from the presumptive heart tissue of that species. This effect could not be duplicated by RNA preparations from embryonic liver.

Subsequently, it was demonstrated that RNA preparations from heart tissue also inhibited the development of neural tissues (Niu and Mulherkar, 1970) and, most significantly, could cause beating heart tissue to form from areas of chick blastoderm which do not normally participate in heart formation (Niu and Deshpande, 1973; Deshpande and Siddiqui, 1977). Histological, biochemical, and physiological examination of these induced tissues revealed that the beating cells had many characteristics of normal cardiomyocytes (Niu and Deshpande, 1973; Deshpande and

Siddiqui, 1977; 1978; McLean *et al.*, 1977). Since the effect was tissue specific - RNA preparations from embryonic liver (Butros, 1965), brain, kidney, and thymus (Niu and Deshpande, 1973) could not mimic the effect - it was believed that this RNA preparation contained the inducer of heart differentiation.

In the series of studies that followed, it was determined that the inductive effects were caused by a small (7S) polyadenylated RNA (Niu and Deshpande, 1973; Deshpande and Siddiqui, 1977; Deshpande *et al.*, 1977). This RNA species was untranslatable *in vitro*, but could inhibit the translation of other, non-cardiac mRNA's (Deshpande *et al.*, 1977). In addition, a complementary DNA clone of the 7S RNA was apparently found to hybridize to chick repetitive DNA, and also to the 3' untranslated regions of chick myosin heavy and light chain genes (Khandekar *et al.*, 1984; Siddiqui *et al.*, 1986).

While it has been suggested that this RNA species has a function in regulating the expression of muscle-specific genes during chick heart development, it is unlikely that it is the inducing agent for several reasons. First, the 7S RNA is isolated from fully-formed hearts, and not from the tissues which initially induce cardiogenesis. As well, neither the presence of the RNA in the inducing tissues, nor its secretion by these cells has been demonstrated. In fact,

this RNA was found only in the heart and not in other tissues examined by these groups.

Therefore, it appears that, rather than being the primary inducing agent, the 7S RNA (if it is indeed secreted or otherwise passed to neighbouring cells) may be an agent which can prevent the translation of other (non-heart) genes by heart tissues. In this way, it may function as an internal secondary messenger, produced in response to the induction, which then promotes the final differentiation of heart tissue.

However, a recent report may preclude even a secondary role for this RNA. Desjardins and colleagues (1989) have reported that the 7S RNA is highly (99%) homologous to a portion of the chick mitochondrial ATPase 6 gene. No homologous sequences could be detected in either the chick genome or RNA transcripts from it, although chicken mitochondrial sequences were easily recognized. This strongly suggests that this RNA species is nothing more than a degradation product of mitochondrial RNA accidentally produced during the isolation. If true, it is difficult to imagine how the 7S RNA described by Khandekar *et al.* (1984) could play a role in the regulation of expression of muscle-specific genes.

Despite this evidence that the RNA of Khandekar *et al.* (1984) is artifactual, Desjardins *et al.* (1989) do not

attempt to explain why supposedly identical RNA preparations from chick tissues other than the heart do not have inductive ability, even though they presumably contain the same mitochondrial RNA. It can only be surmised that if the RNA described by Khandekar *et al.* (1984) is mitochondrial, then the active fraction must contain some other component which co-purifies with the RNA.

1.4 The cardiac-lethal mutant:

One potentially promising model system for the study of heart induction is the cardiac-lethal (*c*) mutation in the Mexican axolotl (*Ambystoma mexicanum*). This is a simple recessive developmental mutation which causes an absence of heart function in affected embryos (Humphrey, 1968; 1972). Embryos homozygous for *c* develop apparently normally until stage 35, when the heart should begin to beat. (For developmental stage series of axolotl embryos, refer to Appendix I; Bordzilovskaya and Dettlaff, 1979; Bordzilovskaya *et al.*, 1989.) In mutant embryos, the heart never begins to beat, and the embryos become severely edemic and microcephalic, and the gills develop poorly. As well, these embryos do not feed, or grow as quickly as their wild-type siblings (Humphrey, 1972; see Fig. 1-3). These defects are believed to be secondary, caused by the lack of circulation. Although the heart does not beat, skeletal muscles are not

Figure 1-3: Photomicrographs of a) wild-type and b) cardiac-lethal mutant (c/c) larvae, taken shortly after hatching. Note the swollen shape, shorter length, reduced gills, and contracted melanocytes on the trunk of the mutant. These characteristics are secondarily caused by the lack of circulation. Anterior is to the left in both cases. Bars = 2 mm.

a
b

affected, as embryos have normal reflexes, and even severely edemic larvae can swim normally.

Histological and ultrastructural studies of c/c hearts have revealed that the cardiomyocytes retain their lipid droplets longer than they normally would (Lemanski *et al.*, 1970; Lemanski, 1973a, b) and, although myofibrillar proteins are present, they do not form organized myofibrils (Lemanski and Fuldner, 1977; Hill and Lemanski, 1979). As well, there are fewer endocardial and mesenchymal cells in mutant hearts, and the cardiac jelly (extracellular matrix) underlying the endocardium becomes enlarged in older mutant hearts (Lemanski and Fitzharris, 1989).

Biochemical and immunocytochemical examination of c/c hearts has revealed that levels of troponin-T (Fuldner *et al.*, 1984), desmin (Shen and Lemanski, 1989), vimentin and vinculin (Shen and Lemanski, 1986) are normal, and that actin, myosin, and α-actinin are present in near-normal amounts (Lemanski and Fuldner, 1977; Lemanski *et al.*, 1975; Lemanski, 1978; Starr *et al.*, 1989). Levels of tropomyosin have been reported to be greatly reduced in mutant hearts (Lemanski, 1978; 1979; Moore and Lemanski, 1982; Starr *et al.*, 1985; 1989). However, a close examination of the available data reveals that this is true only in older mutant hearts and, at the time when heartbeat should begin, the levels of tropomyosin are comparable to those found in

beating wild-type hearts of the same stage (Starr *et al.*, 1989). Consequently, it is unlikely that the decreased level of tropomyosin is responsible for either preventing myofibrillogenesis or the initial failure of mutant hearts to beat.

Therefore, in *c/c* embryos, morphologically normal hearts form (Humphrey, 1972; Lemanski, 1973a; Fransen and Lemanski, 1988), which appear to contain the necessary contractile proteins, yet these hearts fail to form regular sarcomeric arrays and never begin to beat.

What makes this mutant interesting is a series of experiments reported in the initial description of *c/c* embryos (Humphrey, 1972). When Humphrey (1972) transplanted mutant heart-forming mesoderm into normal hosts, heart development proceeded normally, and fully functional hearts developed. However, wild-type heart-forming mesoderm transplanted into mutant hosts failed to begin beating. Humphrey (1972) suggested that this was indicative of either a failure of normal inductive processes, or an active inhibition of the heart in mutant embryos. Since wild-type embryos parabiotically joined to *c/c* ones were not adversely affected, and since the hearts in the mutant members of these pairs did not begin to beat, Humphrey (1972) concluded that these inductive or inhibitory influences must be localized and not systemic.

In a subsequent series of studies, Lemanski and collaborators produced evidence to support the inductive failure hypothesis. They demonstrated that mutant hearts explanted alone, and therefore presumably isolated from any inhibitory influences present in the embryonic milieu, did not begin to beat (Hill and Lemanski, 1979). While this finding has been disputed (Kulikowski and Manasek, 1977; 1978; Justus, 1978), possibly due to the use of different strains of mutant animals (Justus, 1978; Lemanski, 1978; Epstein and Lemanski, 1980; Hill and Lemanski, 1979), Lemanski *et al.* (1979) suggested that the failure of *c/c* hearts to beat *in vitro* proved that there was no inhibitor in the mutant embryos. In addition, it was demonstrated that explanted mutant hearts (stage 35) begin to beat when cultured with wild-type anterior endoderm from stage 30 (Lemanski *et al.*, 1979) or younger (Justus, 1978) embryos, or with medium conditioned by stage 30 endoderm (Davis and Lemanski, 1983; 1987). This suggested that failure of the endoderm to properly induce the heart-forming mesoderm was why mutant hearts did not beat.

In an ultrastructural study of anterior endoderm from *c/c* embryos, Lemanski *et al.* (1977) suggested that this tissue was morphologically abnormal and resembled older tissue than that found in wild-type embryos of the same stage. They proposed that the endoderm in mutant embryos

differentiates more rapidly than normal, and thus rapidly and prematurely passes the point where it is capable of inducing the heart-forming mesoderm. Based on these studies, Lemanski and coworkers have suggested that the *c* mutation affects the inducing tissue (the anterior, or pharyngeal, endoderm) such that its inductive activity is reduced or abolished (Lemanski *et al.*, 1977; 1979; Davis and Lemanski, 1987). If true, this would mean that *c/c* heart-forming mesoderm must be normal and only indirectly affected by the mutation.

Recently, Davis and Lemanski (1987) have reported that an RNA-containing preparation from axolotl anterior endoderm is capable of causing *c/c* hearts to begin beating in culture. As well, they have reported that similar RNA-containing preparations from sheep heart can also cause *c/c* hearts to begin beating *in vitro* (LaFrance *et al.*, 1989), whereas RNA from embryonic axolotl liver and neural tube is ineffective (Davis and Lemanski, 1987). On the basis of these results, they have proposed that this RNA is the substance responsible for heart induction in the axolotl (Davis and Lemanski, 1987).

If the proposal that the *c* mutation causes inductive failure is true, and the RNA preparation of Davis and Lemanski (1987) contains the inducer, the cardiac-lethal mutant would be a potentially powerful model system for studying the mechanisms of heart induction. However, there

are several major flaws in this interpretation. Humphrey's (1972) heart mesoderm transplants were all performed on late tailbud stage embryos (stages 27-29). While the timing of normal heart induction has never been investigated in the axolotl, Jacobson and Duncan (1968) report that induction is complete by stages 24-26 in *Taricha torosa* and even earlier in *Ambystoma tigrinum* (a species closely related to the axolotl; see Section 1.3). This finding strongly suggests that the wild-type heart-forming mesoderm transplanted into *c/c* by Humphrey (1972) should have been fully induced, prior to the operation, and capable of differentiating without any further inductive stimulus.

If true, there are two obvious corollaries. The first is that the wild-type heart-forming mesoderm transplanted into the *c/c* hosts must have been actively inhibited. Therefore, removing the mutant mesoderm from the inhibitory influence of the *c/c* embryonic milieu may be all that is required to allow it to complete its developmental repertoire. The second is that, since little inductive activity should remain in the endoderm at these late stages (see Fig. 1.2; Jacobson and Duncan, 1968; Justus, 1978), the *c/c* hearts, which began to beat in wild-type hosts, must have been induced while still in the donor. Therefore, mutant endoderm must be normal in its ability to induce heart-forming mesoderm.

The proposal that mere removal of *c/c* hearts from the presence of an inhibitor is sufficient to allow its development to continue is contradicted by reports that, when explanted into a presumably neutral environment, such hearts do not begin to beat (Justus and Hollander, 1971; Hill and Lemanski, 1979; Lemanski *et al.*, 1979). Since only fully-formed (stage 35) hearts were used, however, it could be argued that the explants were performed too late to reverse the effects of a prior inhibition. As well, other workers have reported that mutant hearts do, in fact, begin to beat when explanted (Kulikowski and Manasek, 1977; 1978; Justus, 1978).

Another major problem with the hypothesis that the inductive endoderm is abnormal arises from studies of *c/c* embryos. In these embryos, the heart forms completely normally until the onset of heartbeat. The cardiomyocytes contain most or all of the necessary contractile proteins and are morphologically identical to normal cardiomyocytes. (Lemanski *et al.*, 1970; Lemanski, 1973a, b; 1976). This strongly suggests that all inductive influences necessary for the formation of the heart and for the early differentiation of cardiomyocytes are, in fact, present in mutant embryos.

However, these hearts never begin to beat. This may indicate that, while early heart induction occurs normally in the mutant, a final "push", to cause myofibrillogenesis and

initiate beating, is all that is lacking. If the findings of Lemanski and colleagues (1977; 1979; Davis and Lemanski, 1987) are verified, it would appear that this push may also be supplied by the anterior endoderm, albeit at later stages than when it acts as the primary inductor of heart differentiation.

Therefore, the cardiac-lethal mutation in the axolotl is a valuable model system for studying heart induction. While it may not be a mutation which affects the initial induction of the heart-forming mesoderm, as suggested by some (Lemanski *et al.*, 1977; 1979; Hill and Lemanski, 1979; Davis and Lemanski, 1987; Jacobson and Sater, 1988; Sater and Jacobson, 1989), it may provide evidence for: i) a previously unsuspected second step required for the final cytodifferentiation of cardiomyocytes, and/or ii) the existence of a specific inhibitor of this differentiation.

1.5 Nature of this study:

As indicated previously, while Jacobson and Duncan (1968) have described some of the interactions affecting heart differentiation in *Taricha torosa*, and have briefly indicated that they occur even earlier in *Ambystoma tigrinum*, the parameters governing heart induction in the axolotl (*Ambystoma mexicanum*) have never been examined. Of particular importance is the timing of the induction in wild-

type axolotl embryos, since such information is vital in interpreting studies of the c mutant (see Section 1.4).

Therefore, the first part of this project was designed to elucidate the nature and timing of the inductive interactions responsible for heart induction in the axolotl. Subsequent to this, two complementary studies were initiated. The first was an examination of the nature of the inducing agent responsible for initiating cardiomyocyte differentiation, with a view to characterizing and (if possible) isolating it. The second was a re-examination of the nature of the defect in cardiac-lethal mutant embryos. In this way, the mechanisms involved in controlling the later stages of cardiomyocyte differentiation could also be determined.

CHAPTER II

MATERIALS AND METHODS

2.1 Animals:

All embryos were obtained from spawnings between adult axolotls (*Ambystoma mexicanum*) maintained at the University of Ottawa Axolotl Colony. Adults of known genealogy were kept in 50% Holtfreter's solution (Table 2-1) in individual plastic mouse cages or glass bowls. The solution was changed on alternate days, at which time the animals were fed strips of raw beef heart. Larvae and young juveniles were changed daily and fed live brine shrimp (*Artemia salina*) until they reached approximately 5 cm in length, at which time they were fed small pieces of beef heart and occasionally small larvae.

2.2 Spawnings and maintenance of embryos:

Spawning of animals was performed essentially as described by Armstrong *et al.* (1989). Briefly, females were injected with 250 I.U. of human chorionic gonadotropin (Sigma Chemical Co., St. Louis, Mo., USA) on the evening prior to spawning. Occasionally, males were also injected. The following morning, the animals were placed together (in pairs) in plastic dish pans containing 25% Holtfreter's solution and several rocks (to facilitate deposition of

TABLE 2.1

Culture Media

*Holtfreter's medium:**

NaCl	3.46 g
KCl	0.05 g
$CaCl_2$	0.10 g
$MgSO_4 \cdot 7H_2O$	0.20 g
$NaHCO_3$	0.20 g

per litre dechlorinated tap water.
pH 7.4.

Steinberg's medium:

NaCl	3.40 g
KCl	0.05 g
$CaCl_2$	0.05 g
$MgSO_4 \cdot 7H_2O$	0.21 g
Tris	0.56 g

per litre distilled water.
pH 7.7.

* Use 50% for adults and larvae, 25% for embryos.

spermatophores and fertilized eggs), and spawning was allowed to proceed normally.

Eggs were deposited within 24 hours of spawning. These were collected and sorted, and fertile eggs were placed in glass finger bowls containing 25% Holtfreter's medium supplemented with 100 mg/L each penicillin and streptomycin sulphate. Since the rate of development can be slowed by cooling, embryos were kept at 20°, 10°, or 4°C, as required. This facilitated obtaining the desired developmental stages and coordinating the development of embryos from separate spawnings. However, prior to the beginning of neurulation, embryos were never placed at temperatures lower than 10° C, since lower temperatures sometimes cause abnormal gastrulation.

Embryos were staged according to the normal tables of Bordzilovskaya and Dettlaff (1979; see also Bordzilovskaya *et al.*, 1989; Appendix I). Note that these tables are comparable (usually ± 1 stage) to the tables of Twitty and Bodenstein (in Rugh, 1962), Harrison (1969), and Schreckenberg and Jacobson (1975) used by other workers (Table 2-2).

TABLE 2.2

Comparison of Developmental Staging Tables*

Taricha torosa Twitty and Bodenstein	*A. maculatum* Harrison	*Ambystoma mexicanum* Schreckenberg and Jacobson	*Ambystoma mexicanum* Bordzilovskaya and Dettlaff
1	1	1	1
---	---	---	2-
2-7	2-7	2-7	2-7
8	8	8	---
---	---	---	8
9	9(?)	8+	9/9.5
10	10/10-	9	10
---	---	10	10.5
11	11	11	10.75
12	---	---	11
---	---	---	11.5
13	---	12	12
---	12	---	---
---	---	13	12.5
---	13	---	13-
---	13+	14	13
14	14	14+	14
15-22	15-22	15-22	15-22
---	23	23	23
23	24	24	24
---	25	---	25
24	26	---	26
25	27	25	27
26	28	26	28
27	29	27	29
28(?)	---	28(?)	---
29(?)	---	29(?)	---
30-32	30-32	30-32	30-32
---	33	33	33
33	34	---	34
34	35	34	35
35	35	35	35
36	36	36	36
---	36	37	36
37	37	38	37
38	38	39	38
39	?	---	39
---	?	---	40
40	?	40	41

* See text for references.

2.3 Preparation of embryos for surgery:

Surgical instruments (sharpened watchmakers' forceps, tungsten needles, hair loops, glass ball tips, glass bridges, transfer pipettes, and operating dishes) were prepared and sterilized as previously described (Asashima *et al.*, 1989; Graveson, 1990).

Immediately prior to surgery, embryos were mechanically divested of their jelly coats and vitelline membranes using sharpened watchmakers' forceps, and passed through 3 rinses of filter-sterilized 100% Steinberg's medium (Table 2-1) containing 50 µg/ml gentamycin sulphate. Subsequently, the embryos were transferred to operating dishes lined with a 4:1 (w:w) mixture of permoplast (American Art Clay Co., Indianapolis, Ind., USA) and paraplast (Lancer, St. Louis, Mo., USA). Depressions in this lining were made with glass ball tips to hold the embryos during surgery.

2.4 Transplantations:

In this study, explants were always taken from embryos involved in the transplantation operations. For this reason, all transplants were performed in 100% Steinberg's medium (with gentamycin) rather than in the calcium-free Steinberg's solution usually used for transplantations (see Asashima *et al.*, 1989).

To transplant heart mesoderm in early (stage 20) embryos, the overlying ectoderm was first peeled back on the donor embryo. This was accomplished by making incisions just ventral to the cranial neural fold and along a line running ventrally from the junction of the cranial and trunk neural folds, and then pulling the resulting triangular piece of ectoderm antero-ventrally. In this way, the heart mesoderm could be exposed without cutting the antero-ventral ectoderm under which the mesoderm subsequently migrates. The ectoderm was then similarly retracted from the host embryo, and the appropriate area of mesoderm was removed and placed in a depression in the operating dish to heal (see Section 2.4). The same area of mesoderm was then removed from the donor embryo and placed, in the same orientation, in the host, after which the host ectoderm was replaced over the graft and held in place with a glass bridge. If the donor embryo was also to be kept, its ectodermal flap was similarly replaced. After 15-30 min, the glass bridge was removed, and the embryo was left undisturbed for a further 30-60 min to allow healing to continue. Subsequently, the embryos were transferred to disposable plastic 24-well tissue culture dishes lined with sterile 1% or 1.5% Noble agar and containing either 100% Steinberg's or 25% Holtfreter's medium (supplemented with gentamycin). If Steinberg's medium was used, it was changed to 25% Holtfreter's solution after 1-2 days.

When heart area transplants were performed on stage 28-29 embryos, the overlying ectoderm was usually completely removed from the host embryo prior to explanting the heart mesoderm. Ectoderm and mesoderm from the donor were then removed together and placed on the host to heal as described above. This procedure allowed for better healing at these stages than leaving the host ectoderm intact. The results were the same whether donor or host ectoderm was used.

Following surgery, the embryos were checked every 1-2 days for the presence of a beating heart and circulation. The embryos were kept until they died. Those which survived were fed brine shrimp, as described previously (Section 2.1).

2.5 Anaesthetic:

When it was necessary to closely observe or photograph older embryos (after about stage 37) or larvae, they were anaesthetized. This was accomplished using a stock solution of 0.5% benzocaine in 95% ethanol which was diluted to 0.005% in Steinberg's or Holtfreter's medium immediately prior to use (Vanable, 1985).

2.6 Explantation cultures:

Heart area mesoderm and any other tissues required were removed from embryos of the appropriate stages and placed in small depressions in the lining of the operating dish. These tissues were allowed to heal into a ball and let injured cells fall away. The explant cultures were then transferred to small (10-20 µl) drops of sterile 100% Steinberg's medium on the undersides of the lids of 60 mm disposable plastic Petri dishes. The lids were then inverted over the lower parts of the dishes (which contained a few ml of sterile water) and sealed with petroleum jelly (Vaseline). This provided a sterile, humid environment for the hanging drops. The drops were maintained at room temperature (18°-20°C) for two weeks and were checked daily with a dissecting microscope for the presence of spontaneously pulsating cells. This provided a relatively easy assay system for the presence of fully differentiated, functional myocardial cells.

Wild-type embryos younger than stage 28 provide two areas of heart mesoderm each (one from each side; see Section 1.1) and, as often as possible, control and experimental cultures were taken from the opposite sides of the same embryos. In all cases, control cultures were taken from embryos of the same spawnings. When *c/c* embryos were used, only one heart area was removed from each embryo, and the

embryos were allowed to heal and develop until their phenotypes could be identified with certainty. This was necessary since c/c embryos cannot be distinguished from their +/c and +/+ siblings prior to the onset of heartbeat at stage 35.

2.7 Vital staining of explanted tissues:

When it was necessary to distinguish between two tissues in the same culture, one of the explants was vitally stained prior to placing it in culture with the other. This was accomplished by transferring the explanted tissue through a solution of Neutral Red (0.01% in 100% Steinberg's medium, 5-10 min) followed by 3 washes of medium without the stain. In this way, the development of tissues from different donor embryos could be followed even though they often healed into single balls in culture.

2.8 Preparation and assay of conditioned media:

To produce media conditioned by pharyngeal endoderm, the tissues were surgically removed from embryos of the appropriate stages and transferred to BEEM capsules (J.D. EM Services, Montréal, Qué.) containing 10 µl of 100% Steinberg's medium (with gentamycin) per endoderm. These cultures were allowed to incubate for 2-3 days at room temperature (18°-20°C). At this time, the medium was

decanted into sterile 1.5 ml microfuge tubes and centrifuged to remove any particulate matter (5-15 min in a benchtop microfuge). The medium was then immediately assayed for inductive activity by using it as the culture medium for hanging drop cultures (10 µl per drop) containing the appropriate test tissue. This is similar to the method described by Davis and Lemanski (1987).

2.9 Preparation and assay of tissue homogenates:

Tissue homogenates were produced by surgically removing the appropriate tissues from embryos of the desired stages. These were transferred to sterile glass tissue homogenizers containing 10 µl of 100% Steinberg's medium (plus gentamicin) per tissue fragment. The tissues were immediately homogenized with 20 strokes of the pestle, transferred to sterile 1.5 ml microfuge tubes and centrifuged to remove large particulate matter (5 min in a benchtop microfuge). The homogenates were then assayed for inductive activity by using them as culture media for hanging drop cultures containing the appropriate test tissue. Each drop contained 10 µl of homogenate.

2.10 Preparation and assay of embryonic RNA:

RNA extracts from embryonic endoderm were prepared using the procedure of Berger (1987). Briefly, the desired emdoderm was surgically removed from embryos of the appropriate stages and homogenized in a glass tissue homogenizer containing ACE extraction buffer (8 mM sodium acetate, 2.4 mM EDTA, 40 mM NaCl, final concentrations) with 2% SDS. The homogenate was extracted with phenol, phenol-chloroform, and chloroform, and the RNA was precipitated from ice-cold 95% ethanol. The RNA was stored at -20°C in ethanol until needed, whereupon it was lyophilized to remove the ethanol, and resuspended in 100% Steinberg's medium at a concentration of one endoderm equivalent per 10 µl. To assay the activity of each preparation, 10 µl aliquots were used as the culture medium for hanging drop cultures containing the appropriate test tissue. One aliquot from each preparation was also run on a 1% agarose preparative gel to verify the presence of RNA.

2.11 Analysis of data from explantation cultures:

In most cases involving only wild-type tissues, approximately 30 explants from at least 3 different spawnings were used. In experiments involving c/c embryos, surgery was usually performed before these embryos could be distinguished

from their *c/+* and *+/+* siblings. Therefore, sufficient cultures were started to provide approximately 10 containing *c/c* tissues.

Both the proportion of cultures which began beating, and the time (number of days) for these cultures to begin beating was recorded. In cases where there were differences in one of these parameters and not the other (usually cultures containing *c/c* tissues), only these data (± SE) are shown. However, in most instances the data for each type of culture were pooled, and analyzed using a modification of the heart differentiation coefficient (HDC) of Jacobson and Duncan (1968). This is defined as:

$$HDC = 3 \times \frac{\text{percent cultures which began to beat}}{\text{mean number of days to begin beating}}$$

The factor "3" reflects the number of days required (at 18–20°C) for an intact embryo to go from anywhere in neurulation to stage 35, when heartbeat commences. Therefore, a HDC of 100 would indicate that 100% of the cultures began beating on the third day (as would be expected *in situ*). Any decrease in the HDC thus reflects a decrease in the proportion of cultures which contain beating cells and/or an increase in the average time for these cultures to begin to beat.

As a measure of error, Jacobson and Duncan (1968) used

an arbitrary "relative confidence factor" calculated as follows:

$$\text{Confidence interval} = \frac{50}{n}$$

where "n" was the number of cultures. However, this has not been shown to have any statistical or biological significance. Therefore, the standard error of the mean (SE) was calculated for both component factors of the HDC, and these were subsequently used to calculate the SE of the HDC.

2.12 Transmission electron microscopy:

To observe the ultrastructure of the beating cells in culture, explants were fixed overnight at room temperature in 2% paraformaldehyde, 2.5% glutaraldehyde, 0.03% picric acid in 0.1 M sodium phosphate buffer, pH 7.4. The cultures were then rinsed in buffer (3 times 20 min), post-fixed in OsO_4 (1% in phosphate buffer, 1 hour), dehydrated in a graded ethanol series (50%, 70%, 80%, 90%, 5 min each; 95%, 15 min; 3 times 100%, 30 min each) and propylene oxide (3 times 30 min each), and embedded in an Epon substitute (JEMBED 812, J.B. EM Services, Montréal, Qué.). Thin sections were stained with uranyl acetate (2% in 70% methanol, 10 min) and lead citrate (J.B. EM Services; 0.02% in 1 N NaOH for 2-3 min; see Venable and Coggeshall, 1965),

and observed with a Philips 201 transmission electron microscope operated at 60 kV.

CHAPTER III

HEART INDUCTION IN WILD-TYPE AXOLOTLS

As described previously (Chapter I), the interactions responsible for the induction of the heart-forming mesoderm are only poorly understood for any system, and have not been examined at all in the Mexican axolotl (*Ambystoma mexicanum*). Of particular importance is the timing of these interactions in the axolotl, since it has important implications for the interpretation of studies on the cardiac-lethal (c) mutant. Therefore, the first portion of this study consists of an elucidation of the timing and tissues involved in heart induction in wild-type axolotl embryos. Additionally, possible cell-free sources of the inducing agent(s) were examined. Portions of this study have been reported previously (Smith and Armstrong, 1990).

3.1 Timing of heart induction:

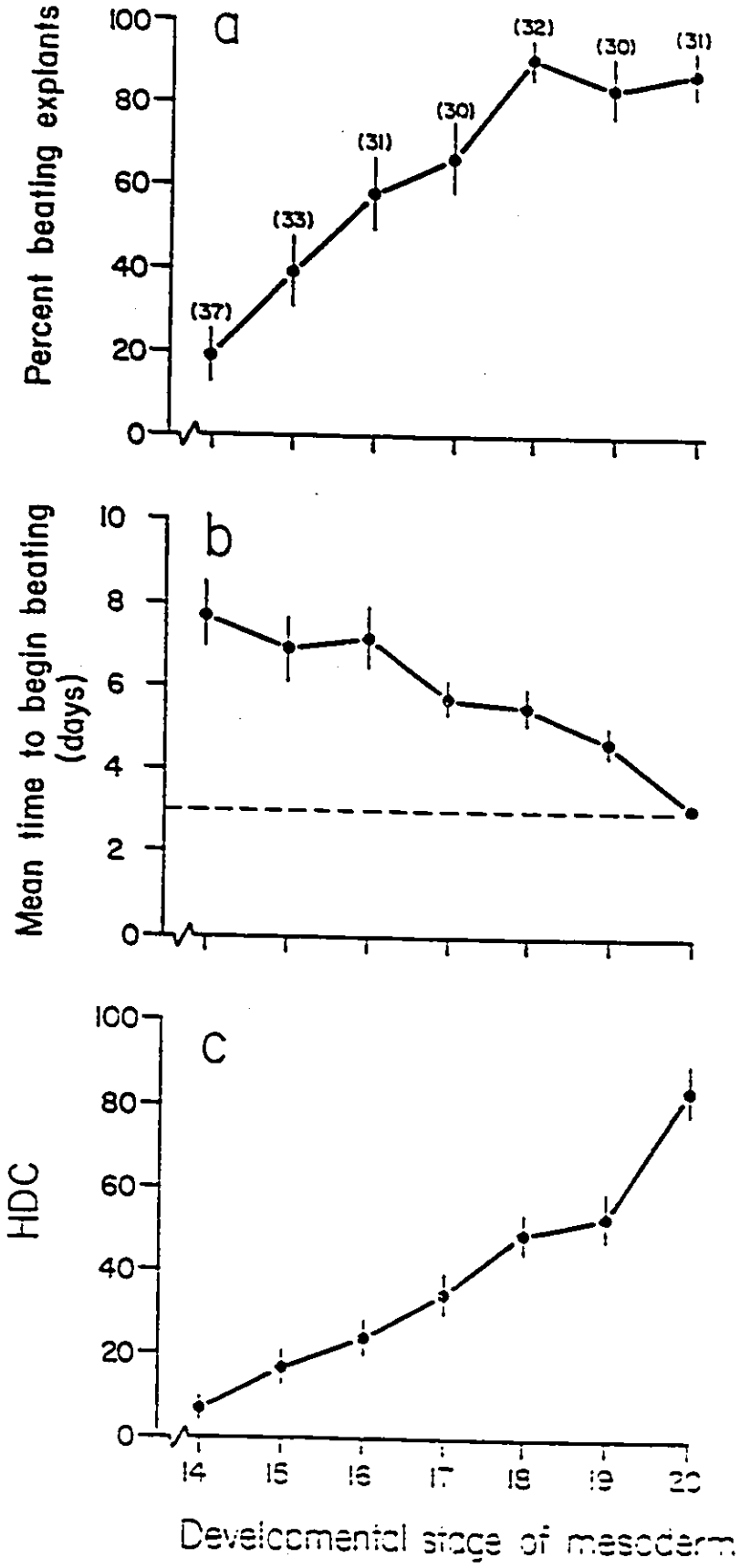
The first step in defining the parameters governing heart induction was to determine when heart-forming mesoderm was able to differentiate into a functional myocardium in the absence of further inductive stimuli. Therefore, heart-forming mesoderm was surgically removed from embryos at different stages of neurulation (stages 14-20) and cultured

alone (Fig. 3-1). Mesoderm removed from early neurulae (stage 14) has only a low heart differentiation coefficient (HDC). This increases gradually, and reaches a maximum at the end of neurulation (stage 20). At this time, the ability of the explanted heart-forming mesoderm to differentiate into a functional (beating) myocardium approaches that observed *in vivo*. The increase in the HDC is the result of both a gradual increase in the proportion of cultures which began to beat (Fig. 3-1a) and a decrease in the time required for them to begin beating (Fig. 3-1b). This strongly suggests that, in the axolotl, the heart-forming mesoderm is gradually induced, and is fully induced and capable of completing its developmental repertoire as early as the end of neurulation.

The low HDC of stage 14 heart mesoderm makes it an ideal test tissue for inductive interactions. By culturing this tissue with other tissues and substances, the inductive potential of the latter can be determined. Any tissue or substance which increases the HDC of stage 14 heart-forming mesoderm (relative to control cultures) would, presumably, contain inductive ability. Similarly, stage 20 heart mesoderm makes an ideal test tissue for inhibitory interactions. Any tissue or substance which decreases the HDC of this tissue would presumably contain an inhibitor of heart differentiation.

It should be noted that left and right heart primordia

Figure 3-1: a) Proportion of explant cultures which began beating within 14 days *in vitro*. All cultures consisted of heart mesoderm removed at the appropriate stage and cultured alone. b) Average time required for the cultures shown in (a) to begin beating. The broken line at 3 days represents the time required for heart differentiation to be completed *in vivo*. c) Heart differentiation coefficients of these cultures, derived from (a) and (b) as described in Section 2.11. The numbers in parentheses at the top of this and subsequent figures is the number of cultures used to obtain the result. All points ± standard error (SE).


a
Percent beating explants
100
80
60
40
20
0
(37)
(33)
(31)
(30)
(32)
(30)
(31)
b
Mean time to begin beating (days)
10
8
6
4
2
0
c
HDC
100
80
60
40
20
0
14
15
16
17
18
19
20
Developmental stage of mesoderm

were cultured separately, in approximately equal numbers, in this and all subsequent assays. The ability to form functional myocardia was independent of the side from which the primordia were removed. As well, it should be noted that the entire heart field was removed, since embryos (from which both heart areas had been explanted) which were allowed to heal and develop were incapable of forming hearts from the remaining mesoderm.

To verify that the beating tissue observed was, indeed, myocardial tissue, mesoderm from four distinct locations (Fig. 3-2) was removed from stage 20 embryos and cultured separately. Only mesoderm from the presumptive heart area (as determined by Wilens, 1955) was ever observed beating (Table 3-1). Therefore, the beating tissue observed in these cultures presumably could not be due to the formation of, for example, skeletal muscle by other mesoderm included in the explant. Furthermore, the failure of other mesoderm to form beating tissue in culture indicates that this mesoderm does not normally undergo the inductive interactions necessary to form heart tissue. This suggests that the inductive interaction is spatially restricted, by having only a limited area of endoderm with inductive potential, and/or a limited area mesoderm capable of responding.

The ultrastructure of beating tissue in cultured heart mesoderm from stage 20 embryos was also examined. Maturing

Figure 3-2: Diagram of the right side of a stage 20 embryo (with the ectoderm removed), showing the regions of mesoderm (stippled) explanted to test for the ability to form rhythmically contracting tissue *in vitro*. The areas are: somitic (s), presomitic (ps), posterior lateral plate (lp), and heart area (h) mesoderm. A: anterior; P: posterior.

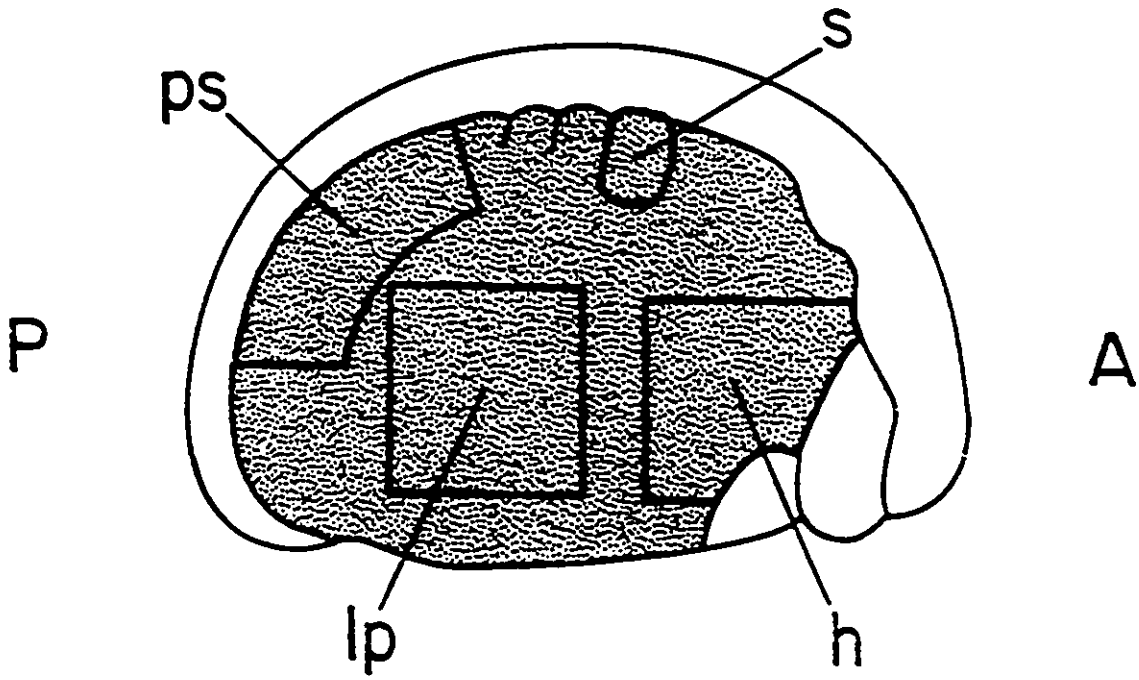

s
ps
P
A
lp
h

TABLE 3-1

Ability of Different Areas of Mesoderm to Begin Beating*

Type of Mesoderm	Number of Cultures	Percent Beating (±SE)
Heart area	30	96.7 ± 3.3
Somitic	30	0 ± 0
Pre-somitic	30	0 ± 0
Posterior lateral plate	30	0 ± 0

* All tissues removed from the same stage 20 embryos.

sarcomeric myofibrils were found in these cultures as early as 5 days after explantation (Fig. 3-3a, c).

3.2 Distribution of inductive tissues:

While it has long been known that anterior (pharyngeal) endoderm is the major inductor of heart differentiation, it is unclear whether the inducing activity is a ubiquitous property of all anterior endoderm (as suggested by Fullilove, 1970), or if the activity is localized (see Section 1.2). Therefore, the distribution of inductive ability in the endoderm was determined. This was accomplished by co-culturing stage 14 heart mesoderm (which has only a low HDC; Fig. 3-1) with endoderm from different areas of stage 14 embryos (Fig. 3-4).

Unlike the reports of previous workers (Jacobson and Duncan, 1968; Fullilove, 1970; see Section 1.2), the ability to induce myocardial differentiation was found to be distinctly localized within the endoderm (Fig. 3-5). The best inductor was mid-ventral pharyngeal endoderm. This is the tissue which lies adjacent to the fully-formed heart at later stages. Mid-lateral pharyngeal endoderm, over which the mesodermal primordia migrate during neurula and early tailbud stages, was also capable of inducing the differentiation of heart mesoderm, albeit to a lesser extent. The third area of pharyngeal endoderm tested (from beneath

Figure 3-3: Electron micrographs of portions of cells found in 5-day-old cultures of stage 20 heart mesoderm (a, c) and 10-day-old cultures of stage 14 heart mesoderm cultured with inductive (mid-ventral) endoderm (b, d). Cells containing developing sacromeric myofibrils are found in both types of culture (a, b). Cross-sectioned fibrillar bundles display the hexagonal arrangement of myofibrils (c, d). Z: Z-band material associated with fibrils. Bars = 0.1 μm.

Figure 3-4: Diagram of the right side of a stage 14 embryo, showing the areas of endoderm tested for inductive activity. Uninduced (stage 14) heart mesoderm was cultured with endoderm from the roof (r), mid-lateral (ml) or mid-ventral (mv) walls of the pharyngeal cavity, or with yolky (y) endoderm from the flank. The broken line indicates the approximate anterior limit of the mesoderm. A: anterior; P: posterior.

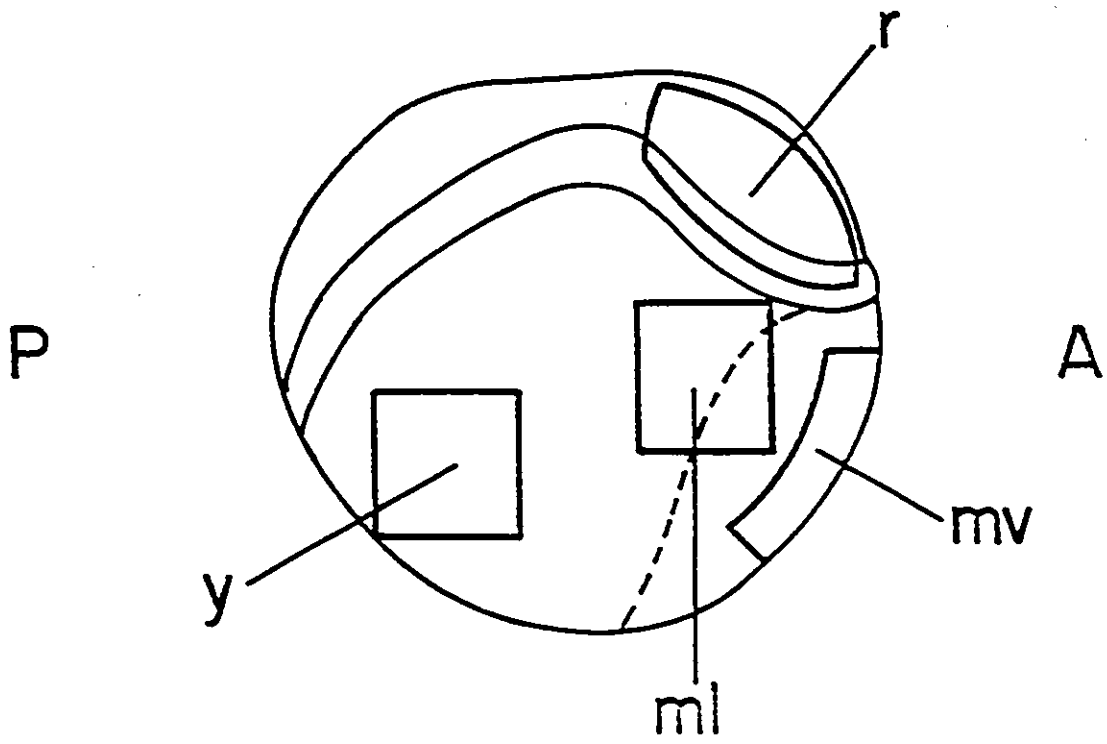
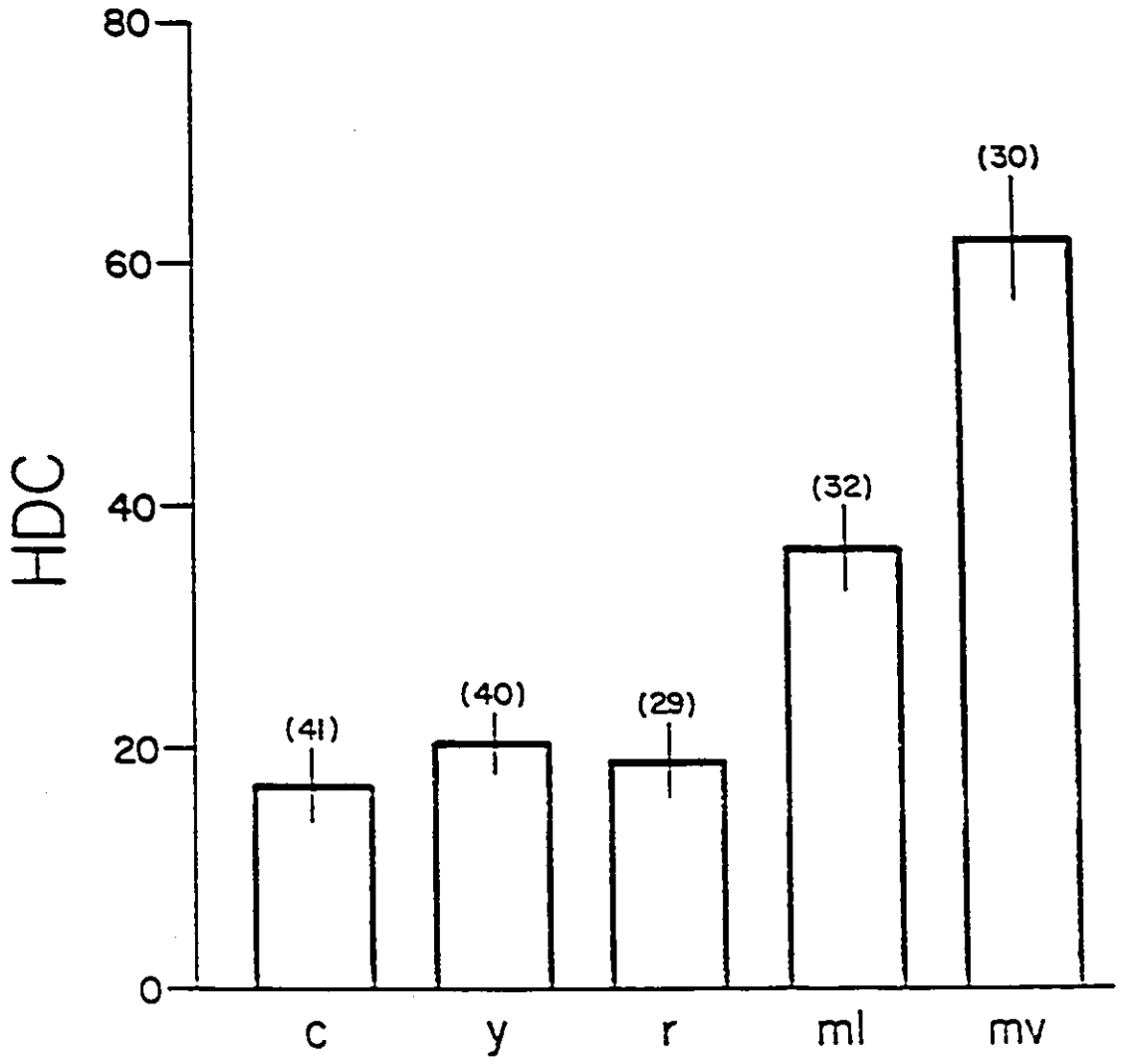

r
P
A
y
mv
ml

Figure 3-5: Inductive ability of different regions of endoderm. The HDC of stage 14 mesoderm, cultured with endoderm from the areas depicted in Fig. 3-4, are shown. c: control mesoderm cultured alone; all other abbreviations as described in Fig. 3-4.


HDC
80
60
40
20
0
(41)
(40)
(29)
(32)
(30)
c
y
r
ml
mv

the neural plate), and non-pharyngeal (yolky) endoderm from the flank contained little, if any, inductive ability.

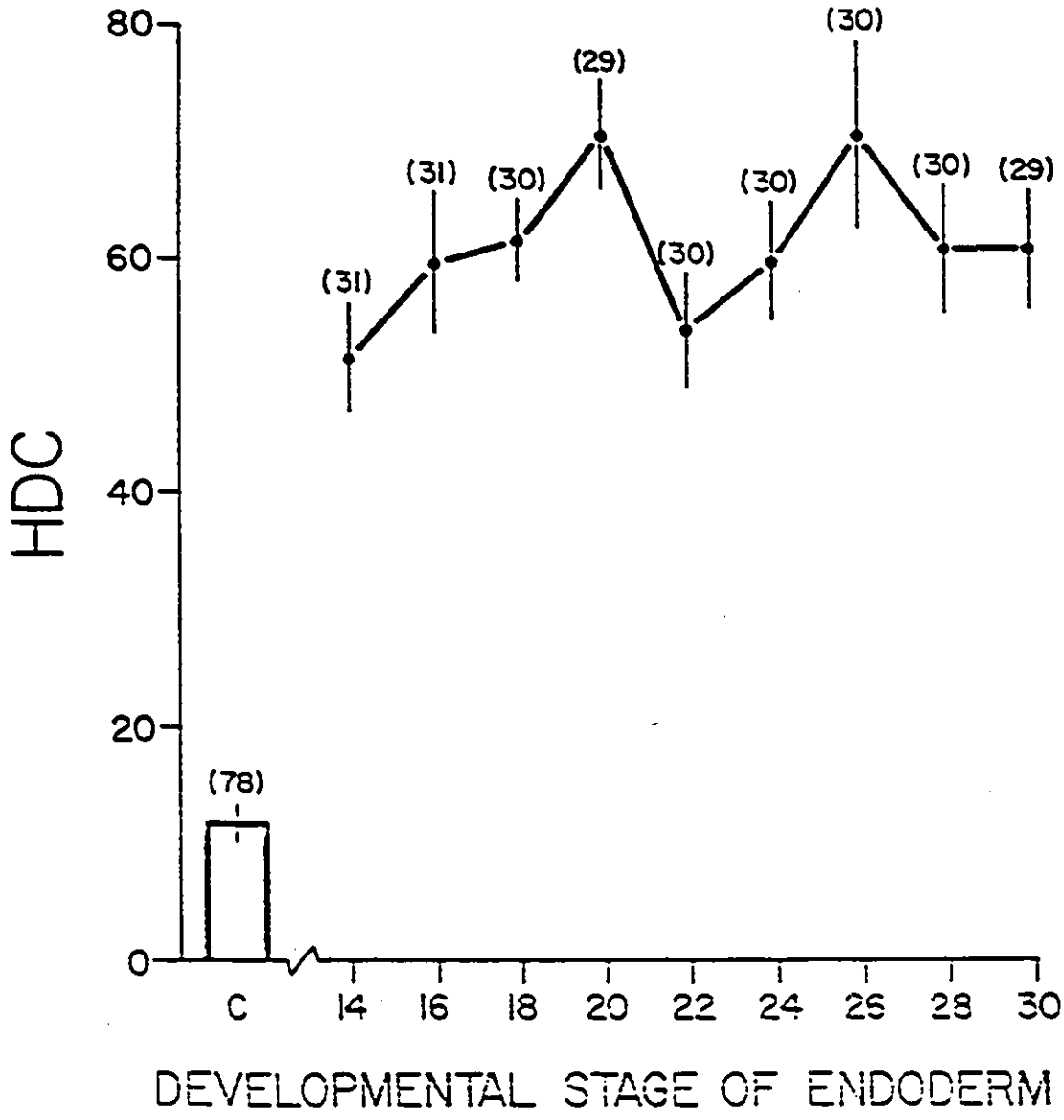
When the ultrastructure of this mesoderm induced *in vitro* was examined, developing sarcomeric myofibrils were again observed (Fig. 3-3b, d).

3.3 *Timing of the inductive interaction:*

Subsequent to determining the distribution of the inductive activity in early neurulae, the persistence of the inductive activity was determined. This was accomplished by culturing uninduced (stage 14) heart-forming mesoderm with endoderm from embryos of different stages. In all cases, mid-ventral (future heart site) endoderm was used. Surprisingly, the inductive ability of this endoderm was found to remain relatively constant over a wide range of neurula and tailbud stages (Fig. 3-6).

Since the inductive period of the endoderm does not appear to be regulated, the timing of the mesodermal ability to respond to the induction was also determined. Heart-forming mesoderm was removed from stage 14 embryos and cultured alone for 1-2 days. This effectively isolated the tissue from the inductive influences it would normally have experienced *in situ*. After 1 and 2 days, mid-ventral pharyngeal endoderm taken from embryos of the appropriate developmental stage (stages 24-25 and 29-30, respectively)

Figure 3-6: Inductive ability of mid-ventral pharyngeal endoderm from embryos of different stages. The the HDCs of stage 14 heart mesoderm, cultured with endoderm from embryos of the appropriate stages, are shown. c: control cultures of stage 14 mesoderm alone.


80
60
40
20
0
HDC
(78)
(31)
(31)
(30)
(29)
(30)
(30)
(30)
(30)
(29)
C
14
16
18
20
22
24
26
28
30
DEVELOPMENTAL STAGE OF ENDODERM

was added to some of these cultures. As shown in Table 3-2, the responsiveness of the heart-forming mesoderm decreased by approximately half during post-neurula development. While diminished, the ability to respond was never completely eliminated, however.

3.4 *Inhibitory interactions:*

Jacobson (1960; 1961; Jacobson and Duncan, 1968) reported that the presence of neural tissues could inhibit heart differentiation. However, some question remains as to whether such an inhibitor really exists, since its effects could not be detected in explant cultures containing neural tissues or extracts of it (Jacobson and Duncan, 1968; see Section 1.2).

Therefore, various tissues were assayed for their ability to inhibit the differentiation of heart-forming mesoderm *in vitro*. The tissues (all from stage 20 embryos) were tested by placing them in explant cultures containing fully induced (stage 20) heart mesoderm. None of the tissues tested was able to prevent heart mesoderm from forming beating tissue (Table 3-3). Cranial neural fold and, to a lesser extent, cranial neural plate were able to delay the onset of beating slightly, but neither could prevent differentiation from occurring. Therefore, little inhibitory activity could be detected in any of the tissues tested.

TABLE 3-2

Timing of Mesodermal Responsiveness

Time Before Addition of Endoderm (Days)*	Number of Cultures	HDC ± SE
0	30	61.6 ± 5.1
1	31	32.3 ± 5.0
2	32	34.5 ± 3.9
control	33	11.9 ± 2.9

* Heart-forming mesoderm was explanted from stage 14 embryos and cultured alone for the indicated number of days before the addition of mid-ventral endoderm of the appropriate stage. 0 = stage 14; 1 = stage 24-25; 2 = 29-30; control = no endoderm.

TABLE 3-3

Inhibitory Ability of Various Tissues*

Tissue	Number of Cultures	Percent Beating (± SE)	Mean Time to Begin Beating (Days ± SE)	HDC ± SE
Nil (control)	22	95.5 ± 4.4	3.1 ± 0.2	92.4 ± 7.3
Cranial neural plate	12	100 ± 0	3.8 ± 0.2	78.9 ± 4.2
Cranial neural fold	12	100 ± 0	4.3 ± 0.5	69.8 ± 8.1
Placodal ectoderm	12	100 ± 0	2.8 ± 0.1	107.1 ± 3.8
Belly ectoderm	12	100 ± 0	3.1 ± 0.2	96.8 ± 6.2
Anterior notochord	10	90.0 ± 9.5	3.3 ± 0.4	81.8 ± 13.2

* Stage 20 heart mesoderm was cultured with the appropriate tissue from stage 20 wild-type embryos.

Interestingly, placodal (otic area) ectoderm, as defined by Smith *et al.* (1988), slightly increased the rate at which heart mesoderm differentiates. No increase occurred when non-placodal (belly) ectoderm was used. Although the increase in the rate of differentiation was slight, it may indicate that the "general stimulatory factor", which Jacobson and Duncan (1968) reported as being present in ectoderm, may also have a restricted distribution.

3.5 Characterization of the inductive agent:

Once the spatial and temporal distribution of the inducer had been determined, the next logical step was to attempt to determine the nature of the inductive agent(s). Therefore, three series of experiments were designed to test cell-free extracts of inductive endoderm for the presence of inductive activity. This was accomplished using medium conditioned by inductive endoderm, whole cell homogenates of endoderm, and RNA preparations from this tissue.

Conditioned media:

Media conditioned by anterior endoderm have previously been reported to be capable of "inducing" stage 35 hearts from c/c embryos to begin beating (Davis and Lemanski, 1983; 1987). Therefore, it was of interest to test similar preparations for their ability to stimulate uninduced heart mesoderm. Media conditioned by inductive endoderm were produced as previously described (Section 2.7). In cases where stage 14 or 20 endoderm was tested, the entire area of inductive endoderm (mid-ventral and both lateral pharyngeal areas) was removed from the donor embryos. For stage 28 endoderm, only the mid-ventral area was used.

As shown in Table 3-4, few cultures of uninduced (stage 14) heart-forming mesoderm formed beating tissue when cultured in the conditioned media. This suggests that no inductive activity is present in such media.

Tissue homogenates:

As well as conditioned media, homogenates of inductive endoderm and other tissues were tested for the presence of inductive activity. In all cases, tissues from stage 14 embryos were homogenized as described in Section 2.8. Again, inductive endoderm included all mid-ventral and lateral pharyngeal endoderm. In addition to this, non-inductive

TABLE 3-4

Efects of Media Conditioned by Inductive Endoderm[a]

Stage of Endoderm	Number of Cultures	HDC ± SE
Nil (control)[b]	31	6.3 ± 2.3
14	22	3.8 ± 2.1
28	8	0 ± 0

[a] Conditioned media produced as described in Section 2.7 and tested on heart mesoderm from stage 14 embryos.

[b] Control cultures were the heart mesoderm from the opposite sides of the same embryos as the experimental cultures.

yolky endoderm, posterior lateral plate mesoderm, and cranial neural plate ectoderm were homogenized as controls.

While homogenates of inductive endoderm appeared to have a slight inductive influence on stage 14 heart mesoderm, all of the homogenates tested had a similar effect (Table 3-5). This included homogenates of cranial neural plate, a tissue believed to have inhibitory properties (Jacobson and Duncan, 1968; Section 3.4). Therefore, the slight inductive effect of these preparations is presumably due to some non-specific property of all embryonic tissues, and not to the presence of a specific inducer of heart differentiation.

RNA preparations:

Finally, the inductive ability of RNA extracted from inductive endoderm was tested. Similar preparations cause fully-formed (stage 35) hearts from *c/c* embryos to begin beating (Davis and Lemanski, 1987). Based on this ability, it has been suggested that the RNA preparations contain the inducer of heart differentiation (Davis and Lemanski, 1987; see Section 1.4). Therefore, similar total RNA extracts were prepared from the inductive endoderm of stage 14 and 30 embryos as described in Section 2.9. Mid-ventral and both lateral areas of endoderm were used in all cases. These preparations were tested on both stage 35 *c/c* hearts (to ensure that the stimulatory activity described by Davis and

TABLE 3-5

Inductive Activity of Tissue Homogenates*

Homogenized Tissue	Number of Cultures	HDC ± SE
Nil (control)	123	6.9 ± 1.1
Inductive endoderm	39	24.4 ± 3.0
Yolky endoderm	32	23.1 ± 3.4
Posterior lateral plate mesoderm	30	17.3 ± 3.8
Cranial neural plate	40	28.1 ± 3.5

* Homogenates prepared as described in Section 2.8 and tested on stage 14 heart mesoderm.

Lemanski, 1987, was present), and on uninduced stage 14 heart-forming mesoderm.

Both RNA preparations caused c/c hearts to begin beating, although the preparation from stage 14 endoderm was somewhat less active (Table 3-6). However, neither preparation induced stage 14 heart mesoderm to form functional (beating) myocardial tissue. This clearly demonstrates that the RNA described by Davis and Lemanski (1987) cannot be the primary inducer of heart differentiation.

TABLE 3-6

Activity of Total RNA Preparations

Test Tissue (Stage)	Treatment*	Number of Cultures	Percent Beating (± SE)
c/c heart	Nil	7	0 ± 0
c/c heart	RNA-14	5	40 ± 22
c/c heart	RNA-30	5	80 ± 18
+/+ heart mesoderm	Nil	12	17 ± 11
+/+ heart mesoderm	RNA-14	10	0 ± 0
+/+ heart mesoderm	RNA-30	10	0 ± 0

* Explanted tissues were placed with RNA preparations from stage 14 (RNA-14) or stage 30 (RNA-30) inductive endoderm. The same preparations were tested on both tissues.

CHAPTER IV

CONTROL OF HEART DIFFERENTIATION IN THE CARDIAC-LETHAL MUTANT

The hypothesis that the *c* gene affects the inductive endoderm, and thus causes a failure to induce the (essentially normal) heart mesoderm is dependent on two lines of evidence. The first is the timing of the induction in another species (*Taricha torosa*; Jacobson and Duncan, 1968). The second is the interpretation of Humphrey's (1972) transplantation experiments based on this timing (see Sections 1.4 and 5.1).

However, I have demonstrated that wild-type heart mesoderm is fully induced and capable of differentiating in isolation as early as stage 20 (Section 3.1). This is well before the stages at which Humphrey (1972) performed his transplants of heart mesoderm (stages 27-29). These results suggest that, in Humphrey's (1972) experiments, wild-type hearts must have been actively inhibited from completing their development by the mutant environment. Furthermore, I have shown that total RNA preparations similar to those reported to be capable of "inducing" *c/c* hearts to begin beating (Davis and Lemanski, 1987) contain no detectable inductive activity when tested on uninduced stage 14 heart mesoderm (Section 3.5). This implies that there is no

inductive failure in *c/c* embryos, but rather that a later stage of heart differentiation is affected in the cardiac-lethal mutant. Therefore, a re-examination of heart induction and development in *c/c* mutants was conducted. Portions of this chapter have been described previously (Smith and Armstrong, 1990; Smith and Armstrong, manuscript submitted).

4.1 Explantation of c/c *heart mesoderm:*

Since it appeared probable that the differentiation of wild-type hearts was inhibited in *c/c* embryos, it was also possible that *c/c* hearts could be capable of completing their differentiation if isolated from their inhibitory environment. Therefore, heart area mesoderm was removed from one side of 52 stage 20 embryos from *+/c* X *+/c* spawnings and placed in hanging drop cultures. In this way, it was hoped that the fully induced mesoderm would be removed before it could be affected by any *in situ* inhibitor. The embryos were allowed to heal and develop until the *c/c* phenotype could be identified with certainty (as described in Section 2.6).

The results (Table 4-1) indicate that mesoderm isolated from *c/c* embryos seldom begins to beat *in vitro*. The single culture in which rhythmically contracting cells were observed contained only a few cells which beat for a short time (2-3 days). Most control cultures (from *+/c* and *+/+* siblings)

TABLE 4-1

Ability of Stage 20 c/c Heart Mesoderm to Begin Beating

Genotype of Embryo	Number of Cultures	Percent Beating (± SE)	Mean Time to Begin Beating (Days ± SE)	HDC ± SE
+/c & +/+[a]	43	97.7 ± 2.3	4.0 ± 0.2	74.2 ± 4.1
c/c	9	11.1 ± 10.5[b]	5.0 ± 0.0	6.7 ± 6.3

[a] Siblings of c/c embryos.

[b] A few pulsating cells were observed in one culture.

contained extensive areas of contractile tissue which began to beat vigorously within a few days after explantation, and continued to beat until the cultures were discarded.

4.2 Inductive ability of c/c endoderm:

The proposal that the cardiac-lethal mutation affects the pharyngeal endoderm (Lemanski *et al.*, 1977; 1979; Davis and Lemanski, 1987; Jacobson and Sater, 1988), but not the mesoderm, has never been tested directly. Therefore, the inductive capacity of mutant endoderm was examined by culturing a portion of it with uninduced (stage 14) wild-type heart mesoderm. Mid-ventral pharyngeal endoderm was used in all cases, and the embryos were allowed to heal and develop until their phenotypes could be positively identified.

The results show that wild-type heart mesoderm is as capable of differentiating into beating tissue when induced by *c/c* endoderm as when *+/+* endoderm is used (Table 4-2). This unequivocally demonstrates that cardiac-lethal mutant endoderm is as inductive as wild-type endoderm. Therefore, the failure of mutant hearts to begin beating cannot be caused by a deficiency of inductive stimuli produced by the pharyngeal endoderm.

TABLE 4-2

Inductive Ability of Mutant Endoderm[a]

Phenotype of Endoderm [b]	Number of Cultures	Percent Beating (± SE)
wt	29	86.2 ± 6.4
c	11	90.9 ± 8.7

[a] The mean time to begin beating was the same for both sets.

[b] wt = wild-type (+/c and +/+); c = mutant (c/c).

4.3 Ability of c/c heart mesoderm to respond to induction:

Since the ability of mutant endoderm to induce myocardial differentiation is clearly normal, the ability of *c/c* heart mesoderm to respond to normal induction was also examined. To accomplish this, heart area mesoderm was removed from one side of each of a series of embryos and placed in culture with a piece of endoderm from another donor. Both sets of donors were from +/c X +/c spawnings, and all were allowed to heal and develop until their phenotypes could be determined.

The results (Table 4-3) confirm that mutant endoderm is, indeed, capable of inducing wild-type heart mesoderm. Furthermore, they demonstrate that *c/c* heart mesoderm is unable to respond normally to the inductive signals produced by wild-type inductive endoderm.

4.4 Bilateral transplantation of heart area mesoderm:

The *c* mutation clearly affects the heart mesoderm directly, and not the inductive endoderm. Furthermore, the ability of mutant endoderm to induce heart differentiation *in vitro* suggests that this tissue cannot be the source of the inhibitor implied by Humphrey's (1972) heart transplantation experiments. This suggested that replacement of the heart area mesoderm in *c/c* embryos with wild-type mesoderm might

TABLE 4-3

Identification of Defective Mutant Tissue[a]

Phenotype [b]		Number of Cultures	Percent Beating (± SE)
Mesoderm	Endoderm		
wt	wt	43	93.0 ± 3.9
wt	c	12	100.0 ± 0.0
c	wt	10	10.0 ± 9.5 [c]
c	c	5	20.0 ± 17.5 [c]

[a] The mean time to begin beating was the same for all sets.

[b] wt = wild-type (+/c and +/+); c = mutant (c/c).

[c] A few cells were observed twitching for a short time.

provide a means of rescuing mutant animals if performed early enough.

Therefore, the heart area mesoderm was removed from both sides of a series of stage 20 embryos from +/c X +/c spawnings, and was replaced with heart mesoderm from stage 20 +/+ donors. The explanted mesoderms were placed separately in hanging drop cultures to identify the phenotypes of the host embryos. In this way, the donor mesoderm was fully induced, and the maximum time between surgery and the onset of heartbeat (3 days) was available for healing.

As shown in Table 4-4, beating hearts formed in most of the host embryos, including mutant ones. The hearts contracted rhythmically in an apparently normal manner, and circulation was established in most of the embryos. Once present, circulation continued until the embryos died.

The rescued *c/c* embryos did not exhibit most of the secondary characteristics associated with the mutant phenotype (Fig. 4-1). No edema was evident, and all had well-developed gills, normal pigmentation patterns, and morphologically normal head structure. However, one important mutant characteristic was retained by these embryos; they were unable to feed.

Some of the wild-type hosts had defects resulting from incomplete post-operative healing. This was apparent as misshapen opercula (often including missing primary gill

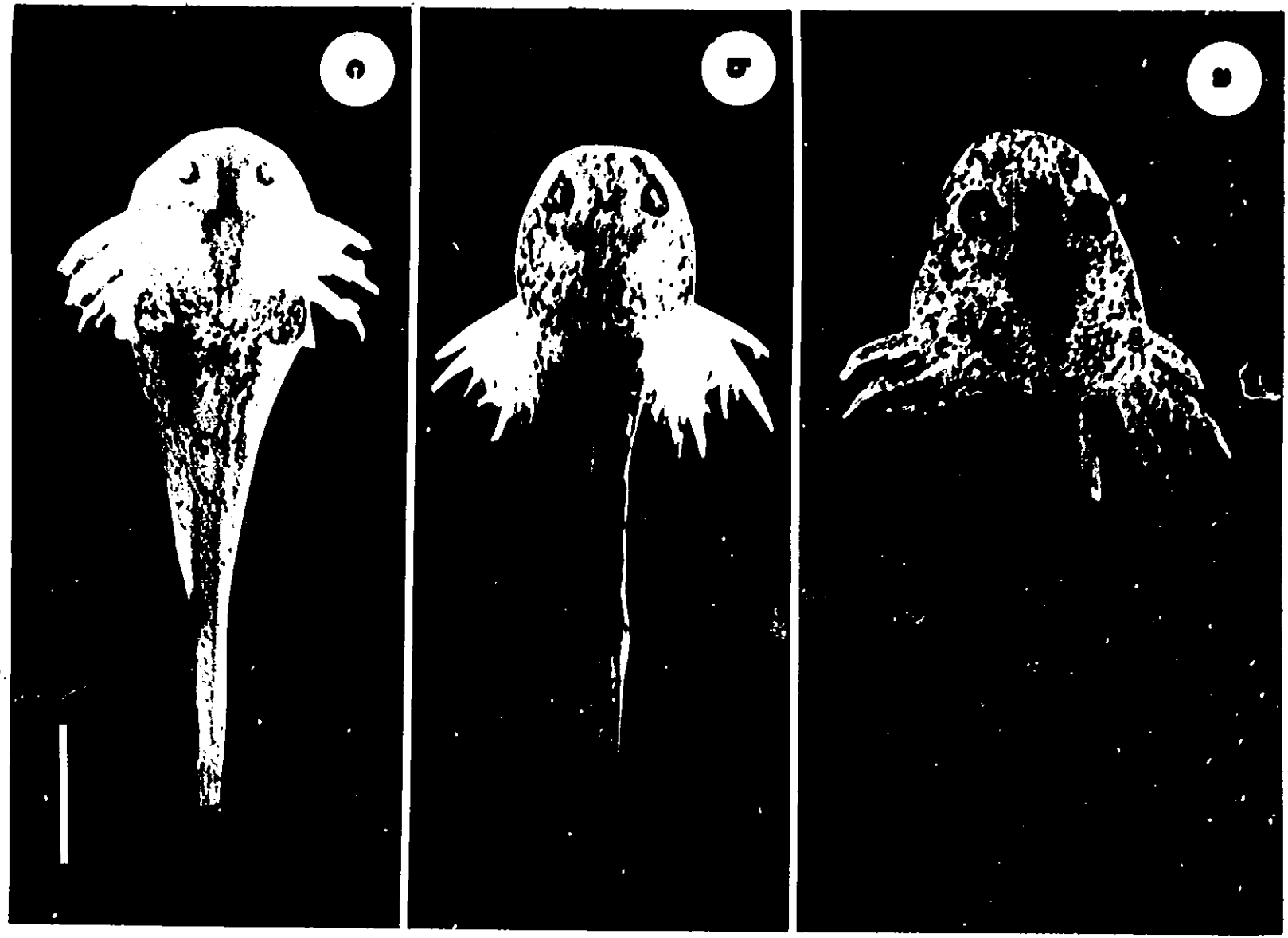
TABLE 4-4

Bilateral Transplantations of Heart Mesoderm

Host Phenotype	Number of Embryos	Number with Beating Heart	Number with Circulation *
wt	33	31	28
c	7	6	6

* Circulation continued until death in all embryos.

Figure 4-1: a) Dorsal view of a c/c larva containing a bilateral transplant of wild-type heart mesoderm. Circulation was present; note the absence of edema, improved gill development, and the normal pigmentation pattern. b) Wild-type larva. c) Unoperated (control) c/c larva. Anterior is to the left in all cases. Bar = 2 mm.


a
b
c

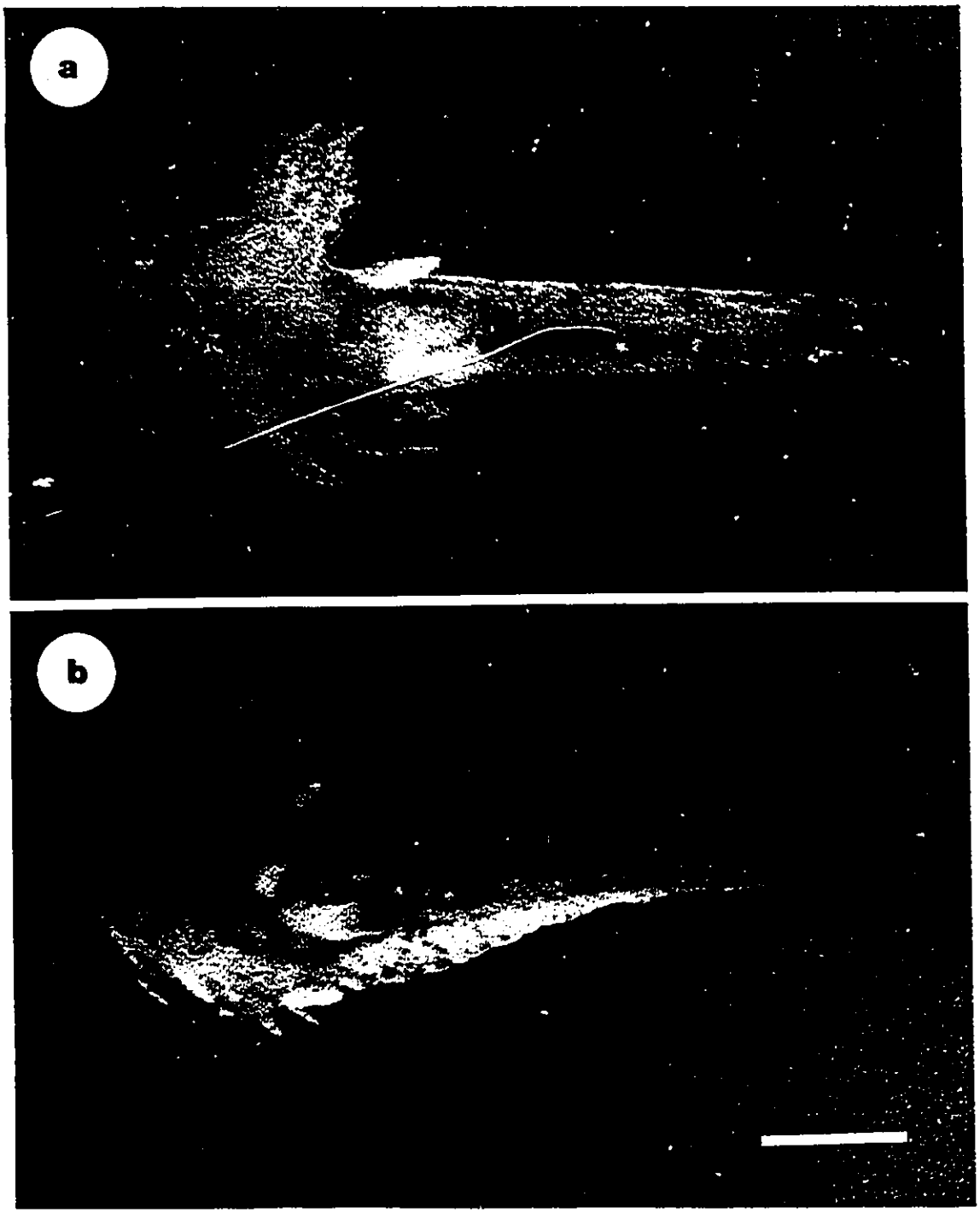
filaments) and slightly extruded organs (usually the liver) immediately posterior to the opercula. These embryos were often unable to feed. However, wild-type embryos without such obvious external defects were able to feed, and grew normally.

Of the 6 *c/c* hosts with normal circulation, 3 had such clearly visible surgical defects. But, as mentioned above, 2 of the 3 remaining *c/c* larvae were also unable to feed. Although brine shrimp were visible in their mouths, these embryos were apparently unable to swallow, and they starved to death. Only one of these larvae was able to swallow some food (although less than its wild-type counterparts), but this came from an atypical spawning, in that unoperated mutant larvae were also able to swallow a little food (Fig. 4-2). All of the *c/c* larvae containing wild-type heart mesoderm continued to have normal circulation but died within 2 weeks, due to their inability to feed.

4.5 Autoregulation of heart differentiation:

Since it was clear, from the preceding sections, that the heart mesoderm is directly affected by the *c* gene, the effects of co-culturing mutant and wild-type heart mesoderm were examined. To accomplish this, fully induced (stage 20) heart mesoderm was removed from one side of a series of stage 20 embryos from *+/c* X *+/c* spawnings. Half of the explants

Figure 4-2: a) Ventral view of a c/c larva containing a bilateral transplant of wild-type heart mesoderm. Of three such embryos, only this one was able to swallow. b) Control c/c embryo from the same spawning. This was an atypical spawning, in that the mutant embryos were able to feed a little. Brine shrimp are orange, and can be observed in the gut. Anterior is to the left in both cases. Bar = 2 mm.


a
b

were vitally stained (as described in Section 2.7), and placed in explant culture. Each culture contained one stained and one unstained mesoderm. In this way, the development of the tissues from each embryo could be followed, even though the tissues often healed into single balls in culture. As before, the donor embryos were allowed to heal and develop until their phenotypes could be determined.

As shown in Table 4-5, all of the mesodermal primordia formed beating tissue. Although the *c/c* explants took almost twice as long to begin beating, all of them did begin beating. This strongly suggests that wild-type heart mesoderm contains some factor capable of stimulating heart differentiation in *c* mutant mesoderm.

4.6 Unilateral transplantation of heart area mesoderm:

From the preceding section, it is obvious that wild-type heart area mesoderm provides sufficient stimulus to cause mutant mesoderm to begin beating *in vitro*. Therefore, the equivalent experiment was conducted *in vivo*. A series of unilateral transplantations of heart area mesoderm was performed on stage 20 embryos. By replacing the heart mesoderm on only one side, hearts containing both *c/c* and wild-type tissue would form when the two primordia fused at the ventral midline. In this way, it was hoped that the

TABLE 4-5

Stimulatory Activity of Wild-type Mesoderm

Tissues [a] Comb.	Ind.	Number of Cultures	Percent Beating (± SE)	Mean Time to Begin Beating (Days ± SE)
wt+wt	wt	24	100 ± 0	3.8 ± 0.2
wt+c	wt	10	100 ± 0	4.0 ± 0.5
	c	10	100 ± 0	6.3 ± 0.4
wt alone [b]		43	97.7 ± 2.3	4.0 ± 0.2
c alone [b]		9	11.1 ± 10.5 [c]	5.0 ± 0.0

[a] Comb. = tissue combination in culture; Ind. = individual members of each pair.

[b] After Table 4-1, for comparison.

[c] A few cells were observed twitching for a short time.

mutant embryos could still be partially rescued, but that surgical damage and the resulting complications could be minimized.

Both donor and host embryos were from +/c X +/c spawnings. Thus, the effects of placing a single c/c heart primordium into wild-type hosts could also be observed. The donor embryos (from which only one heart area had been removed), and the host heart mesoderm were maintained post-operatively to determine the phenotypes of the donors and hosts, respectively.

The results of these transplantations are shown in Table 4-6. In 7 cases, wild-type (+/c and +/+) heart mesoderm was transplanted into c/c hosts. Six of these embryos subsequently developed beating hearts. Of these, circulation of the blood was established in four. However, in all of these cases, circulation stopped within a few days. Although their hearts continued to beat vigorously until they died, circulation was never re-established. These embryos became severely edemic (similar to control c/c embryos) and died. Erythrocytes were observed pooling in various capillary beds (particularly the gills, pronephroi, and liver), and often in the heart itself (Fig. 4-3).

A similar phenomenon was observed when c/c heart mesoderm was unilaterally transplanted into wild-type hosts. All 13 hosts developed beating hearts, and 10 apparently had

TABLE 4-6

Unilateral Transplantations of Heart Mesoderm

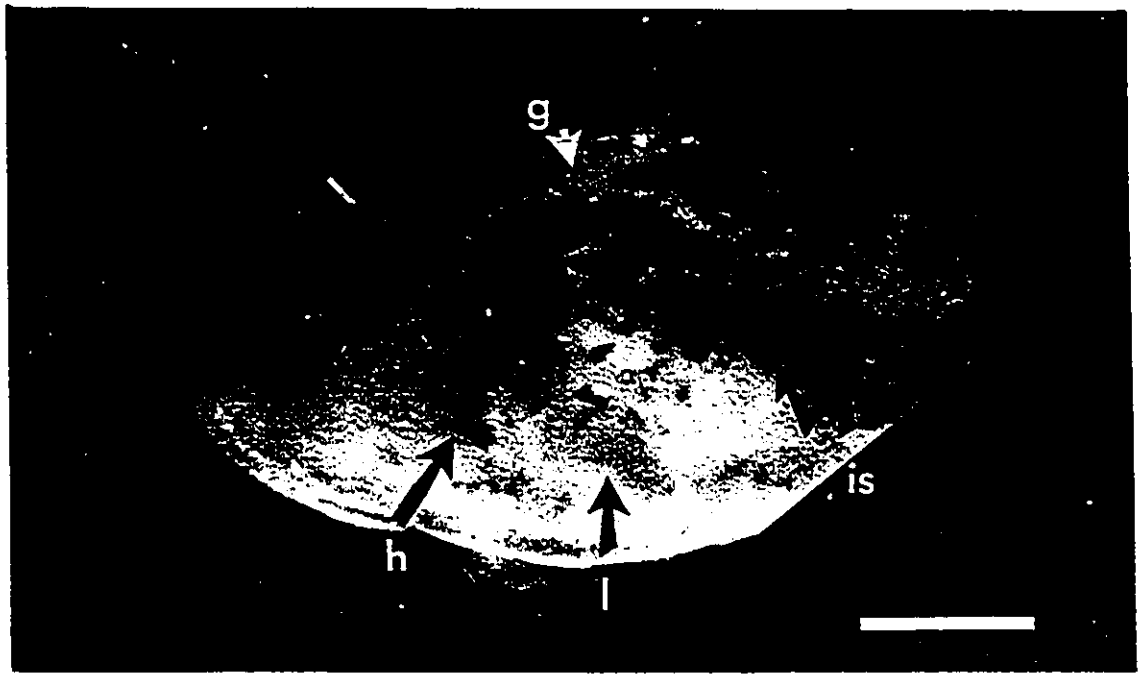
Phenotype [a]		No. of	No. with	No. with	No. with Circulatory
Donor	Host	Embryos	Beating Heart	Circulation	Arrest
wt	wt	35	35	32	0
wt	c	7	6	2	4 [b]
c	wt	13	13	8	10 [b,c]
c	c	1	0	0	0

[a] wt = wild-type (+/c and +/+); c = mutant (c/c).

[b] In 4 cases, blood was found pooled even though circulation was never actually observed.

[c] In one case, circulation was re-established after a few days.

Figure 4-3: Left antero-ventral view of wild-type larva which received a unilateral transplant of c/c heart mesoderm. Circulation has arrested, and blood is pooled in the gills (g), liver (l), some intersegmental vessels (is) in the trunk and the heart (h). The heart continued to beat vigorously, moving the blood between its chambers. Note the edema in the thoracic area and the reduced gills. Bar = 1 mm.


g
is
h
l

circulation, but the circulation later arrested in 10 of these embryos. Circulation remained blocked until death in 9 of the 10 embryos. In one case, circulation was later re-established. This embryo, and in one where the circulation never arrested, were allowed to grow, and both were able to feed normally.

In 35 cases, wild-type heart mesoderm was transplanted into wild-type hosts. All developed beating hearts, and in all but 3, circulation was established. In contrast to the hosts containing *c/c* mesoderm, circulatory arrest was never observed in embryos containing only wild-type tissue.

4.7 Transplantations of heart mesoderm in older embryos:

In his initial description of the cardiac-lethal mutant, Humphrey (1972) reported that wild-type heart mesoderm transplanted into *c/c* hosts did not begin to beat (see Section 1.4). *A priori*, this seems to contradict the results of the bilateral and unilateral heart transplants (Sections 4.4 and 4.6). Therefore, it became necessary to verify Humphrey's (1972) results in older embryos.

In his paper, Humphrey (1972) reported that he transplanted the heart mesoderm from the area described by Copenhaver (1950). However, Copenhaver (1950) delineated two areas of heart mesoderm: the heart-forming mesoderm, which actually develops into heart tissue, and the heart-field

mesoderm, which includes the heart-forming mesoderm, and also a peripheral area of mesoderm which is capable of forming heart tissue, but normally does not (Fig. 4-4). Since it is unclear which area Humphrey (1972) had used, two series of transplantations were performed, involving mesoderm from both areas.

Transplantations of heart mesoderm were performed as described in Section 2.4. Stage 29 embryos were used in all cases; wild-type embryos were used as donors, and the hosts were from +/c X +/c spawnings. Once removed, the host heart mesoderms were maintained in explant culture to identify the phenotypes of the hosts.

In 8 of 31 cases where the entire heart field (defined as all of the mesoderm between the posterior edges of the mandibular arches; Fig. 4-4, region A) was transplanted, the hosts were c/c embryos (as determined by the explant cultures). All of these embryos developed vigorously beating hearts (Table 4-7). In only one case was circulation of the blood ever established, and in this case the circulation arrested permanently within a few days.

Of 23 wild-type hosts, all developed vigorous heartbeats, and in 12 of these circulation was established. Curiously, in three of the 12, the circulation arrested and, although it recommenced in one case, the arrest was permanent in the other two.

Figure 4-4: Diagram of the antero-ventral half of a stage 29 embryo (with the ventral trunk ectoderm removed), showing the areas of heart mesoderm transplanted in these embryos. A: heart-field mesoderm; B: heart-forming mesoderm, as delineated by Copenhaver (1955). The mesoderm was usually transplanted together with the overlying ectoderm. The presence or absence of this ectoderm did not affect the results. MA: mandibular arch; GB: gill bulge; Ant.: anterior; Post.: posterior.

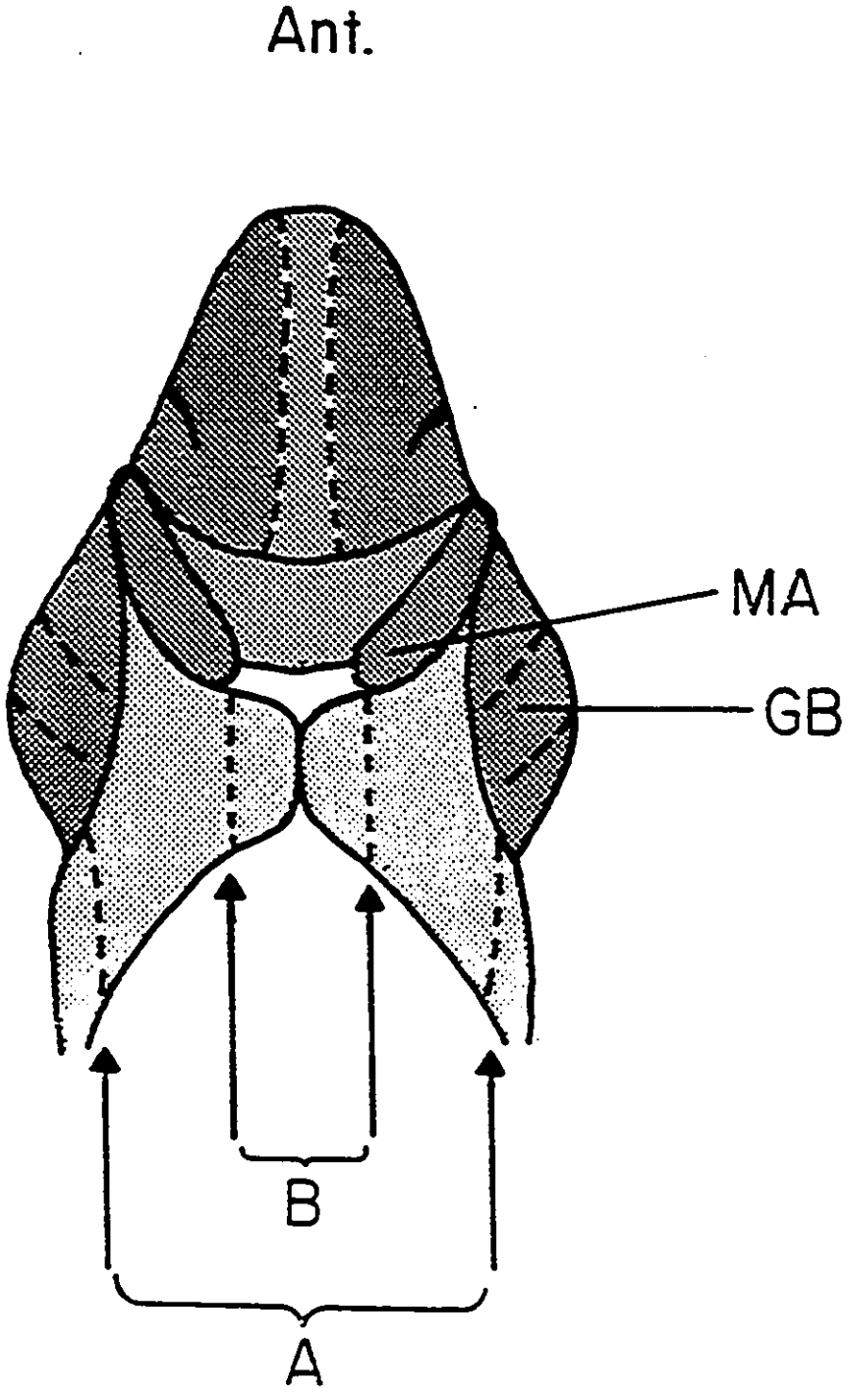

Ant.
MA
GB
B
A
Post.

TABLE 4-7

Transplantation of Heart-Field Mesoderm[a]

Host Phenotype[b]	No. of Embryos	No. with Beating Heart	No. with Circulation	No. with Circulatory Arrest
wt	23	23	12	3 [c]
c	8	8	1	1

[a] Heart-field mesoderm is defined as: all mesoderm between the ventral edges of the gill bulges (see Fig. 4-4; Copenhaver, 1955).

[b] wt = wild-type (+/c and +/+); c = mutant (c/c).

[c] In one case, circulation was re-established after 5 days.

In addition to transplanting the entire heart field, transplantations involving only the heart-forming mesoderm (Fig. 4-4, region B) were performed. Again, the explanted mesoderms were maintained in culture to determine the phenotypes of the hosts.

It is important to note, however, that the extremely small size of these explants frequently led to their death. Therefore, while all of the explants which began beating were, obviously, from wild-type hosts, those which were not observed beating presumably included both those from *c/c* hosts, and some wild-type ones.

Of 31 transplantations performed, 15 were into known wild-type hosts (Table 4-8). All of these embryos developed vigorously beating hearts, and in 7 of these cases, circulation was permanently established. No cases of circulatory arrest were observed.

In 16 cases, the explanted mesoderm was not observed to beat. Of these, vigorous heartbeats were observed in only 8, of which 4 developed circulation. Circulatory arrest was never observed. The 8 cases where no heartbeat was observed corresponds well to the expected proportion of *c/c* embryos (8 of 31 total cases = 25.8%).

These results strongly suggest that if only the heart-forming mesoderm is transplanted into cardiac-lethal mutant embryos, a beating heart cannot form and the mutant phenotype

TABLE 4-8

Transplantation of Heart-Forming Mesoderm[a]

Host Phenotype[b]	No. of Embryos	No. with Beating Heart	No. with Circulation	No. with Circulatory Arrest
wt	15	15	7	0
c(?) [c]	16	8	4	0

[a] Heart-forming mesoderm is defined as: all mesoderm between the postero-ventral tips of the mandibular arches (see Fig. 4-4; Copenhaver, 1955).

[b] wt = wild-type (+/c and +/+); c = mutant (c/c).

[c] Many explants of host mesoderm were exceedingly small, and did not begin to beat due to their small size, or because they died. Therefore, these embryos presumably consist of both c/c hosts, and wild-type ones which could not be identified by their explant.

persists. However, if the entire heart field is included in the transplant, a functional heart can form, and the mutant can be partially rescued.

CHAPTER V

DISCUSSION

5.1 Heart induction in wild-type axolotl embryos:

One major goal of this study was to examine the nature and timing of the inductive interactions responsible for normal heart differentiation in the axolotl. Therefore, the initial phase consisted of determining when heart area mesoderm could complete its developmental repertoire in the absence of further inductive stimuli. To accomplish this, heart mesoderm was removed from embryos at different stages (14-20) during neurulation, and was cultured *in vitro* in a simple saline medium.

In this presumably neutral environment, the proportion of cultures which were capable of forming rhythmically contracting tissue increased gradually as the age of the donors increased. As well, the average time for the cultures to begin beating decreased gradually as the donor age increased. By the end of neurulation (stage 20), most such cultures contained beating tissue which commenced beating at about the same time as would be expected *in situ* (3 days post-operatively). The beating cells observed in these cultures were, indeed, myocardial tissue, since mesoderm from other areas was not capable of forming beating tissue *in*

vitro. In addition, ultrastructural examination of cultures containing beating tissue revealed the presence of sarcomeric myofibrils, a characteristic of functional cardiomyocytes (see Lemanski, 1973b; 1976). Non-beating cultures of stage 14 heart mesoderm did not contain such myofibrils.

These results indicate that the induction to form a functional myocardium is a gradual, cumulative process. The gradual nature of the induction is consistent with the findings of Jacobson and Duncan (1968) for *Taricha torosa*. However, the induction in the axolotl is essentially completed by the end of neurulation (stage 20), much earlier than in *T. torosa* (stage 24-26; Jacobson and Duncan, 1968).

The timing of the induction appears to be consistent with Jacobson and Duncan's (1968) finding that heart induction is completed earlier in *Ambystoma tigrinum*, although they present little data for this species. Combined with Sater and Jacobson's (1989; 1990) report that heart induction is completed even earlier (during gastrulation) in the anuran *Xenopus laevis*, this indicates that there is a great variability in the timing of the induction in different amphibian species and emphasizes the importance of clearly delineating the parameters governing the induction for any species prior to examining the factors affecting later stages of heart differentiation.

While Jacobson (1960; 1961; Jacobson and Duncan, 1968)

has determined that anterior endoderm is the major inducer of heart differentiation, it remained unclear whether the inductive ability was a ubiquitous property of all endoderm, or of all pharyngeal endoderm, or if it had a restricted distribution. Therefore, the spatial distribution of the inductive activity was determined by co-culturing stage 14 heart mesoderm with different areas of endoderm. The results demonstrate clearly that, in the axolotl, the inductive activity is highly localized within the pharyngeal endoderm.

This apparently contradicts the findings of both Jacobson and Duncan (1968) and Fullilove (1970). These workers reported that the inductive activity is broadly based within the anterior (pharyngeal) endoderm. However, when their results are examined closely, it becomes apparent that only low levels of inductive activity were present in any of the areas tested by them (see Section 1.2). Furthermore, Fullilove (1970) only tested the lateral pharyngeal walls; the inductive ability of mid-ventral pharyngeal endoderm was never examined. Similarly, Jacobson and Duncan (1968) found little inductive activity in their "anterior dorsal endoderm" (which they report is the most inductive area) when it was cultured with very early heart mesoderm. Slight differences only became apparent when older mesoderm was used. This suggests that only low levels of inducer (capable only of augmenting an induction already in progress, but not of

commencing one) are present in the areas tested by Jacobson and Duncan (1968) and Fullilove (1970). Interestingly, Mangold (1956; as cited by Jacobson, 1960; 1961) reported the presence of an inductive influence in both dorsal and ventral endoderm. As well, Bacon (1945) reported that the inductive activity was present "... in the tissues surrounding the final heart position" (emphasis mine).

My results, however, strongly suggest that there is a distinct difference in the inductive activity of different regions of pharyngeal endoderm. Mid-ventral endoderm is the most potent inducer of heart differentiation, followed by the lateral pharyngeal walls. Yolky (posterior) endoderm and pharyngeal endoderm from beneath the cranial neural plate contain little, if any, inductive ability.

At first, it is unclear why the lateral pharyngeal endoderm is not a more active inductor of heart differentiation, since the heart-forming mesoderm migrates over this area during neurulation. It may be that prolonged, intimate contact between the mesoderm and the subjacent lateral pharyngeal endoderm is sufficient to gradually cause the former to become determined.

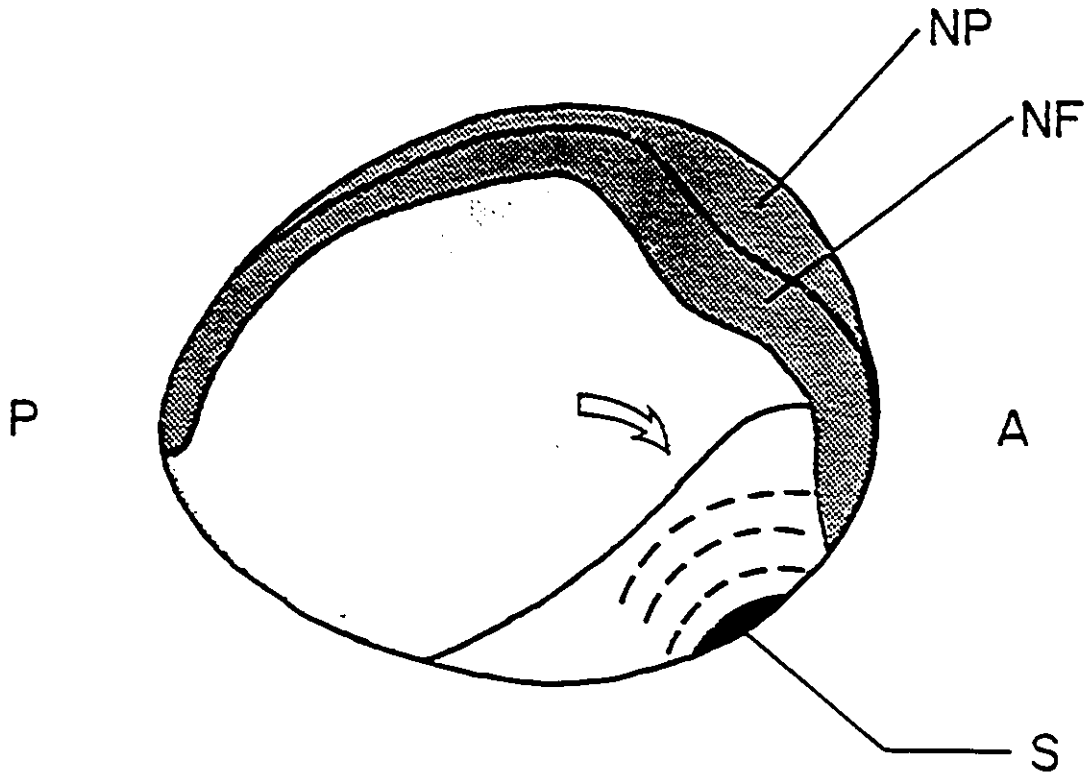
Alternatively, the differing inductive abilities of the pharyngeal endoderm may explain why the induction is gradual and cumulative (see Jacobson and Duncan, 1968; Section 3.1), rather than an all-or-nothing phenomenon. The distribution

of inductive activity appears to increase, within the endoderm, from ventral to dorsal. This suggests that the inducing agent forms a gradient, being most concentrated antero-ventrally and decreasing with distance from this area (Fig. 5-1). The heart area mesoderm, during its antero-ventral migration, would encounter gradually increasing concentrations of inducer. Thus, the heart mesoderm would gradually become fully induced as the migration progressed.

A further advantage of the gradual induction hypothesis is that the leading edges of the mesodermal mantle would encounter higher levels of inductive influence sooner than more posterior areas. Therefore, the heart-forming mesoderm, which is at the anterior-most edge (Copenhaver, 1955; Wilens, 1955), could become more rapidly and highly induced than the rest of the heart field. The anterior edge could thus become more likely to actually form the heart than more posterior regions. This distinction between heart-forming and other heart-field mesoderm becomes important when examining the later phases of heart differentiation (see Section 5.4).

Given that the induction appears to be completed *in vivo* by stage 20, it is perhaps surprising that the inductive ability of mid-ventral endoderm remains relatively constant throughout neurula and tailbud stages, at least up to stage 30. Copenhaver (1955) also noted that the inductive activity persisted long after it was no longer necessary. While the

Figure 5-1: Right side view of a stage 16 neurula (with the ectoderm removed), demonstrating how the heart mesoderm could become gradually induced. As the mesoderm (lightly stippled) migrates antero-ventrally, in the direction of the arrow, it encounters increasingly higher concentrations of inducer (broken lines). The heart-forming mesoderm, at the anterior edge, would encounter higher concentrations sooner than more posterior areas of the heart field. Therefore, it could be induced sooner, and/or more highly than the latter, and a gradient of activator and inhibitor sources could be established within the mesoderm. The presence of a repressor of induction in the neural tissues (heavily stippled) may also be involved in preventing induction in the dorsal-most mesoderm. S: "source" or area of highest inducer concentration; NP: neural plate; NF: neural fold; A: anterior; P: posterior.


NP
NF
P
A
S

reason for this is uncertain, it may be that the inducing activity may be required to prevent an inhibition of heart formation (see below). Equally surprising was the finding that heart area mesoderm retained some (albeit reduced) ability to respond to inductive signals after reaching relatively advanced stages in the absence of such signals. The reason for this prolonged period of responsiveness is unclear.

Like Jacobson (1960; 1961; Jacobson and Duncan, 1968), I have demonstrated that there is a slight inhibitory influence present in the anterior neural fold and plate. Although slight, an effect was evident *in vitro*. Jacobson (1960; 1961; Jacobson and Duncan, 1968) was unable to demonstrate such inhibitory effects in culture (see Section 1.2). While the role of this inhibitory influence is uncertain, it may be that inhibition by the dorso-anterior tissues serves to further restrict the ability of the mesoderm to form the heart (as suggested by Jacobson, 1960; 1961).

In addition to determining the temporal and spatial distribution of the inducer, I attempted to further characterize it by culturing stage 14 heart area mesoderm with various cell-free preparations of the inductive endoderm. Unfortunately, these preparations contained little or no inductive activity. While whole-cell homogenates of inductive endoderm appeared to contain some inducer, all of

the tissues tested had a similar effect. This strongly suggests that the slight effects observed were non-specific, similar to the results obtained by Jacobson and Duncan (1968).

However, it may be possible to glean some information about the nature of the inducer from these experiments. The finding that the inductive agent is not present in either homogenates or in medium conditioned by inductive endoderm may indicate that it is neither diffusible nor in a form which becomes soluble when the cells are lysed. If so, this may mean that the induction is mediated only through direct cell-cell contact between the inducing and responding tissues.

Alternatively, it may be that the inducer is diffusible, but is so labile as to be undetectable in these preparations. The latter explanation would, perhaps, be more consistent with the inducer-gradient model presented earlier, since the properties of diffusibility and lability could both be involved in producing a gradient of inductive ability. This is not the only possibility, however, since it is also conceivable that a stable gradient of a cell-surface-bound agent is present on the endoderm. At present, it is not possible to distinguish between these alternatives.

Of greater significance is the finding that neither conditioned media nor RNA extracted from the inductive

endoderm contains any detectable inducing activity when tested on uninduced (stage 14) heart mesoderm. Both types of preparation have been reported to stimulate the formation of functional myocardia in fully-formed (stage 35) hearts of cardiac-lethal embryos (Davis and Lemanski, 1987). Differences in the procedures used to extract RNA could not account for these results, since both contained a similar activity when tested on mutant hearts. This strongly suggests that, contrary to the proposal of Davis and Lemanski (1987), these preparations do not contain the inducer of heart differentiation. Rather, they appear to contain a substance(s) which controls a later phase of cardiomyocyte differentiation, namely, myofibrillogenesis.

5.2 Heart differentiation in cardiac-lethal mutant embryos:

As described in Chapter I, the c mutant is a potentially important model system for studying the regulation of heart development. Previous studies of this mutant have concluded that the failure of *c/c* hearts to begin to beat is due to a failure of the anterior (pharyngeal) endoderm to properly induce the heart-forming mesoderm, resulting in a failure of myofibrillogenesis in the heart (Lemanski *et al.*, 1977; 1979; Davis and Lemanski, 1987). However, this conclusion is based on the assumption that the timing of heart induction is the same in *Ambystoma mexicanum* as in another species (*Taricha*

torosa, Jacobson and Duncan, 1968). The inductive failure model is also based on the finding that wild-type hearts do not beat when transplanted into *c/c* host embryos (Humphrey, 1972). While Humphrey's (1972) observation appears to be valid, the conclusion (Lemanski *et al.*, 1977; 1979) is absolutely dependent on the previous assumption. Furthermore, the suspected inability of *c/c* endoderm to induce heart mesoderm has never been directly demonstrated.

From Section 5.1, it is clear that heart area mesoderm in the axolotl is fully induced and capable of self-differentiation (in the absence of further inductive stimuli) by stage 20. This is well before the late tailbud stages (27-29) used by Humphrey (1972) in his transplantation experiments. Therefore, the wild-type heart mesoderm which Humphrey (1972) transplanted into *c/c* host embryos must have been actively inhibited from completing their differentiation in this environment. Indeed, this alternative was proposed by Humphrey (1972), but was largely ignored in subsequent studies. Furthermore, RNA extracts which are capable of stimulating beating in *c/c* hearts, clearly do not have any inductive activity. Therefore, I have critically examined the proposed failure of the inductive process in cardiac-lethal embryos.

Since an inhibitor of heart differentiation must be present in *c/c* embryos, it seemed possible that mutant heart-

forming mesoderm might be capable of forming beating tissue if isolated from its deleterious environment early enough. This was attempted by Hill and Lemanski (1979), using fully-formed (stage 35) mutant hearts. However, it could be argued that the explantation was performed too late to reverse the effects of the inhibition. Therefore, fully induced (stage 20) heart mesoderm was explanted from c/c embryos. This is well before the differentiation of cardiomyocytes and the beginning of heartbeat (stage 35, 3 days later). Despite the early isolation, mutant heart mesoderm almost never formed beating tissue.

This may indicate that the inhibitory effects on heart differentiation begin very early in the development of mutant embryos (during or before neurulation). If true, the obvious corollary is that isolation from the source of the inhibitor is not sufficient to reverse the effects of a prior inhibition. Alternatively, these results may indicate that the heart mesoderm is, itself, the source of the inhibition. If this were the case, explantation would have no effect on the ultimate fate of mutant mesoderm, since the inhibitor would still be present *in vitro*. Whatever the source of the inhibitory agent, it is unlikely that a novel inhibitor is produced by the mutation, particularly since wild-type tissues are capable of reversing its effects (Lemanski *et al.*, 1979; Justus, 1978).

The cornerstone of the hypothesis that the *c* gene affects the endoderm, is that this tissue is rendered incapable of inducing heart differentiation. This implies that mutant heart mesoderm is normal, and is only indirectly affected by the mutation. The only evidence to support this hypothesis are demonstrations that mutant hearts begin to beat when placed with wild-type inductive endoderm (Justus, 1978; Lemanski *et al.*, 1979) and ultrastructural studies of mutant endoderm (Lemanski *et al.*, 1977), which vaguely suggest that there are morphological abnormalities in this tissue. However, despite the key nature of the predictions mentioned above, neither one has previously been criticaly tested.

Therefore, I have directly tested the inductive ability of mutant endoderm, and the ability of mutant mesoderm to respond to the induction, by combining these tissues *in vitro*. The results are unequivocal; the inductive ability of the pharyngeal endoderm is not affected by the *c* gene. Mutant and wild-type endoderm have the same inductive capacity. Rather, the mutation directly affects the heart mesoderm, rendering it incapable of responding to normal inductive signals. This again suggests that the RNA preparations of Davis and Lemanski (1987) contain, not the inducer, but rather an "activator" which controls a later phase of heart differentiation (*i.e.* the assembly of

sarcomeric myofibrils).

Based on the timing of heart induction in wild-type embryos, and on the suspected presence of a specific activator and inhibitor, Armstrong (1989) proposed that the later phases of heart differentiation are controlled by a two-morphogen reaction-diffusion mechanism similar to that believed to control head (and foot) formation and regeneration in *Hydra* (Gierer and Meinhardt, 1972; Bode and Bode, 1984). According to this model (see Section 5.4), the reaction-diffusion system is established within the heart mesoderm itself, in response to the induction. In c mutant embryos, the heart mesoderm would either underproduce the activator, and/or overproduce the inhibitor in an abnormal response to a normal induction. The resulting relative overabundance of the inhibitor would prevent myofibrillogenesis, and the heart would not begin to beat even though most (or all) of the contractile proteins are present.

This hypothesis makes two critical predictions. The first is that the inductive signal is normal in c/c embryos and that only the response to that signal is affected. This is clearly true. The second is that the heart mesoderm is the source of both the activator and the inhibitor. In an effort to detect the presence of one of these substances, fully induced (stage 20) c/c and wild-type heart mesoderms

were explanted and allowed to develop, together, *in vitro*. Although the *c/c* explants in these cultures took almost twice as long as wild-type tissues to begin beating, all of them did, eventually, begin to beat. This strongly suggests that wild-type heart mesoderm produces an activator-like substance capable of stimulating myofibrillogenesis in mutant tissue. It is interesting to note that Ekman (1925, as cited by Copenhaver, 1955) reported that there was an "... inductor in the heart primordium" itself.

Since the defect in *c/c* embryos appears to affect only the mesoderm, it seemed plausible that mutant embryos could be rescued by replacing their heart mesoderm with wild-type tissue. When both areas of pre-cardiac mesoderm were replaced in stage 20 mutant embryos, almost all formed vigorously beating hearts. It is important to note that the entire heart field was replaced in these experiments. Therefore, *all* of the tissue capable of forming myocardium, not merely that which actually does (under normal circumstances), was wild-type.

This result has two important implications for heart development (see Section 5.3 for other implications). First, it provides *in vivo* confirmation that the only defect caused by the *c* gene (with respect to heart differentiation) lies in the heart mesoderm. Neither the inductive tissues, nor any other tissues involved in heart formation (*i.e.* the inductive

endoderm, or the neural crest; see Section 5.3) are directly affected by the mutation.

The second implication is a corollary of the first; if an inhibitor is produced by mutant tissues, it must be produced by the heart mesoderm, and not by adjacent tissues. This, combined with the findings that explanted c/c heart mesoderm does not begin to beat (whether isolated early, Section 4.1, or late, Hill and Lemanski, 1977), seems to indicate that the heart mesoderm is the source of the inhibition. This is consistent with the prediction of Armstrong's (1989) reaction-diffusion model.

The results of the *in vitro* mesoderm recombination experiments suggested that enough activator might be present in a single primordium to rescue the mutant half of a fully-formed heart. Therefore, in addition to replacing both heart primordia in stage 20 mutant embryos, unilateral transplantations of heart mesoderm were performed. These transplantations had the added advantage of minimizing the surgical damage to the host embryos. Again, vigorously beating hearts developed in nearly all c/c hosts. Heartbeat continued, and remained strong, until the embryos died.

The results of these experiments provide *in vivo* corroboration of the *in vitro* mesodermal recombinations. Sufficient activator is present in wild-type heart mesoderm to stimulate myofibrillogenesis in mutant mesoderm, even in

the environment of a *c/c* host.

The results of the bilateral and unilateral transplantations of heart mesoderm appear to contradict the results of Humphrey's (1972) transplantation experiments, which were performed on older (stage 27-29) embryos. Therefore, Humphrey's (1972) experiments were repeated. Both the heart-forming mesoderm, and wider areas of heart-field mesoderm, as delineated by Copenhaver (1955), were transplanted. This was necessary because Humphrey (1972) did not specify which area he transplanted (see Section 4.7).

When the entire heart field was replaced in *c/c* embryos, beating hearts formed in all cases. This is consistent with the results of the transplantations of pre-cardiac mesoderm at early stages. However, when only the actual heart-forming mesoderm (the tissue between, and posterior to, the tips of the mandibular arches) was transplanted, beating hearts did not form in 8 of 31 cases (25.8%). This corresponds well to the expected proportion of *c/c* embryos, and is consistent with Humphrey's (1972) results. This is not likely to be due to a random effect, since beating hearts formed in all cases where the host embryos could be conclusively identified (by the presence of beating tissue in the explants of host tissue) as being wild-type.

Three conclusions may be drawn from these results. First, Humphrey (1972) apparently transplanted only the

actual heart-forming mesoderm, and not the entire heart field. Second, an inhibitor of wild-type cardiomyocyte differentiation must be present in *c/c* embryonic tissues. Finally, it is evident that the inhibition is no longer present when all of the mutant heart-field mesoderm is replaced. All of this evidence clearly indicates that the inhibitor must be present within the heart mesoderm itself, as predicted by the reaction-diffusion model of Armstrong (1989).

5.3 Other effects of the c *gene:*

While the phenotypic lesion caused by the *c* gene clearly affects the heart mesoderm, there is some evidence that other tissues are directly or indirectly affected as well. When wild-type heart mesoderm was bilaterally transplanted into *c/c* host embryos, most of the mutant characteristics assumed to be secondarily caused by the lack of circulation (*i.e.* retarded growth, microcephaly, stunted gills, abnormal pigmentation patterns; see Humphrey, 1972) were corrected. However, one characteristic of mutant embryos was retained in those with normal circulation. Of three apparently normal, undamaged *c/c* embryos, 2 were completely unable to feed. Although food could be seen in their mouths, they appeared to be unable to swallow. The single case in which a little food was swallowed was from an atypical spawning; control *c/c*

embryos could also swallow a little.

This indicates that, while the other characteristics of the mutation can be attributed to the failure of the heart to beat and the resulting lack of circulation, the inability to feed must be caused by a separate, previously unsuspected, effect of the *c* gene. Although it is uncertain what tissue is affected, an obvious choice is the mandibular mesoderm, which is at the anterior-most edge of the mesodermal mantle, immediately adjacent to the heart area (see Hirakow *et al.*, 1987; Fig. 1-1). This tissue was not replaced during my transplantation experiments; mutant mandibular mesoderm was left *in situ*.

Another effect of the *c* gene was observed in the unilateral transplantations of heart mesoderm. In those embryos containing hearts composed of both mutant and wild-type mesoderm, and where circulation commenced, the circulation of the blood later arrested, and the blood pooled in a variety of capillary networks (particularly the gills, liver, and pronephroi). This happened in both *c/c* host embryos and in all wild-type hosts containing a single mutant primordium (although among the latter, circulation was eventually re-established in one case). This could not have been caused by surgical damage, since circulatory arrest was never observed in embryos containing only wild-type tissues. This strongly suggests that there is a morphological defect

(distinct from the failure of myofibrillogenesis and the resulting functional defect) in hearts containing c/c tissue. This defect is not corrected, even when myofibrillogenesis is stimulated.

The nature of this suspected morphological defect remains unclear. However, observations of the early stages of heartbeat in normal embryos suggests a possibility. At stage 35, heartbeat commences. Initially, the contractions are weak and appear to be propogated in smooth waves, almost peristaltically, along the length of the heart tube. The contractions gradually become stronger and, at about stage 36, circulation is first estabilshed. In slightly older embryos, however, the contractions become more characteristic of mature hearts; different segments of the heart begin to contract separately and in succession. A co-ordinated, but alternating, beat is evident.

At the earlier stages, heart valves may not be necessary to ensure proper movement of the blood, since the sweeping, peristaltic contractions of the heart would be sufficient to keep the blood flowing in one direction. However, at later stages, these valves would become necessary to prevent the blood from flowing back and forth between the alternately contracting chambers of the heart. Indeed, blood moving in this manner could be directly observed in many cases of circulatory arrest. Furthermore, the resulting loss of

arterial pressure would presumably also cause the blood to pool on the arterial side of capillary networks, since there would be insufficient systolic pressure to force the blood cells through the narrow capillaries. This suggests that c/c hearts either do not form valves, or only form defective ones. Hearts containing both mutant and wild-type mesoderm would also be defective, since a partial valve would presumably not function normally.

This hypothesis may be supported by a recent morphological study of c/c hearts. Lemanski and Fitzharris (1989) found that the number of endocardial and mesenchymal (cardiac cushion) cells is greatly reduced, and the extracellular cardiac jelly is greatly expanded in the anterior regions of mutant hearts. The cardiac cushion mesenchyme forms the septa and valves of the heart (Patten *et al.*, 1948; Manasek *et al.*, 1984; Krug *et al.*, 1985; Hirakow, 1986).

Interestingly, it has been demonstrated that neural crest cells contribute to the walls of the large arteries in the pharyngeal area (LeLièvre and LeDouarin, 1975) and to the cardiac cushion tissue (Phillips *et al.*, 1987). Indeed, extirpation of the so-called "cardiac" (*viz.* branchial area) neural crest causes a variety of septal defects in chick embryos (Kirby and Stewart, 1983; Kirby *et al.*, 1983; 1985; Kirby and Bockman, 1984; Besson *et al.*, 1986; Nishibatake *et*

al., 1987). These morphological defects are often accompanied by a decrease in arterial blood pressure (Stewart *et al.*, 1986).

The neural crest also appears to contribute to normal heart development in the axolotl, since a gene (premature death; *p*) which affects some subpopulations of the neural crest (Graveson, 1990; Graveson and Armstrong, manuscript submitted) also causes morphological abnormalities in the endocardium (Trottier and Armstrong, 1977). As well, complete extirpation of the neural crest in the axolotl often causes the blood to pool in the gills (Graveson, 1990; Graveson and Armstrong, in preparation).

It is unlikely that the neural crest cells are directly affected by the *c* gene for two reasons. First, normal hearts (providing normal circulation) can form in *c/c* embryos if the entire heart mesoderm (both primordia) is replaced at stage 20. This is prior to the beginning of neural crest cell migration (see Hörstadius, 1950; Graveson and Armstrong, 1987; Löfberg *et al.*, 1989; Hall and Hörstadius, 1988). Therefore, *c/c* neural crest cells can apparently participate in normal heart development when in the presence of wild-type mesoderm. The second reason is that circulatory arrest was also observed in wild-type embryos containing a single *c/c* primordium, even though all of the neural crest in these embryos would have been wild-type.

Therefore, if the neural crest contribution to the heart is abnormal in *c/c* embryos, and in beating hearts containing mutant mesoderm, it may indicate that the heart-forming (and/or other heart field) mesoderm plays a previously unsuspected role in controlling the formation of heart-associated structures by the branchial neural crest. If this is the case, then *c/c* heart mesoderm may also be defective in its ability to properly signal these neural crest cells to fulfill their normal function(s). Indeed, Krug *et al.* (1985) have demonstrated that protein extracted from myocardial tissue stimulates endocardial cell differentiation, suggesting that this signal may be reduced or absent in mutant myocardium.

Indeed, the re-establishment of circulation in one embryo containing both *c/c* and *+/+* heart mesoderm may be due to the presence of wild-type tissue (supplying normal signals) and the dramatic regulatory ability of the neural crest cells (see Hörstadius, 1950; Hall and Hörstadius, 1988; Graveson and Armstrong, in preparation). These two factors may allow the (delayed) formation of normal valves in some *c/c:+/+* hearts (perhaps those containing a somewhat larger contribution of wild-type mesoderm).

Curiously, this hypothesis may also be supported by the seemingly incongruous results of the transplantations of heart mesoderm into older (stage 29) embryos (Section 4.7).

When the entire heart-field mesoderm was transplanted, circulatory arrest was observed in one *c/c* host and *also* in 3 wild-type hosts. (One of the latter eventually recovered.) Stage 28-29 corresponds to the time when the neural crest cells of the branchial area are beginning to migrate into the branchial mesoderm in the axolotl (Graveson and Armstrong, 1987). Therefore, it is distinctly possible that the incisions made immediately ventral to the gill bulges (see Fig. 4-4) would disrupt the normal migration of the neural crest cells into the heart area.

Conversely, transplantations involving only the heart-forming mesoderm required that incisions be made further from (more ventral to) the branchial mounds. As well, these grafts tended to heal more quicky and completely, since they were in a flattened area of the embryo and not a curved one (like the heart field grafts). In these embryos, circulatory arrest was never observed. This suggests that transplanting only the heart-forming area caused less disruption of normal neural crest cell migration than transplanting the entire heart field.

If abnormal signals from mutant mesoderm to the neural crest are, in fact, involved, it may also provide an explanation for the inability of mutants to swallow, since many skeletal and mesenchymal derivatives of the head and neck regions are formed by the cranial neural crest

(Hörstadius, 1950; Weston, 1970; LeDouarin, 1975; 1982; Hall and Hörstadius, 1988).

5.4 A comprehensive model for heart development:

In this thesis, evidence has been presented which demonstrates the presence of a specific activator and inhibitor of heart differentiation. These agents are produced by the heart area mesoderm itself, and appear to be distrubuted throughout the heart field, suggesting that they are diffusible. Furthermore, the very existence of a morphogenetic field for the heart (all of which is capable of forming heart tissue, but only one side of which normally does) indicates that some mechanism must exist to distinguish the heart-forming mesoderm from the rest of the field. That mechanism must, at the same time, promote organ formation and differentiation by the heart-forming mesoderm and suppress it in the rest of the mesoderm *but* be adaptable enough to allow the pattern to be re-established in the remaining tissue should the former be removed. This evidence suggests that the later (post-inductive) phases of heart development are controlled by a two-morphogen reaction-diffusion system such as that proposed by Armstrong (1990).

This represents a previously unrecognized level of control over heart development. Combined with what is known of the induction, it allows a comprehensive model for the

control of vertebrate heart development to be formulated.

Heart development appears to be controlled by a two-step process. The first step is the induction proper, which occurs (in the axolotl) during neurulation (stages 14-20). The inducer is present in the highest concentrations in the mid-ventral pharyngeal endoderm, and appears to be distributed in a gradient. As the heart mesoderm migrates antero-ventrally over the pharyngeal endoderm, it gradually encounters higher concentrations of the inducer and becomes induced. The leading edges of the mesodermal mantle (which contain the heart-forming areas) would be induced sooner, and possibly to a slightly greater extent, than the more posterior areas of the heart field mesoderm. The induction is completed, in the axolotl, by stage 20.

The induction appears to have two direct results. First, it may trigger the production of the appropriate contractile proteins by the mesodermal cells. This is not certain, however, since non-beating (uninduced?) explants of wild-type stage 14 heart mesoderm contain at least one muscle-specific contractile protein. Muscle-specific myosin has been detected immunohistochemically in such cutrures (M. McIsaac, personal communication).

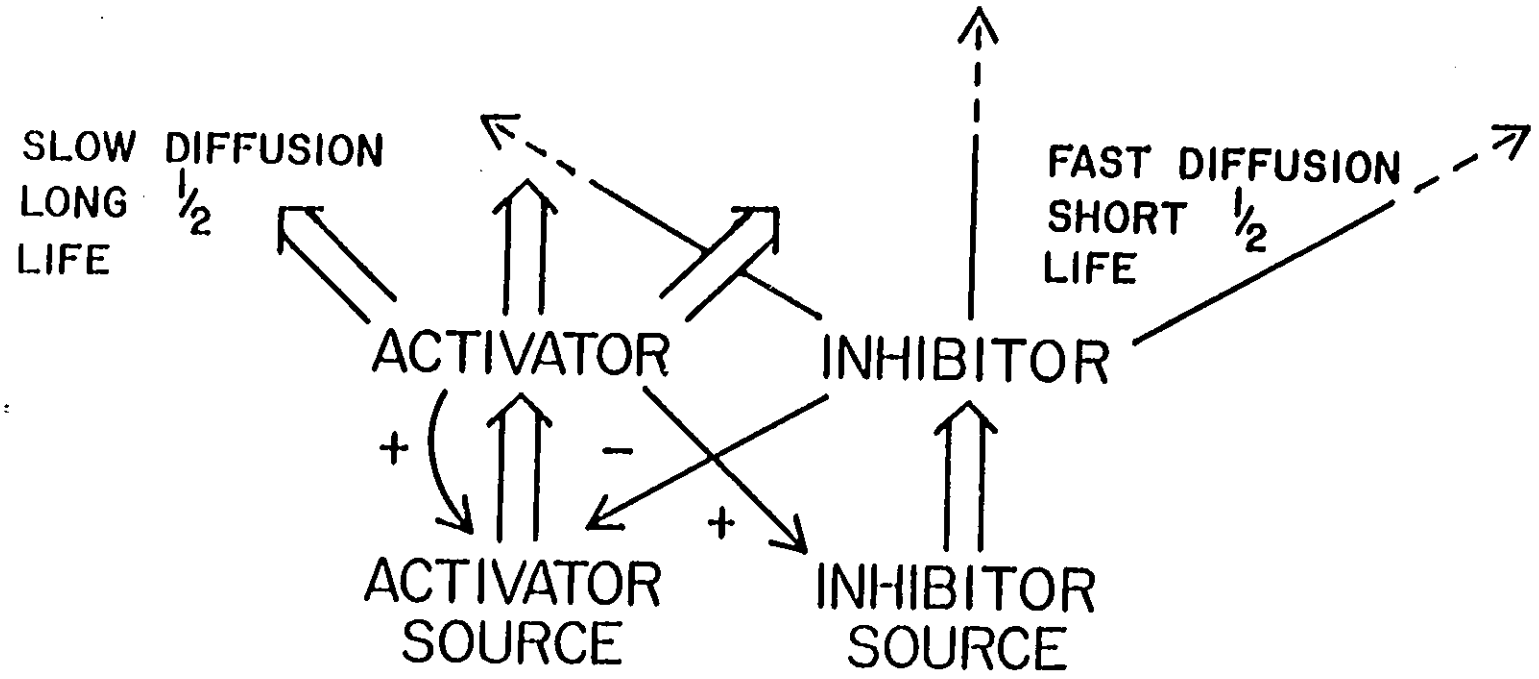
The second result of the induction is more certain: the heart-field mesoderm begins to produce both an activator and an inhibitor. The distribution of these agents may be

controlled by a reaction-diffusion mechanism, as proposed by Armstrong (1989, based on the equations of Gierer and Meinhardt, 1972).

The model of Gierer and Meinhardt (1972), formulated to explain the control of head and foot development and regeneration in *Hydra*, is one of several variants of the reaction-diffusion mechanism first proposed by Turing (1952). The basic premise of Turing models is that a patterned distribution of an activator and inhibitor can form as the result of auto- and cross-catalysis (reaction) and diffusion of the agents. Furthermore, these models (including that of Gierer and Meinhardt, 1972) are based on the idea of "short-range activation, long-range inhibition". The inhibitor is believed to be a smaller, readily diffusible, somewhat labile substance, while the activator is believed to be larger, less diffusible, but more stable molecule. In this way, the activator tends to be most effective a short distance from its sources, and the inhibitor is most effective at a greater distance.

As well as affecting development, the two components affect their own production (Fig. 5-2). The activator stimulates its own production and also that of the inhibitor. Conversely, the inhibitor acts cross-catalytically to suppress the production of activator. With these properties, the equations of Gierer and Meinhardt (1972) predict that

Figure 5-2: Diagrammatic representation of the basic properties of a two-morphogen reaction-diffusion system (see Turing, 1952; Gierer and Meinhardt, 1972; Bode and Bode, 1984). Activator and inhibitor are produced by their sources. The activator diffuses slowly, is more stable, and stimulates both the production of more activator and inhibitor, and the differentiation of the "activated" tissue. The inhibitor diffuses more quickly, is more labile, and inhibits activator production and differentiation. With these properties, a mechanism of "short-range activation, long-range inhibition" is established. (Figure courtesy of J.B. Armstrong.)

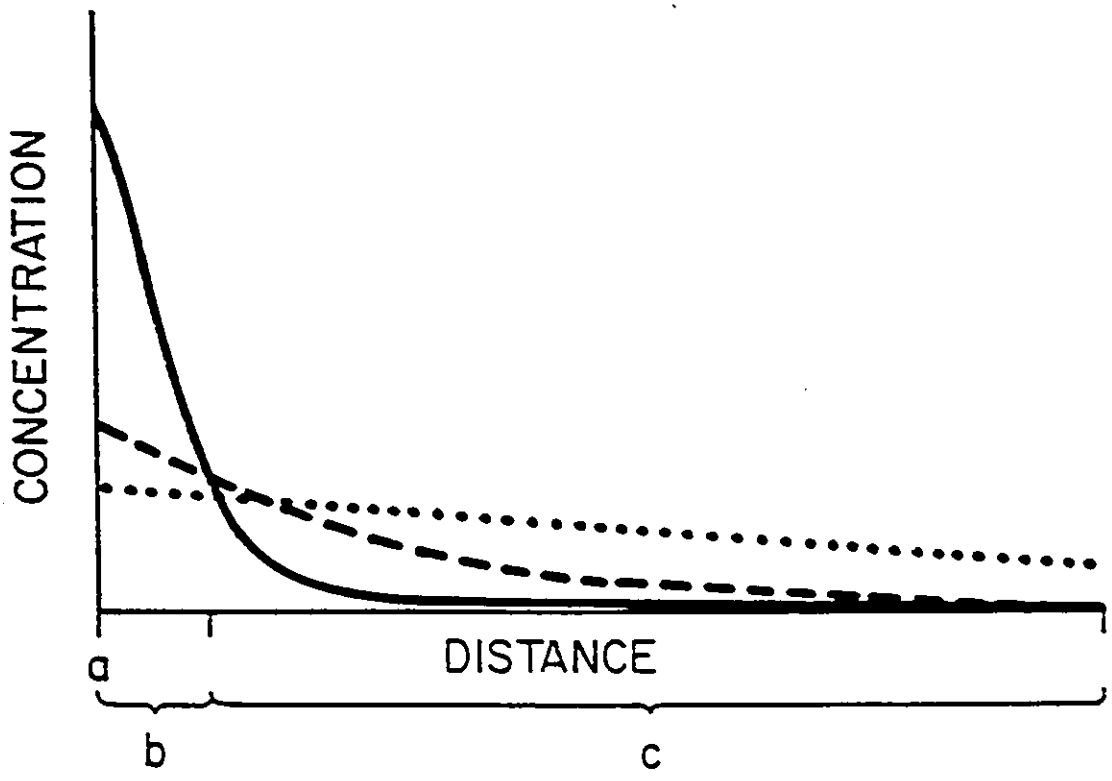

SLOW DIFFUSION
LONG ½
LIFE
FAST DIFFUSION
SHORT ½
LIFE
ACTIVATOR
INHIBITOR
+
−
+
ACTIVATOR
SOURCE
INHIBITOR
SOURCE

even a very shallow gradient of sources of the two components is sufficient to cause a stable pattern of activator and inhibitor. This is achieved when the production, diffusion, and breakdown of the two agents reach a dynamic equilibrium. At equilibrium, the maximal concentrations of both activator and inhibitor will be at the higher end of the source gradient.

During heart development, it is possible that there is a slightly higher level of sources of activator and inhibitor in the heart-forming mesoderm, since this area of the mesodermal mantle is exposed to a slightly longer induction. This slight gradient of sources would, therefore, cause a stable pattern of activator and inhibitor to form within the heart-field mesoderm (Fig. 5-3). In the heart-forming mesoderm, the high concentration of activator would promote the organization of the contractile proteins into functional sarcomeres and possibly also the initial formation of the heart tube. Later morphogenetic changes in the heart are undoubtedly the result of other processes, since the formation of beating tissue and looping of the heart can occur independently (see Bacon, 1945; Manasek and Monroe, 1972; Manasek *et al.*, 1984).

In the rest of the heart-field mesoderm, the excess of inhibitor would prevent this organization. In this way, the tissue which actually forms the heart becomes distinct from

Figure 5-3: Graphic representation of the concentrations of activator (——————) and inhibitor (- - - - -) with the properties shown in Fig. 5-2, if a shallow gradient of sources (.....) is present. As predicted by the equations of Gierer and Meinhardt (1972), stable gradients of activator and inhibitor are produced at the location of the highest concentration of sources. In the heart mesoderm, if the leading (anterior-most) edge of the mesodermal mantle were at the left side of the graph (a); (b) would be the heart-forming mesoderm, and (c) would be the region of the heart field where the formation of heart tissue is normally inhibited. Together, (b) and (c) comprise the entire heart field.


CONCENTRATION
DISTANCE
a
b
c

the rest of the heart field. As well, the formation of overly large and/or ectopic hearts could be prevented, even though a large area of heart-field tissue, capable of forming myocardium, is present. However, if the heart-forming area is extirpated, the gradients can be re-established in the adjacent heart field and a new heart-forming area generated (see Copenhaver, 1955). In this way, the final differentiation of the cardiomyocytes and some aspects of the initial morphogenesis of the heart could be co-ordinately controlled. Later during heart development, neural crest cells invade the heart area, and begin to form appropriate structures, possibly under the direction of the heart mesoderm.

In the hearts of cardiac-lethal mutant embryos, the production of the activator is suppressed and/or the production of the inhibitor is augmented. Alternatively, it is possible that normal gradients of abnormal components (totally inactive, or missing only some functions) are formed. Thus, cardiomyocyte differentiation is never completed, even though the contractile proteins are present, and the hearts do not begin to beat.

At this point, the question becomes: what is the nature of the activator and inhibitor? While there is no data to indicate the nature of the inhibitor, it is possible that the RNA-containing preparations of Davis and Lemanski (1987)

contain the activator. This is likely, since such preparations are capable of causing myofibrillogenesis in c/c hearts (see also Section 3.5). However, the active RNA preparations were isolated from anterior endoderm, rather than from the heart mesoderm itself. This may indicate that the pharyngeal endoderm plays a role in sequestering the activator (and/or inhibitor), and thus participates in the formation of the gradients.

The reaction-diffusion model (Armstrong, 1989) predicts, and my data (Sections 4.7; 5.2) shows, that the heart mesoderm is the most likely source of the activator and inhibitor. Therefore, the endoderm is probably not the original source of the activator. Recently, LaFrance *et al.* (1989) have shown that RNA-containing preparations from sheep heart are also capable of stimulating myofibrillogenesis in c mutant hearts. This indicates that myocardial tissue probably is the source of the activator. It also suggests that a very similar, or identical, mechanism may regulate mammalian heart development.

Similarly, RNA from chick heart tissue has been shown to stimulate the formation of functional myocardial tissue by chick blastoderm (Niu and Deshpande, 1973; Deshpande *et al.*, 1977; Khandekar *et al.*, 1984; Siddiqui *et al.*, 1986). Desjardins *et al.* (1989) have suggested that this is artifactual. But if not, it may indicate that heart

development is similarly regulated in birds.

A priori, RNA might not seem to be the obvious choice for the activator and inhibitor. Indeed, some proteinaceous or other contaminant of Davis and Lemanski's (1987) preparations could be responsible for the induction. However, it is interesting to note that just such roles have been found for small RNAs during the development of brine shrimp (*Artemia salina*; Lee-Huang *et al.*, 1977). Although these authors are apparently unaware of reaction-diffusion mechanisms, they describe the characteristics of 2 RNA species (which they call the activator and inhibitor), and show how the two interact to promote and repress (respectively) the translation of mRNA.

Despite the fact that Lee-Huang *et al.* (1977) did not associate their descriptions with reaction-diffusion models, some of the characteristics of their RNAs bear a remarkable resemblance to that predicted by Turing-like models. For example, the activator is 50% larger than the inhibitor (M_r 9000 and 6000, respectively). The RNAs are produced and degraded by opposite RNases (RNase A degrades inhibitor and produces activator; RNase T1 performs the opposite functions). Furthermore, Lee-Huang *et al.* (1977) believe that the the activator selectively binds the inhibitor to neutralize it. Therefore, different species of RNA may, in fact, be valid candidates for the roles of activator and

inhibitor.

Variations of the reaction-diffusion mechanism, initially proposed by Turing (1952), have been used to explain the development of a variety of biological patterns (see Lacalli and Harrison, 1979; Harrison, 1982; 1987; Meinhardt, 1982, for reviews). This includes pattern formation during head and foot development and regeneration in *Hydra* (Gierer and Meinhardt, 1972; Bode and Bode, 1984), slime mould differentiation (Lacalli and Harrison, 1978), shell formation in molluscs (Meinhardt and Klingler, 1987), plant growth (Meinhardt, 1984), and the development of a variety of systems in *Drosophila* (Kauffman *et al.*, 1978; Meinhardt, 1986; Lacalli *et al.*, 1988). Among vertebrates, similar models have been used to explain pigmentation patterns in mammals (Murray, 1981), and chick limb development (Newman and Frisch, 1979). However, most of these examples are based solely on morphological correlations with computer-generated patterns. In almost none (the notable exception being *Hydra*; see Bode and Bode, 1984) has the presence of the activator or inhibitor been experimentally demonstrated.

The data presented in this thesis support the proposal that a two-morphogen reaction-diffusion system controls the later phases of heart development in urodeles, and possibly in other vertebrates as well. This is, to my knowledge, the

first experimental evidence for such a mechanism in any vertebrate organ system. As well, while the models mentioned above all address problems of pattern formation, they remain vague on how cellular differentiation is co-ordinated with these larger morphogenetic processes. The model presented here provides an explanation for how the regulation of these two fundamental developmental processes could be linked.

REFERENCES

Amano, H. (1961). Le rôle de l'entoblaste dans la formation du coeur chez l'Urodèle. C.R. Soc. Biol. 155: 2218.

Armstrong, J.B. (1989). A Turing model to explain heart development. Axolotl Newsletter 18: 23-25.

Armstrong, J.B., S.T. Duhon, and G.M. Malacinski. (1989). Raising the axolotl in captivity. In: *Developmental Biology of the Axolotl.* (J.B. Armstrong and G.M. Malacinski, eds.) Oxford University Press, NY, USA, *pp.* 220-227.

Asashima, M., G.M. Malacinski, and S.C. Smith. (1989). Surgical manipulation of embryos. In: *Developmental Biology of the Axolotl.* (J.B. Armstrong and G.M. Malacinski, eds.) Oxford University Press, NY, USA, *pp.* 255-263.

Bacon, R.L. (1945). Self-differentiation and induction in the heart of *Amblystoma*. J. Exp. Zool. 98: 87-121.

Balinsky, B.I. (1939). Experiments on total extirpation of the whole endoderm in *Triton* embryos. C. R. Acad. Sci. URSS 23: 196-198.

Berger, S.L. (1987). Isolation of cytoplasmic RNA: Ribonucleoside-vanadyl complexes. In: *Guide to Molecular Cloning Techniques (Methods in Enzymology, Vol. 152.)*

(S.L. Berger and A.R. Kimmel, eds.) Academic Press, Inc., Orlando, Fla., USA, *pp.* 231-232.

Besson, W.T. III, M.L. Kirby, L.H.S. Van Mierop, and J.R. Teabeaut II. (1986). Effects of the size of lesions of the cardiac neural crest at various embryonic ages on incidence and type of cardiac defects. Circulation 73: 360-364.

Bode, P.M. and H.R. Bode. (1984). Patterning in Hydra. In: *Pattern Formation.* (G.M. Malacinski and S.V. Bryant, eds.) Macmillan Press, NY, USA, *pp.* 213-241.

Bordzilovskaya, N.P. and T.A. Dettlaff. (1979). Table of stages of the normal development of axolotl embryos and the prognostication of timing of successive developmental stages at various temperatures. Axolotl Newsletter 7: 2-22.

Bordzilovskaya, N.P., T.A. Dettlaff, S.T. Duhon, and G.M. Malacinski. (1989). Developmental stage series of axolotl embryos. In: *Developmental Biology of the Axolotl.* (J.B. Armstrong and G.M. Malacinski, eds.) Oxford University Press, NY, USA, *pp.* 201-219.

Bride, M. (1979). Autodifférenciation du mésoderme précardiaque d'Amphibiens Anoures en culture *in vitro*. C.R. Acad. Sci. Paris 278 D: 777-780.

Butros, J. (1965). Action of heart and liver RNA on the differentiation of segments of chick blastoderms. J.

Embryol. Exp. Morphol. 13: 119-128.

Copenhaver, W.M. (1926). Experiments on the development of the heart of *Amblystoma punctatum*. J. Exp. Zool. 43: 103-126.

Copenhaver, W.M. (1955). Heart, blood vessels, blood, and entodermal derivatives. In: *Analysis of Development*. (B.H. Willier, P.A. Weiss, and V. Hamburger, eds.) W.B. Saunders Co., Philadelphia, PA, USA, *pp.* 440-461.

Davis, L.A. and L.F. Lemanski. (1983). Inductive properties of a factor produced by endoderm. J. Cell Biol. 97: 58a (abstract).

Davis, L.A. and L.F. Lemanski. (1987). Induction of myofibrillogenesis in cardiac lethal mutant axolotl hearts rescued by RNA derived from normal endoderm. Development 99: 145-154.

Deshpande, A.K. and M.A.Q. Siddiqui. (1977). A reexamination of heart muscle differentiation in the post nodal piece of chick blastoderm mediated by exogenous RNA. Dev. Biol. 58: 230-247.

Deshpande, A.K. and M.A.Q. Siddiqui. (1978). Acetylcholinesterase differentiation during myogenesis in early chick embryonic cells caused by an inducer RNA. Differentiation 10: 133-137.

Deshpande, A.K., S.B. Jakowlew, H.-H. Arnold, P.A. Crawford, and M.A.Q. Siddiqui. (1977). A novel RNA affecting

embryonic gene functions in early chick blastoderm. J. Biol. Chem. 252: 6521-6527.

Desjardins, P., D. L'Abbé, B.F. Lang, and R. Morais. (1989). Putative chicken "muscle-specific 7 S RNA" is related to the mitochondrial ATPase 6 gene. J. Molec. Biol. 207: 625-629.

Ekman, G. (1925). Experimentelle Beiträge zur Herzentwicklung der Amphibien. Roux' Arch. Entw.-mech. 106: 320-352.

Epstein, M.L. and L.F. Lemanski. (1980). Electrical activity in cardiac mutant axolotl hearts. J. Exp. Zool. 211: 131-136.

Fales, D.E. (1946). A study of double hearts produced experimentally in embryos of *Amblystoma punctatum*. J. Exp. Zool. 101: 281-298.

Fautrez, J. and H. Amano. (1961). Pourquoi le coeur ne se développe-t-il pas après extirpation de l'entoblaste dans une larvae d'Urodèle? C.R. Soc. Biol. 155: 2219.

Fransen, M.E. and L.F. Lemanski. (1988). Myocardial cell relationships during morphogenesis in normal and cardiac lethal mutant axolotls, *Ambystoma mexicanum*. Amer. J. Anat. 183: 245-257.

Fuldner, R.A., S.-S. Lim, M.L. Greaser, and L.F. Lemanski. (1984). Accumulation and localization of troponin-T in developing hearts of *Ambystoma mexicanum*. J. Embryol. Exp. Morphol. 84: 1-17.

Fullilove, S.L. (1970). Heart induction: Distribution of active factors in newt endoderm. J. Exp. Zool. 175: 323-326.

Gierer, A. and H. Meinhardt. (1972). A theory of biological pattern formation. Kybernetic 12: 30-39.

Graveson, A.C. (1990). *Studies on the differentiation of cranio-visceral cartilage in normal and premature death mutant embryos of* Ambystoma mexicanum. Ph.D. Thesis, University of Ottawa, Ottawa, Ont.

Graveson, A.C. and J.B. Armstrong. (1987). Differentiation of cartilage from cranial neural crest in the axolotl (*Ambystoma mexicanum*). Differentiation 35: 16-20.

Graveson, A.C. and J.B. Armstrong. The premature death (*p*) mutation of *Ambystoma mexicanum* affects a subpopulation of the neural crest. (manuscript submitted).

Hall, B.K. and S. Hörstadius. (1988). *The Neural Crest*. Oxford University Press, London, UK.

Harrison, R.G. (1969). Harrison stages and description of the normal development of the spotted salamander, *Ambystoma punctatum* (Linn.). In: *Organization and Development of the Embryo*. (S. Wilens, ed.) Yale University Press, New Haven, CT, USA. *pp*. 44-66.

Harrison, L.G. (1982). An overview of kinetic theory in developmental modeling. In: *Developmental Order: Its Origin and Regulation*. (S. Subtelny and P.B. Green,

eds.) Alan R. Liss, Inc., NY, USA, *pp.* 3-33.

Harrison, L.G. (1987). What is the status of reaction-diffusion theory thirty-four years after Turing? J. Theor. Biol. **125**: 369-384.

Hill, C.S. and L.F. Lemanski. (1979). Morphological studies on cardiac lethal mutant salamander hearts in organ cultures. J. Exp. Zool. **209**: 1-20.

Hirakow, R. (1986). Development of the vertebrate heart and the extracellular matrix. Congen. Anom. **26**: 205-212.

Hirakow, R., S. Komazaki, and T. Hiruma. (1987). Early cardiogenesis in the newt embryo. Scanning Microsc. **1**: 1367-1376.

Hommes, O.R. (1957). *Primary Entodermal Defects, Development of Body Form and Genital Organs of Acardii in Uni-vitelline Twins*. Jacob van Campen Press, Amsterdam, Holland.

Hörstadius, S. (1950). *The Neural Crest: Its Properties and Derivatives in the Light of Experimental Research*. Oxford University Press, London, UK.

Humphrey, R.R. (1968). A genetically determined absence of heart function in embryos of the Mexican axolotl (*Ambystoma mexicanum*). Anat. Rec. **160**: 475 (abstract).

Humphrey, R.R. (1972). Genetic and experimental studies on a mutant gene (*c*) determining absence of heart action in embryos of the Mexican axolotl (*Ambystoma mexicanum*).

Dev. Biol. 27: 365-375.

Jacobson, A.G. (1960). Influences of ectoderm and endoderm on heart differentiation in the newt. Dev. Biol. 2: 138-154.

Jacobson, A.G. (1961). Heart determination in the newt. J. Exp. Zool. 146: 139-151.

Jacobson, A.G. and J.T. Duncan. (1968). Heart induction in salamanders. J. Exp. Zool. 167: 79-103.

Jacobson, A.G. and A.K. Sater. (1988). Features of embryonic induction. Development 104: 341-359.

Justus, J.T. (1978). The cardiac mutant: An overview. Amer. Zool. 18: 321-326.

Justus, J.T. and P.B. Hollander. (1971). Electrophysiology studies on the cardiac non-function mutation in the Mexican axolotl *Ambystoma mexicanum*. Experimentia 27: 1040-1041.

Kauffman, S.A., R.M. Shymko, and K. Trabert. (1978). Control of sequential compartment formation in *Drosophila*. Science 199: 259-270.

Khandekar, P., C. Saidapet, M. Krauskopf, A.-M. Zarraga, W.-L. Lin, C. Mendola, and M.A.Q. Siddiqui. (1984). Co-ordinate control of gene expression. J. Mol. Biol. 180: 417-435.

Kirby, M.L. and D.E. Bockman. (1984). Neural crest and normal development: A new perspective. Anat. Rec. 209: 1-6.

Kirby, M.L. and D.E. Stewart. (1983). Neural crest origin of cardiac ganglion cells in the chick embryo: Identification and extirpation. Dev. Biol. 97: 433-443.

Kirby, M.L., T.F. Gale, and D.E. Stewart. (1983). Neural crest cells contribute to normal aorticopulmonary septation. Science 220: 1059-1061.

Kirby, M.L., K.L. Turnage, and B.M. Hayes. (1985). Characterization of conotruncal malformations following ablation of "cardiac" neural crest. Anat. Rec. 213: 87-93.

Krug, E.L., R.B. Runyan, and R.R. Markwald. (1985). Protein extracts from early embryonic hearts initiate cardiac endothelial cytodifferentiation. Dev. Biol. 112: 414-426.

Kulikowski, R.R. and F.J. Manasek. (1977). Cardiac mutant salamanders: Evidence for heart induction. J. Exp. Zool. 201: 485-490.

Kulikowski, R.R. and F.J. Manasek. (1978). The cardiac lethal mutant of *Ambystoma mexicanum*: A re-examination. Amer Zool. 18: 349-358.

Lacalli, T.C. and L.G. Harrison. (1978). The regulatory capacity of Turing's model for morphogenesis, with application to slime moulds. J. Theor. Biol. 70: 273-295.

Lacalli, T.C. and L.G. Harrison. (1979). Turing's conditions

and the analysis of morphogenetic models. J. Theor. Biol. 76: 419-436.

Lacalli, T.C., D.A. Wilkinson, and L.G. Harrison. (1988). Theoretical aspects of stripe formation in relation to *Drosophila* segmentation. Development 103: 105-113.

LaFrance, S.M., H. Zou, B. Dunham, and L.F. Lemanski. (1989). Induction of myofibrillogenesis in cardiac mutant axolotls. J. Cell Biol. 109: 167a (abstract).

LeDouarin, N.M. (1975). The neural crest in the neck and other parts of the body. In: *Morphogenesis and Malformation of Face and Brain (Birth Defects: Original Article Series, Vol. XI, No. 7.)*. (D. Bergsma, ed.), Alan R. Liss, NY, USA, *pp.* 19-50.

LeDouarin, N.M. (1982). *The Neural Crest*. Cambridge University Press, Cambridge, UK.

Lee-Huang, S., J.M. Sierra, R. Naranjo, W. Filipowicz, and S. Ochoa. (1977). Eucaryotic oligonucleotides affecting mRNA translation. Arch. Biochem. Biophys. 180: 276-287.

LeLièvre, C.S. and N.M. LeDouarin. (1975). Mesenchymal derivatives of the neural crest: Analysis of chimeric quail and chick embryos. J. Embryol. Exp. Morphol. 34: 125-154.

Lemanski, L.F. (1973a). Morphology of developing heart in cardiac lethal mutant Mexican axolotls (*Ambystoma mexicanum*). Dev. Biol. 33: 312-333.

Lemanski, L.F. (1973b). Heart development in the Mexican salamander, *Ambystoma mexicanum*. II. Ultrastructure. Amer. J. Anat. 136: 487-526.

Lemanski, L.F. (1976). Morphological and biochemical abnormalities in hearts of cardiac mutant salamanders (*Ambystoma mexicanum*). J. Supramolec. Struct. 5: 221-238.

Lemanski, L.F. (1978). Morphological, biochemical and immunohistochemical studies on heart development in cardiac mutant axolotls, *Ambystoma mexicanum*. Amer. Zool. 18: 327-348.

Lemanski, L.F. (1979). Role of tropomyosin in actin filament formation in embryonic salamander heart cells. J. Cell Biol. 82: 227-238.

Lemanski, L.F. and T.P. Fitzharris. (1989). Analysis of the endocardium and cardiac jelly in truncal development in the cardiac lethal mutant axolotl *Ambystoma mexicanum*. J. Morphol. 200: 123-130.

Lemanski, L.F. and R.A. Fuldner. (1977). Immunofluorescent studies for myosin, tropomyosin, and α-actinin in developing hearts of normal and cardiac lethal mutant salamanders (*Ambystoma mexicanum*). J. Cell Biol. 75: 327a (abstract).

Lemanski, L.F., E.M. Bertke, and J.T. Justus. (1970). The ultrastructure of myocardial cells in normal and cardiac

lethal mutant Mexican axolotls (*Ambystoma mexicanum*). In: *Proceedings of the Electron Microscopy Society of America, 28th annual meeting.* Claitor's Publishers, Baton Rouge, La., USA. *pp.* 62-63.

Lemanski, L.F., X. Joseph, and M.R. Iyengar. (1975). Quantitation by radioimmunoassay of absolute amounts of myosin in embryonic hearts of cardiac lethal mutant axolotls (*Ambystoma mexicanum*). J. Cell Biol. **67**: 239a (abstract).

Lemanski, L.F., B.S. Marx, and C.S. Hill. (1977). Evidence for abnormal heart induction in cardiac-mutant salamanders (*Ambystoma mexicanum*). Science **196**: 894-896.

Lemanski, L.F., D.J. Paulson, and C.S. Hill. (1979). Normal anterior endoderm corrects the heart defect in cardiac mutant salamanders (*Ambystoma mexicanum*). Science **204**: 860-862.

Löfberg, J., H.H. Epperlein, R. Perris, and M. Stigson. (1989). Neural crest cell migration: A pictorial essay. In: *Developmental Biology of the Axolotl.* (J.B. Armstrong and G.M. Malacinski, eds.) Oxford University Press, NY, USA, *pp.* 83-101.

Manasek, F.J. and R.G. Monroe. (1972). Early cardiac morphogenesis is independent of function. Dev. Biol. **27**: 584-588.

Manasek, F.J., R.R. Kulikowski, A. Nakamura, Q. Nguyenphuc,

and J.W. Lacktis. (1984). Early heart development: A new model of cardiac morphogenesis. In: *Growth of the Heart in Health and Disease.* (R. Zak, ed.) Raven Press, New York, NY, USA, *pp.* 105-130.

Mangold, O. (1956). Experimente zur Analyse der Herzentwicklung bei *Triton*. Naturwiss. 43: 287.

Meinhardt, H. (1982). Generation of structures in a developing organism. In: *Developmental Order: Its Origin and Regulation*. (S. Subtelny and P.B. Green, eds.) Alan R. Liss, Inc., NY, USA, *pp.* 439-461.

Meinhardt, H. (1984). Models of pattern formation and their application to plant development. In: *Positional Controls in Plant Development*. (P.W. Barlow and D.J. Carr, eds.) Cambridge University Press, Cambridge, UK, *pp.* 1-32.

Meinhardt, H. (1986). Hierarchical inductions of cell states: A model for segmentation in *Drosophila*. J. Cell Sci. Suppl. 4: 357-381.

Meinhardt, H. and M. Klingler. (1987). A model for pattern formation an the shells of molluscs. J. Theor. Biol. 126: 63-89.

McLean, M.J., J.-F. Renaud, M.C. Niu, and N. Sperelakis. (1977). Membrane differentiation of cardiac myoblasts induced *in vitro* by an RNA-enriched fraction from adult heart. Exp. Cell Res. 110: 1-14.

Moore, P.B. and L.F. Lemanski. (1982). Quantification of tropomyosin by radioimmunoassay in developing hearts of cardiac mutant axolotls, *Ambystoma mexicanum*. J. Muscle Res. Cell Motil. 3: 161-167.

Murray, J.D. (1981). A pre-pattern formation mechanism for animal coat markings. J. Theor. Biol. 88: 161-199.

Newman, S.A. and H.L. Frisch. (1979). Dynamics of skeletal pattern formation in developing chick limb. Science 205: 662-668.

Nieuwkoop, P.D. (1947). Experimental investigations on the origin and determination of the germ cells, and on the development of the lateral plates and germ ridges in Urodeles. Arch. Néerl. Zool. 8: 1-205.

Nishibatake, M., M.L. Kirby, and L.H.S. Van Mierop. (1987). Pathogenesis of persistent truncus arteriosus and dextroposed aorta in chick embryos after neural crest ablation. Circulation 75: 255-289.

Niu, M.C. and A.K. Deshpande. (1973). The development of tubular heart in RNA-treated post-nodal pieces of chick blastoderm. J. Embryol. Exp. Morphol. 29: 485-501.

Niu, M.C. and L. Mulherkar. (1970). The role of exogenous heart-RNA in development of the chick embryo cultivated *in vitro*. J. Embryol. Exp. Morphol. 24: 33-42.

Ojeda, J.L. and J.M. Hurle. (1981). Establishment of the tubular heart. Role of cell death. In: *Perspectives in*

Cardiovascular Research, Vol. 5, Mechanisms of Cardiac Morphogenesis and Teratogenesis. (T. Pexieder, ed.) Raven Press, New York, NY, USA, *pp.* 101-113.

Orts-Llorca, F. (1963). Influence of the endoderm on heart differentiation during the early stages of development of the chick embryo. Roux' Arch. Entw.-mech. **154**: 533-551.

Orts-Llorca, F. and D.R. Gil. (1965). Influence of the endoderm on heart differentiation. Roux' Arch. Entw.-mech. **156**: 368-370.

Patten, B.M., T.C. Kramer, and D.A. Barry. (1948). Valvular action in the embryonic chick heart by localized apposition of endocardial masses. Anat. Rec. **102**: 297-312.

Phillips, M.T., M.L. Kirby, and G. Forbes. (1987). Analysis of cranial neural crest distribution in the developing heart using quail-chick chimeras. Circ. Res. **60**: 27-30.

Rugh, R. (1962). *Experimental Embryology.* Burgess Publishing Co., Minneapolis, MN, USA.

Sater, A.K. and A.G. Jacobson. (1989). The specification of heart mesoderm occurs during gastrulation in *Xenopus laevis*. Development **105**: 821-830.

Sater, A.K. and A.G. Jacobson. (1990). The role of the dorsal lip in the induction of heart mesoderm in *Xenopus laevis*. Development **108**: 461-470.

Schreckenberg, G.M. and A.G. Jacobson. (1975). Normal stages of development of the axolotl, *Ambystoma mexicanum*. Dev. Biol. 42: 391-400.

Shen, P.-S. and L.F. Lemanski. (1986). Immunofluorescent studies for desmin, vimentin and vinculin in developing hearts of normal and cardiac mutant Mexican axolotls, *Ambystoma mexicanum*. J. Cell Biol. 103: 123a.

Shen, P.-S. and L.F. Lemanski. (1989). Immunofluorescent, immunogold, and electrophoretic studies for desmin in embryonic hearts of normal and cardiac mutant Mexican axolotls, *Ambystoma mexicanum*. J. Morphol. 201: 243-252.

Siddiqui, M.A.Q., A.M. Zarraga, D. Nicholson, A. Deshpande, C. Saidapet, and C. Mendola. (1986). Control of muscle gene expression: Transcription of myosin light chain gene and the role of repetitive elements in gene regulation. In: *Molecular Biology of Muscle Development*. Alan R. Liss, Inc., York, NY, USA. *pp.* 773-791.

Smith, H.M. (1989). Discovery of the axolotl and its early history in biological research. In: *Developmental Biology of the Axolotl*. (J.B. Armstrong and G.M. Malacinski, eds.). Oxford University Press, NY, USA, *pp.* 3-12.

Smith, S.C., and J.B. Armstrong. (1990). Heart induction in wild-type and cardiac mutant axolotls (*Ambystoma mexicanum*). J. Exp. Zool. (in press).

Smith, S.C., and J.B. Armstrong. The cardiac-lethal mutant: Evidence for a reaction-diffusion model of vertebrate cardiogenesis. (manuscript submitted).

Smith, S.C., M.J. Lannoo, and J.B. Armstrong. (1988). Lateral-line neuromast development in *Ambystoma mexicanum* and a comparison with *Rana pipiens*. J. Morphol. 198: 367-379.

Starr, C.M., J.G. Diaz, and L.F. Lemanski. (1985). Analysis of actin and tropomyosin in cardiac mutant axolotls by two-dimension gel electrophoresis, western blots, and immunofluorescent microscopy. J. Cell Biol. 101: 42a (abstract).

Starr, C.M., J.G. Diaz, and L.F. Lemanski. (1989). Analysis of actin and tropomyosin in hearts of cardiac mutant axolotls by two-dimensional gel electrophoresis, western blots, and immunofluorescent microscopy. J. Morphol. 201: 1-10.

Stewart, D.E., M.L. Kirby, and K.K. Sulik. (1986). Hemodynamic changes in chick embryos precede heart defects after cardiac neural crest ablation. Circ. Res. 59: 545-550.

Trottier, T.M. and J.B. Armstrong. (1977). Experimental studies on a mutant gene (*p*) causing premature death of *Ambystoma mexicanum* embryos. J. Embryol. Exp. Morphol. 39: 139-149.

Turing, A. (1952). The chemical basis of morphogenesis. Phil. Trans. Roy. Soc. Lond., B 237: 37-72.

Vanable, J.W. (1985). Benzocaine: An excellent amphibian anesthetic. Axolotl Newsletter 14: 19-21.

Venable, J.H. and R. Coggeshall, (1965). A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. 25: 407-408.

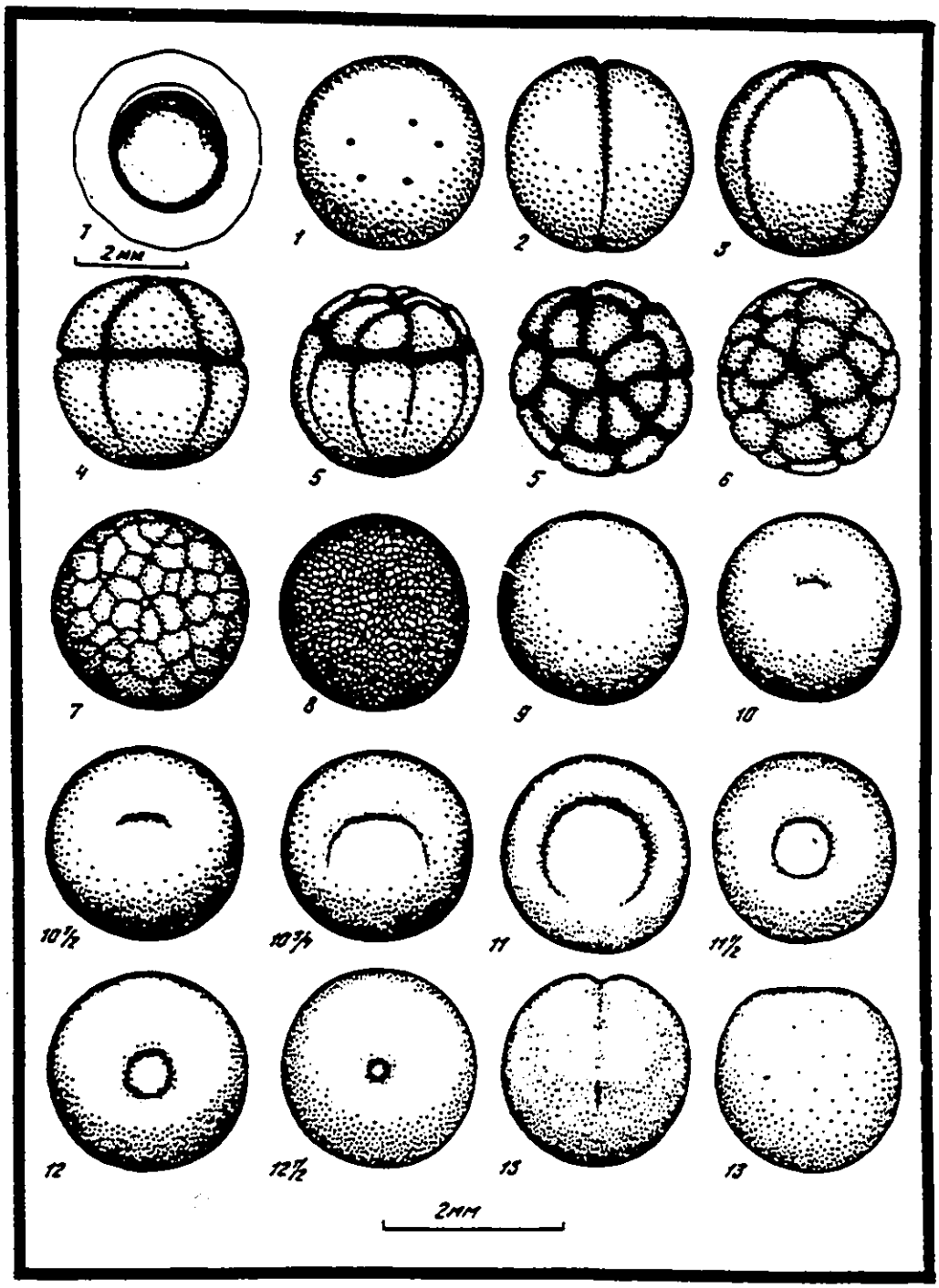
Weston, J.A. (1970). The migration and differentiation of neural crest cells. Adv. Morphogen. 8: 41-114.

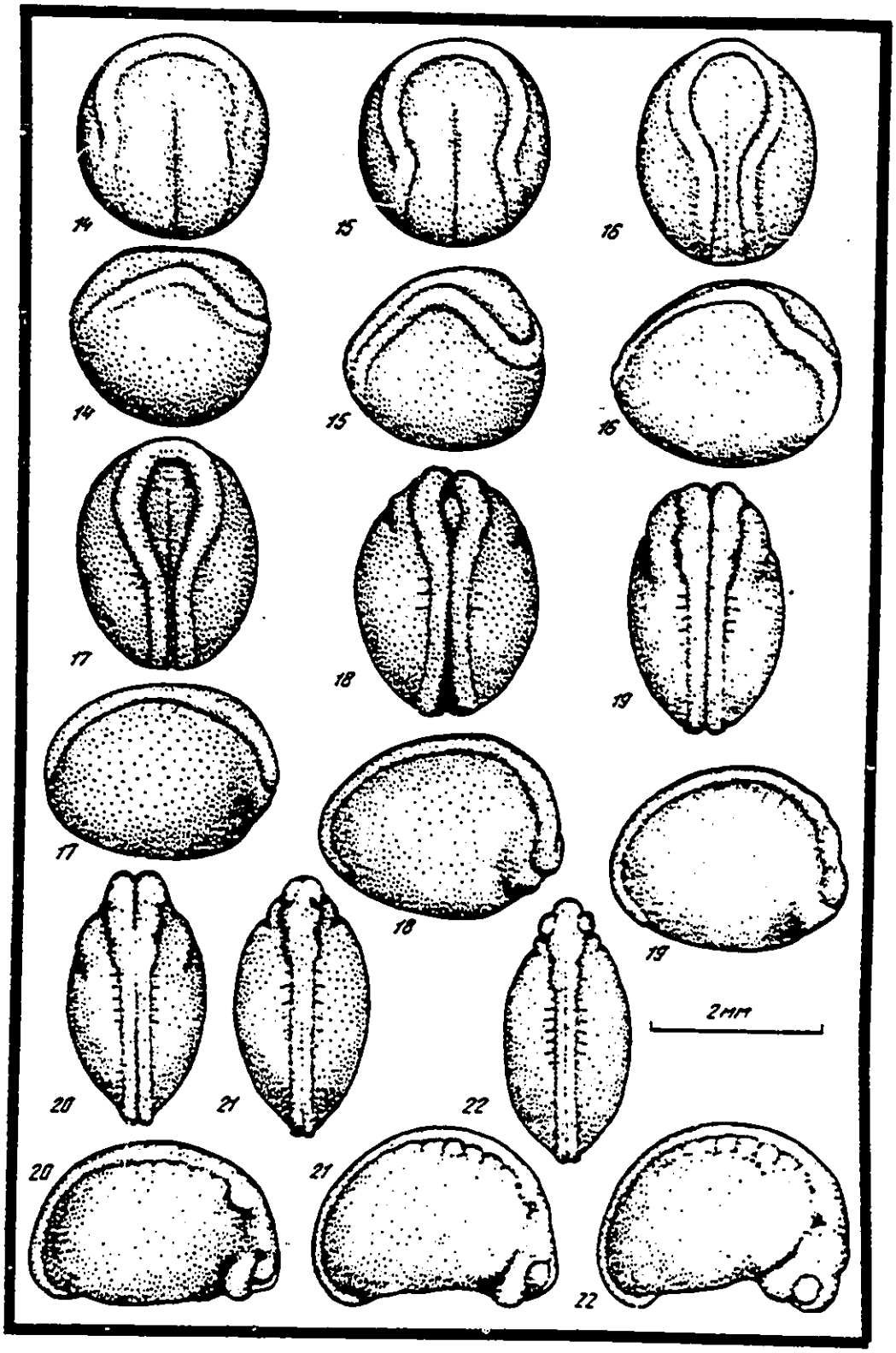
Wilens, S. (1955). The migration of heart mesoderm and associated areas in *Amblystoma punctatum*. J. Exp. Zool. 129: 579?-605.

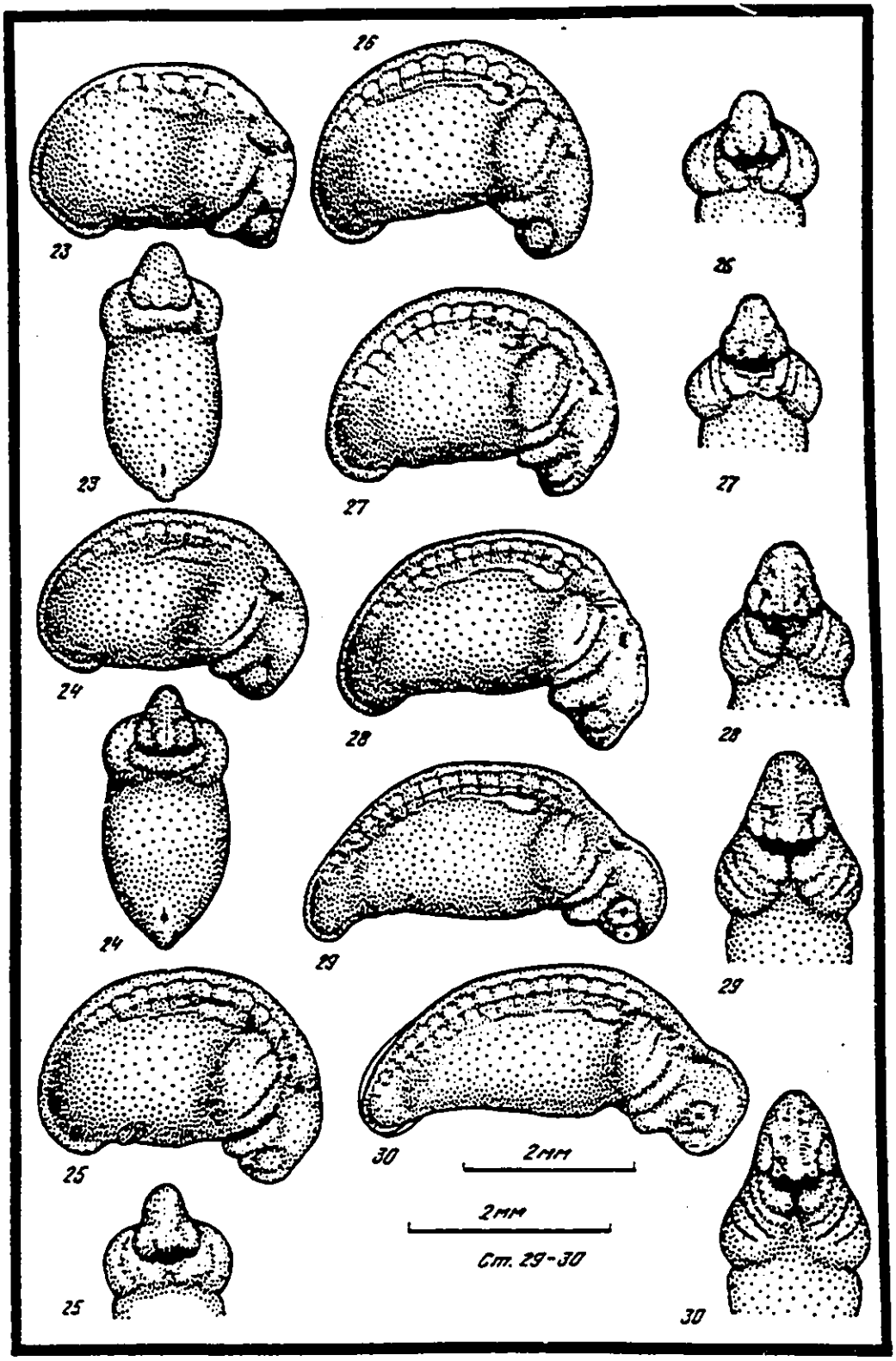
APPENDIX I

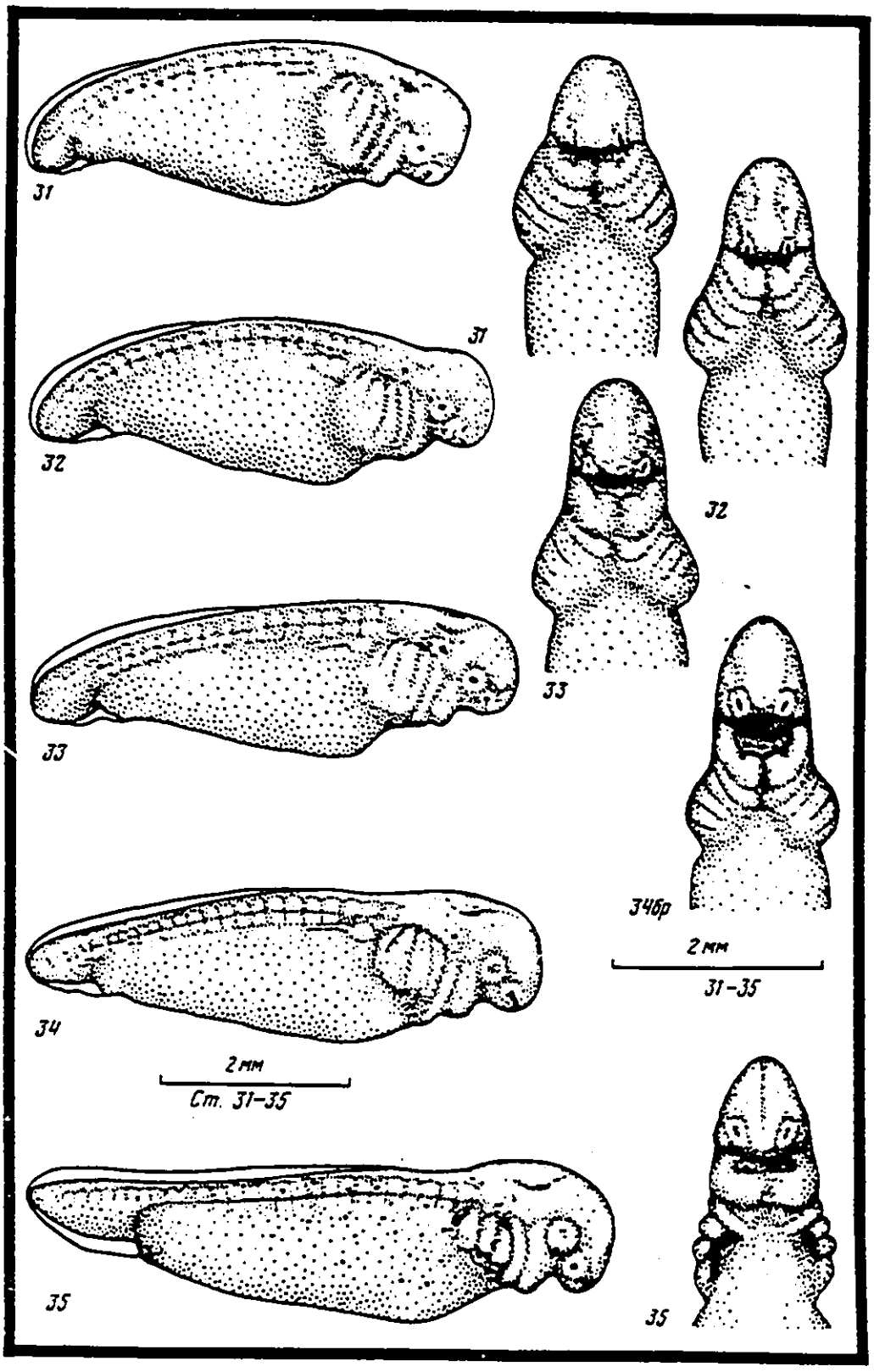
Developmental Staging of *Ambystoma mexicanum* embryos.

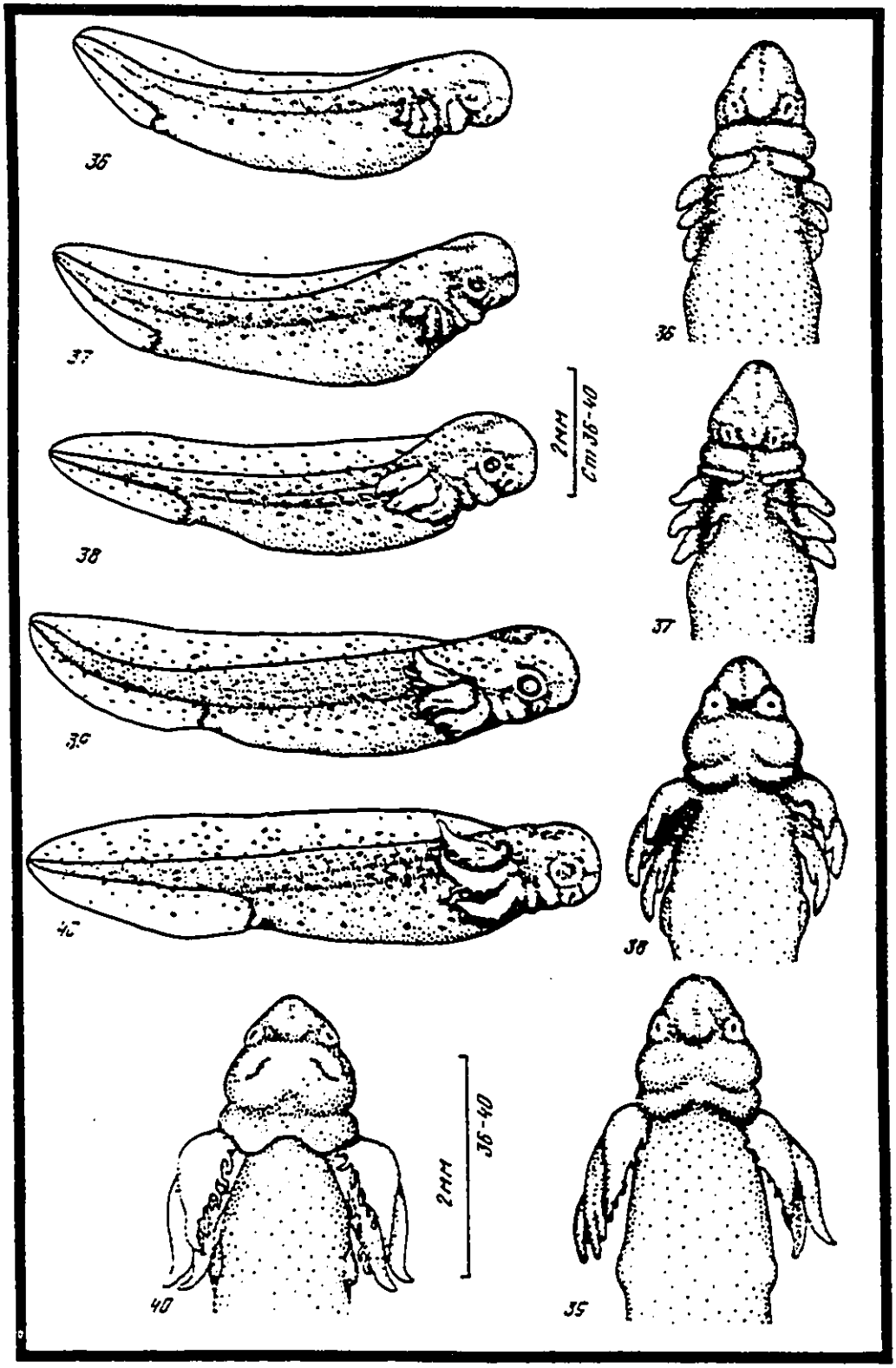
(From: Bordzilovskaya *et al.*, 1989.)

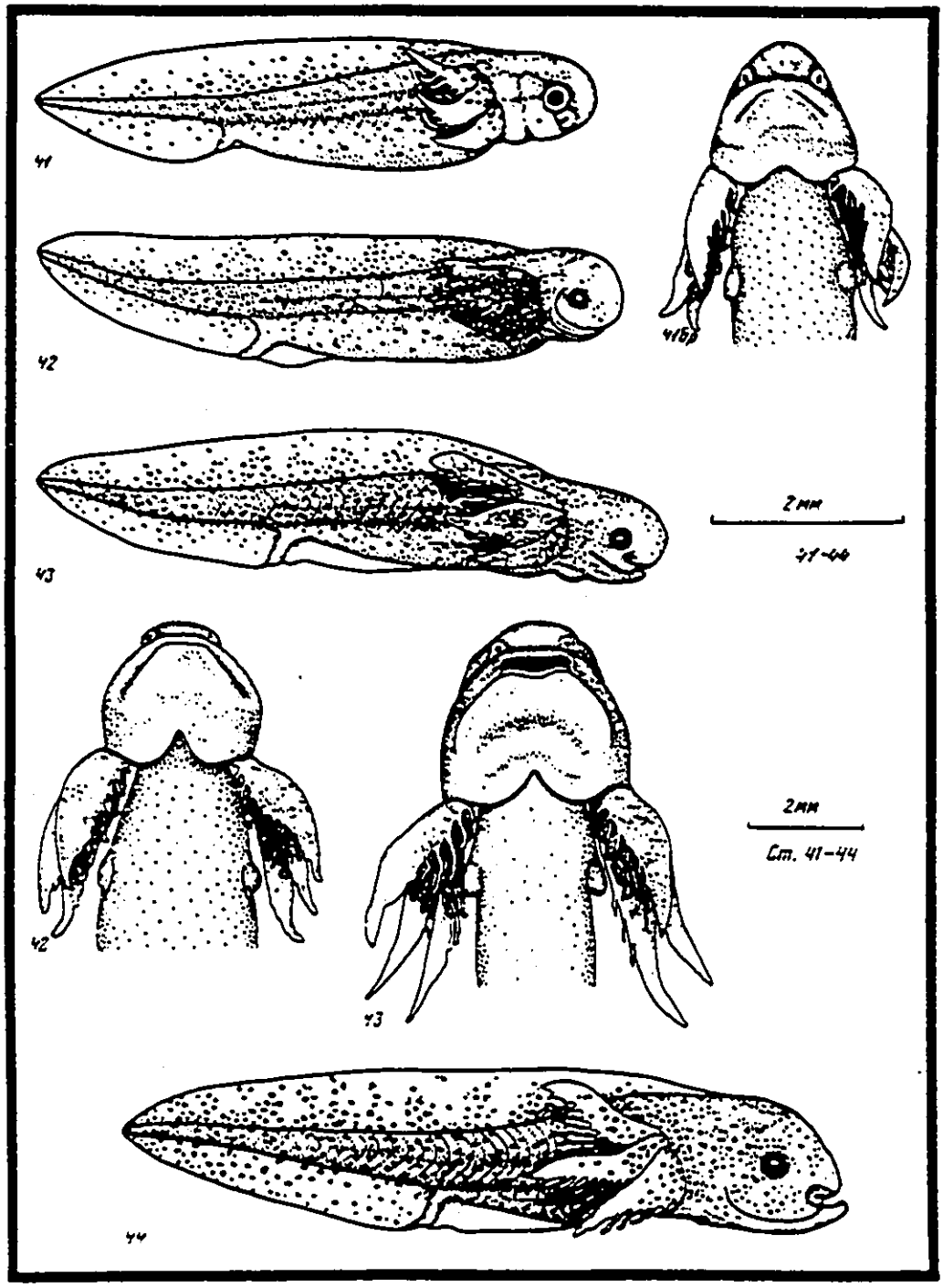

1
2MM
1
2
3
4
5
5
6
7
8
9
10
10½
10¾
11
11½
12
12½
13
13
2MM


14
15
16
14
15
16
17
18
19
17
18
19
2mm
20
21
22
20
21
22


26
23
26
23
27
27
24
28
28
24
29
29
25
30
2мм
2мм
См. 29-30
25
30


31
31
32
32
33
33
34
34б
2 мм
31-35
2 мм
См. 31-35
35
35


36
37
38
39
40
2MM
FIGS 36-40
36
37
38
39
40
2MM
36-40


41
42
41б
43
2мм
41-44
42
43
2мм
См. 41-44
44