Experimental studies on a
mutant gene \((p)\) causing premature death of
Ambystoma mexicanum embryos

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SUMMARY

The premature death \((p)\) mutation is a recessive lethal, which, in the homozygous condition, gives rise to a complex of abnormalities. The mutant embryos develop only to stage 37, at which time disintegration of superficial tissue begins. Many of the abnormalities observed in sections of the stage-37 mutant embryo are related to its failure to establish a functioning circulatory system, or to the resulting edema and/or ascites that distend the abdomen and flanks. There are, however, abnormalities of heart, liver, gill and muscle development which cannot be attributed to lack of circulation and edema. All of these abnormalities can be indirectly related to the endoderm, particularly the anterior and dorsal endoderm. The findings, therefore, suggest that the mutation leads to a fairly general defect of the endoderm.

INTRODUCTION

Amphibians have long been favorite organisms for the study of embryology, and in recent years genetics has been used increasingly as a tool for studying their development. The Mexican axolotl (Ambystoma mexicanum) is the best genetically studied amphibian species; to date, over 30 genes have been described that affect oogenesis or embryonic and larval development (Humphrey, 1975). These genes have been placed into five categories (Briggs, 1973; Malacinski & Brothers, 1974): maternal effect genes causing deficiencies in the egg cytoplasm, genes whose effect is on specific organs or tissues, genes that affect the nucleolus, genes that affect pigment cell differentiation or color pattern, and finally, the so-called ‘autonomous lethals’.

Autonomous lethals are defined as genes which exert lethal effects that cannot be corrected by parabiosis or by grafting organ primordia into a normal host. The effects are thus ‘autonomous’ in the sense that they cannot be corrected by substances capable of being transported from the normal co-twin or host to the mutant co-twin or an implanted organ or tissue. Another feature of most autonomous lethals is that they generally affect some essential function common

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to all cells of a fairly advanced embryo. However, many of the mutants in this
group have not been extensively characterized, and it is quite likely that not all
will fit conveniently into this simple scheme when more is known about them.
For example, recently it has been shown that the stasis mutant (gene st),
erroneously classed as an autonomous lethal on the basis of parabiosis experi-
ments, does not produce a lethal effect on all cells. Transplants of gill and limb
primordia from st/st donors have been found to survive and develop normally
on normal recipients (Humphrey, 1975).

The premature death mutant (gene p), also assigned to this group of auto-
nomous lethals (Briggs, 1973; Malacinski & Brothers, 1974; Humphrey 1975),
was discovered originally by Dr R. Tompkins, then at Princeton University. He
found that mutants survived only to stage 36 of Harrison's series (unbranched
gill rudiments) and noted that heart action continued for a time after dis-
integration of superficial tissue began. We observed what appeared to be the
same mutant phenotype in progeny of a mating between sibs sent to us by
R. R. Humphrey (see below). On inquiring, we found that Tompkins' original
stock had been lost. We are, however, retaining the designation p for our mutant
because of the similarity to Tompkins' description. In what follows, we describe
the morphology and histology of the mutant embryos, and the results of
parabiosis experiments.

MATERIALS AND METHODS

Animals

The parents of these spawnings, kindly donated by R. R. Humphrey, were
sibs designated 3719–2 and 3719–5. In addition to being heterozygous for the
gene p, they were also heterozygous for the gene axanthic (ax). The male was
heterozygous for the gene melanoid (m), while the female was homozygous for
that gene.

Parabiotic twins

Parabiotic combinations were made from embryos at stages 24–25 of the
Schreckenberg & Jacobson (1975) series. Embryos were manually dejellied with
watchmaker's forceps and allowed to remain in calcium-free 10 % Holtfreter's
saline containing 400 mg/l. each of penicillin and streptomycin, for several
minutes before the operation. Ectoderm, plus underlying mesoderm, was
removed from the appropriate sides of the prospective twins, which were then
placed side by side in a narrow groove cut in an agar-lined Petri plate, and
allowed to remain in the calcium-free modified Holtfreter's solution for several
hours while wound healing took place. This calcium-free solution was then
gradually replaced by 10 % Holtfreter's with 400 mg/l. each of penicillin and
streptomycin.
Histology and cytology

For routine histological studies, whole embryos were fixed in Smith's fixative for 24 h, washed in tap water for 24–28 and kept in 5 % formaldehyde. Embryos to be sectioned were dehydrated in a graded ethanol series and were transferred to a 1:1 ethanol-ether mixture for 24 h. Specimens were then infiltrated for 24 h or longer in a 3–5 % collodion solution, and were vacuum embedded in Paraplast. Sections were cut at 10 μm and mounted on albuminized glass slides. Sections were stained with Harris' hematoxylin and eosin, dehydrated and mounted in Permount.

RESULTS

Origin and mode of inheritance of gene \( p \)

A mating of two sibs (3719-2 and 3719-5) obtained from R. R. Humphrey produced a spawning in which 71 of 261 progeny (27.2 %) showed the mutant phenotype described below, and died before hatching. In a second spawning of the same two animals, 162 of 602 were mutants; i.e. a total of 233 of 863, or 27.0 %. These percentages suggest that the gene in question is inherited as a typical Mendelian recessive trait (\( p = 0.170 \)).

The parents of spawning 3719 were both DeLanney stock, descended from animals originally imported from Mexico to Wabash College. The female was from the Indiana colony, while the male was from Dalton's colony at Penn State. Tompkin's original observation of the \( p/p \) phenotype was also in DeLanney stock taken from the Indiana colony.

Morphology of the mutant

The mutant could first be distinguished from the wild-type at about stage 37 of Schreckenberg & Jacobson's (1975) series. Mutants were recognizable by their lateral curvature and cessation of development with simple unbranched gill rudiments (see Fig. 1). In addition, the gills frequently developed a small bulb at the distal end. Mutants survived without growth for about a week, after which disintegration of superficial tissue began. During this period, most showed edema or epidermal blistering of some sort. The swelling was most often localized in the area of the presumptive forelimb or the pronephros but was not restricted to that area, as older mutants often showed blistering along the fin fold and sides, caudal to the pronephric region. The head appeared somewhat microcephalic, and eye structure was not easily distinguished in all of the mutants. The external nares were well developed and the cloacal margins were generally slightly swollen. Chromatophores in the mutant were sufficiently well developed so that \( ax/ax \) embryos could be distinguished from wild-type, but it was difficult to distinguish \( m/m \) embryos from the wild-type. Melanophores tended to be contracted in the caudal areas, but expanded in the cranial areas where
Fig. 1. Parabiotic combination of mutant and normal embryos. This photograph was taken 3 days after the embryos were joined. The normal twin is at stage 38, and the mutant is at the stage where development normally arrests. × 30.

Fig. 2. Parabiotic combination of mutant and normal embryos at 10 days after joining. The normal embryo has reached stage 40, but the mutant partner has not changed appreciably. The arrow indicates an epidermal blister forming on the side of the mutant. × 7.
intermedin from the hypophysis presumably reached them by diffusion through the tissues.

The heart could be seen to be beating feebly from the time the mutant could be identified, but microinjection of a small amount of dye into the heart showed no circulation. The blood islands were distinct, but at no time before death did blood cells appear elsewhere than in the islands. The mutants showed no righting reflex or swimming movements, but could be stimulated to move their tails feebly by irritation of the gill area with a probe.

**Parabiosis**

Of 13 pairs of animals joined in parabiosis, four were of the desired mutant-normal combination. Figure 1 is a photograph taken 3 days after joining the embryos. At this time the mutants could be distinguished from the wild-type by the above criteria. Circulation was well established in the wild-type embryo, but no red blood cells were observed in the circulating fluid. The blood islands were still evident and had become concentrated in the zone where the embryos were joined. The mutant had established a heart beat which was rhythmic and slower than the wild-type, but there was no evidence of a circulating fluid.

Figure 2 is a photograph of the same parabionts taken 1 week later. The wild-type co-twins had progressed to about stage 40, while the mutants had progressed no further than they did when not joined to the wild-type. There were still red blood cells in the blood islands, but fewer in the circulating fluid of the normal partner than in control embryos of the same age. The mutants had begun to blister as previously described, and superficial tissues had begun to disintegrate. All four pairs died shortly after the photograph was taken.

**Histology**

Mutants were examined on the first day they became distinguishable, or after they had continued to ‘develop’ for 2 or 4 days. This gave four groups of embryos for comparative purposes: mutants isolated on the day they were first recognized, designated M-0; wild-type (stage 37) embryos taken the same day, designated N-0; and mutants taken 2 or 4 days later, designated M + 2 and M + 4, respectively. Examination of serial sections revealed that the mutant suffered from several abnormalities, detailed below, and also that the mutant tissues had less affinity for the hematoxylin stain than the wild-type.

One of the major differences in the mutant was the abnormal structure of the heart. The mutant heart possessed a well established sinus venosus which received the vitelline veins much as did the normal. The atrium also showed normal differentiation, but the cephalic portions of the heart (ventricle, conus arteriosus) invariably contained a plug of undifferentiated cells (Fig. 4) in place of the normal thin-walled endocardial lining (Fig. 3). The conus arteriosus ended as a mass of undifferentiated cells at the cephalic limits of the pericardial cavity (compare Figs. 5 and 6). However, neither the myocardium, nor the pericardium, appeared to be substantially less differentiated than the normal.
The aortic arches were generally present, but the dorsal aorta was usually evident only as paired, thin walled vessels ventral to the notochord (Fig. 7). In none of the mutants examined was there any communication between the aortic arches or ventral aortae, which were tubular structures when present, and the conus arteriosus. The major veins, rather than differentiating into tubular structures, remained as thin-walled, undifferentiated sinuses (Fig. 7), while the red blood cells remained in blood islands clearly evident in cross-section as pockets in the yolk endoderm (Fig. 8).

In the mutant, the pharyngeal endoderm appeared to have formed only the closed finger-like projections seen in Fig. 10, instead of the normal outpocketing which led to formation of the gill pouches in the wild-type (Fig. 9). As a result,
the pharyngeal cavity was a more continuous, oval shaped cavity than that found in normal embryos. Generally the liver diverticulum was only a short, slender, blind pocket proceeding caudoventrally from the anterior part of the foregut, rarely extending as far as the presumptive liver cells. Figure 7 shows the best developed diverticulum of all the mutants surveyed, which was still decidedly lacking in organization. The liver itself remained in a primitive state. In sections of the wild-type, the liver showed a high degree of differentiation and organization (Fig. 11), but in the mutant, there was little evidence of a hepatic cavity.
The cells in the presumptive liver area showed almost no differentiation, and it was difficult to distinguish between these cells and the neighbouring endodermal cells.

The midgut lumen was rarely evident in any of the mutants, though on occasion it appeared as a small slit in the dorsal part of the endoderm. The cloaca and the hindgut were usually comparatively normal, with the exception of swelling of the epidermal portions of the cloaca, and continuity between these two was fairly easy to establish. The nephric ducts appeared to open into the cloaca normally.
An examination of myotome structure showed that mutant cells remained less differentiated than those of a normal embryo, and that mutant myotomes underwent considerable progressive degeneration during the observation period (Fig. 12–14). In the normal stage-37 embryo, the nuclei of the myotomal cells had become elongated, and the vertical myocomma was less distinct, as the primitive segmentation of the muscles had regressed somewhat by this time (Fig. 12). In the M-0 mutant, the nuclei were less elongated, the cells were less differentiated and the vertical myocomma was still obvious (Fig. 13). After 2 days, ‘tears’ started to develop between the myotomal cells (Fig. 14), and by 4 days, this progressive degeneration led to a scarcely recognizable structure. There appeared to be no further differentiation of myotomal cells beyond what was observed in the M-0 mutant. The vertical myocomma was still apparent in the M + 2 mutants, but because of the degeneration, this structure was not obvious by the M + 4 stage. A light microscopic examination of myotomal cells revealed that the myofibrils produced by the normal embryo were highly organized and arranged in a longitudinal fashion conforming to the arrangement of the cells themselves. There appeared to be myofibrils in the mutant cells as well, but they were neither as numerous nor as well defined as those in the normal embryo.

During the 4-day period following the time at which the mutants were first recognized, there was increased accumulation of fluid and consequent swelling and distension of the coelom and pronephroi of the mutant. The extent to which this accumulation occurred, and the edema and epidermal blistering caused by it, are clearly evident in Fig. 8.

In none of the serial sections examined was there any indication of abnormalities in the developing nervous system. The brain and spinal cord, as well as associated structures, were essentially the same in both the normal and mutant embryos. A light microscopic examination also revealed no differences that could not be attributed to the differences in staining characteristics. However, this study was done at a fairly gross level and we cannot rule out the possibility that the nervous system, or any other system or tissue, may suffer from more subtle abnormalities.

In addition to those mutants isolated at stage 37, nine embryos were fixed and sectioned at stage 25. All nine of these embryos were essentially identical when examined. All looked normal for an embryo at this stage of development, and possessed a well defined pharyngeal cavity, liver diverticulum, heart primordium, gut and segmented myotomes.

DISCUSSION

Several of the abnormalities in the mutant embryo may be related to the lack of circulation. In the cardiac lethal mutant (c), Humphrey (1972) attributed edema, lack of melanophore expansion on the trunk and poor development of
the gills and gut to the lack of circulation. The fluid imbalance mutant (f) also suffers from edema and blistering, and Humphrey (1960) attributed microcephaly to the lack of circulation. The lack of circulation can be attributed to the plug of undifferentiated endothelial cells which appear to block the cephalic portions of the heart. However, the origin of the plug is perplexing in that the cephalic parts develop first (Lemanski, 1971, 1973). Either the early development of the heart was normal, and the endocardial plug developed later, or the lack of endocardial differentiation does not interfere greatly with subsequent development.

Another abnormality that cannot be explained as arising from lack of circulation is the poor development in the branchial region. Normally, in the stages immediately following the closure of the neural tube, the lateral walls of the pharyngeal cavity open out to form the gill or branchial pouches. When the gill pouches reach the epidermis, the epidermis folds in to form a series of branchial grooves. The gill pouches must reach the epidermis for the external gills to develop (Severinghaus, 1930). In the stage-37 p/p embryo there was no evidence of open branchial pouches, but analogous structures appeared to be solid finger-like projections of endoderm which extended from the pharynx to the epidermis. External gills developed, but appeared to be abnormal beyond what would be expected from the lack of circulation. In the c/c mutant the gills remained short and stunted, but some formation of filaments did occur, whereas the gills of the p/p mutant were short, always lacked filaments, and developed a small bulb at their distal ends.

The myotomes and muscle cells appeared to remain in a more primitive state in the mutant than in the normal embryos. Lemanski (1973) concluded that skeletal muscle in the c/c embryo was normal, based on the facts that embryos were capable of normal swimming movements, and that skeletal muscle could be induced to contract vigorously by electrical stimulation. Thus, the muscle abnormalities in the p/p mutant are probably caused by something other than the lack of circulation.

Though poor liver development cannot be attributed to circulatory malfunction, it is unlikely that it contributes much to the overall poor development of the mutant, as Copenhaver (1943) found that removal of the liver anlage from *Ambystoma* embryos did not produce any detrimental effects during the embryonic stages.

Of the abnormal structures present in the mutant embryo, the gut, liver, and gill pouches are endodermally derived (Copenhaver, 1955; Elias, 1955). The heart and muscles, though mesodermally derived, both depend on inductive interactions with the endoderm for proper development. In the case of the heart, the importance of these interactions was clearly demonstrated by Jacobson (1961) in transplantation experiments with the newt. In the axolotl, the cardiac lethal mutant was found to be defective in the endoderm rather than the mesoderm (Humphrey, 1972). Muchmore (1951) and Nieuwkoop (1973) found
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that in transplantation and explantation experiments, muscle or myotome differentiation was very poor when the endoderm was not included. Both concluded that the dorsal and lateral endoderm was implicated in the induction of the somites.

These observations would seem to justify a tentative hypothesis that the genetic basis for the defects in the p/p mutant somehow involves the endoderm. Further experiments will concentrate on endodermal and endodermally-induced structures.

The authors acknowledge the expert photographic assistance of George Ben-Tchavtchavadze. This work was supported by a grant from the National Research Council of Canada.

REFERENCES


(Received 2 September 1976)