# Mutant Genes in the Mexican Axolotl

Mutant genes of *Ambystoma mexicanum* can be used in classical and biochemical analyses of embryogenesis.

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The amphibian embryo has been a favorite experimental system of developmental biologists for well over a century. The relative ease with which morphogenesis can be observed, the universal availability of one species or another, and the simple laboratory culture techniques required to raise eggs through a complete life cycle have insured the amphibian a permanent position in the annals of embryology. Included among the major contributions to embryology derived from the use of amphibian material are the definitive demonstrations of (i) the cytoplasmic localization of morphogenetic determinants (*1*); (ii) the developmental equivalence of the nuclei of the early embryo (*2*); and (iii) the temporal patterns of gene transcription and translation which accompany embryogenesis (*3, 4*).

Recent developments in the last decade, however, have focused new attention on the amphibian embryo as a tool for experimental embryology (*5, 6*). Not only have advances been made in both technical and conceptual approaches to the analysis of the biochemistry of embryonic development, but large numbers of mutant genes have been discovered in the axolotl and other amphibians and many of these genes can be used in analyses of the stages in amphibian development. In addition, amphibians can be subjected to a variety of genetic manipulations to produce haploid or polyploid individuals (*7*). Parthenogenetic or gynogenetic reproduction provides a valuable tool for mapping gene-kinetochore distances for known loci, uncovering new mutations, and producing homozygous strains (*8*). Nuclear transplantation can be used to produce isogenic strains of amphibians (*9*).

In this article we describe briefly several of the major research areas of contemporary amphibian embryology. We also review the mutant genes in the axolotl and discuss the ways in which the mutant phenotypes can be profitably employed to increase our knowledge of embryology.

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## Research Areas in Which the Axolotl Might Be Particularly Useful

Perhaps one of the oldest research areas in embryology to which work on amphibians has contributed relates to the manner in which the pattern of morphogenesis is contained in the cytoplasm of the unfertilized egg. The so-called "morphogenetic determinants" which are built into the egg cytoplasm during oogenesis serve to direct the major morphological changes that occur during the early (preneurulation) stages of development. Proof of the existence of several morphogens has been available for several years (*10*); purification and characterization has not, however, been achieved for a single morphogenetic determinant. The lack of a quantitative bioassay has been the rate-limiting aspect of progress toward the isolation of specific morphogens. The discovery in the axolotl of genes that exhibit maternal effects and give rise during oogenesis to deficiencies in the egg cytoplasm has led to the development of bioassays that are proving useful in attempts to isolate some determinative substances.

Another important research area that is amenable to study with the amphibian egg, and that brings to a vertebrate system concepts that often have been examined in marine invertebrate systems such as the sea urchin (*11*), is the analysis of the regulation of macromolecular synthesis during embryogenesis. The routine availability of spawnings from amphibia with similar or identical genetic backgrounds, and the convenience with which eggs or embryos can be manipulated, for example, they can be given isotopes or metabolic inhibitors by microinjection (*12*), and can be enucleated and analyzed by nuclear transplantation (*13*), contribute to the general usefulness of the axolotl egg for these types of studies. The availability of mutant genes in the axolotl that control, for example, the size of the nucleolus, indicates further that the axolotl can provide exciting experimental material for the analysis of the synthesis of specific ribonucleic acids (RNA's). This is the same way that *Xenopus* has been exploited in studies of the structural organization of the nucleolar organizer region and of the synthesis of specific RNA's during development (*14*).

A third major research area in embryology concerns the definition of the biochemical, cytological, and anatomical events that occur during the development of the tissues and organs of vertebrates, and during the higher organizational processes of development. The tissue interactions and subsequent molecular events that result in the differentiation of such organs as the eye, heart, kidney, and limbs are now subjects of investigation in several laboratories. Genetic mutations that affect the development of these organs are available in the axolotl and can be employed for direct studies on the control of organogenesis. Several of these mutations should serve as model systems for studies of similar embryological events in human fetal development.

## Life Cycle and Mutant Phenotypes in the Mexican Axolotl

With much foresight, R. R. Humphrey began an inbreeding program in the Mexican axolotl at the State University of New York, Buffalo, some 30 years ago. The axolotl, *Ambystoma mexicanum*, is a neotonous salamander indigenous to a lake near Mexico City, and is normally aquatic for its entire

life cycle (*15*). It is easily maintained in the laboratory and matings of sexually mature animals provide several hundred progeny from a single spawning. Therefore, statistically significant ratios can be quickly obtained for establishing the genetic nature of an altered phenotype.

The various stages in the life cycle of the axolotl (see Fig. 1) are separated, somewhat arbitrarily, into five groups to facilitate the description in this article of the various mutant phenotypes. The life cycle as shown begins with the construction of the egg during oogenesis (group 1) and continues through development to sexual maturity (group 5).

The axolotl has a generation time of approximately 1 year, and sexually mature females in good breeding condition can be mated as often as every 3 months. Recently, it has been discovered that the frequency of successful matings can often be increased by administering appropriate hormones (*16*). Each of the stages in the life cycle can be readily observed and, because of their relatively large size, the egg (2 millimeters in diameter) and embryo can be surgically manipulated. One can also regulate the rate of development over a broad range by altering the environmental temperature.

A list of all the mutant genes currently available in the Mexican axolotl is presented in Table 1. All of these mutants have arisen under "natural" conditions. However, a program to induce new mutations by the application of mutagens is now under way and it is hoped that this will prove to be an even richer source of interesting new mutant genes.

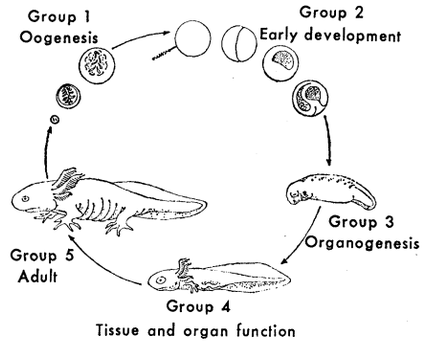



Fig. 1. The developmental life cycle of the Mexican axolotl, *Ambystoma mexicanum*. The various developmental stages are somewhat arbitrarily divided into five groups to facilitate categorization of the mutant genes.

The Mexican axolotl colony was moved in 1957 to Indiana University where eight different strains, including the Wistar, Holtfreter, Dutch, English, DeLanney, Brandon, Tompkins, and Xochimilco strains are now maintained. Closed strain breeding of the Wistar strain for at least 29 generations has yielded many mutant genes, among which are those for "eyeless" and "anemic." Systematic searches for mutants in the progeny of two strains of wild animals imported from Mexico within the past 11 years—the Tompkins and DeLanney stocks—have yielded 13 of the mutant genes listed in Table 1.

The characterization of the phenotypic changes which result from the genetic mutations is a prerequisite for determining the usefulness of each new mutant gene for studies in developmental genetics (*17*). We will therefore discuss several of the procedures, such as embryological, cytological, and biochemical analyses, that have been employed for the characterization of mutant phenotypes in amphibians because they illustrate the degree to which the amphibian embryo can be manipulated, and might suggest some future lines of experimental research.

Direct visual observation of the developing embryo has been useful for determining some of the phenotypic changes caused by the "maternal effect" genes that cause abnormalities during oogenesis (Table 1). Matings of animals heterozygous for either the *o*, *f*, *cl*, or *v* genes (see Table 1) produce offspring that develop to sexual maturity in an apparently normal manner. The female offspring that are homozygous for any of these genes, however, spawn eggs that display characteristic patterns of developmental arrest (Fig. 2). The genes *o*, *f*, and *cl* display 100 percent penetrance—that is, the mutant phenotypes are expressed in 100 percent of the progeny (*18*). In the case of the gene *v*, a variable amount of expression of the mutant phenotype is frequently observed (*19, 20*).

Several of the mutant genes in the axolotl do not give rise to easily observed gross morphological or anatomical abnormalities. Routine cytological analyses of somatic cells and tissues have provided valuable information about the nature of some of these altered phenotypes. For example, variant nucleolar sizes can be ob-

Table 1. List of mutant genes in the Mexican axolotl (*Ambystoma mexicanum*). Histocompatability factors are described in (*52*).

| Gene | Description | References |
|---|---|---|
| | *Group 1: Oogenesis* | |
| *o* | Ova deficient; arrest at gastrulation due to an oöplasmic deficiency | (*24, 27–29, 40, 53*) |
| *cl* | Abnormal cleavage | (*4, 29, 40, 54*) |
| *f* | Fluid imbalance; swelling of the blastocoel | (*21, 29, 40, 55, 56*) |
| *v* | Arrest at blastula stage; variable | (*19, 20, 29, 40, 57*) |
| | *Group 2: Early development* | |
| $n^1$ | Small nucleolus | (*29–32, 40, 58*) |
| $n^2$ | Small nucleolus | (*29–32, 40*) |
| $n^3$ | Small nucleolus (mutation left no descendents) | (*29, 40*) |
| $n^4$ | Reduced amount of rDNA | (*29–31, 40*) |
| | *Group 3: Organogenesis* | |
| *e* | Eyeless; sterile | (*25, 29, 35, 40, 59*) |
| *c* | Cardiac nonfunction | (*22, 23, 29, 33, 40*) |
| *an* | Transitory anemia | (*29, 36, 40*) |
| *micro* | Microphthalmia (semilethal) | (*29, 39, 40*) |
| *s* | Short toes | (*29, 37, 40*) |
| *ph* | Phocomelia | (*29, 40*) |
| *sp* | Spastic | (*20, 29, 38, 40*) |
| *as* | Ascites | (*29, 40*) |
| | *Group 4: Cell and tissue function* | |
| *p* | Premature death | (*20, 29, 40*) |
| *st* | Stasis of blood circulation | (*29, 40*) |
| *q* | Quivering behavior | (*29, 40*) |
| *t* | Twisted gills | (*29, 40*) |
| *u* | Slow growth; abnormal gills | (*29, 40*) |
| *mi* | Microphthalmic lethal | (*29, 40*) |
| *g* | Gill lethal | (*29, 40, 56, 60*) |
| *l* | Small eyes | (*29, 40*) |
| *r* | Renal insufficiency | (*29, 40, 61*) |
| *x* | Fragile gills | (*29, 40*) |
| *y* | Limb development arrested | (*26, 29, 40*) |
| *h* | Foot malformation | (*29, 40*) |
| *b* | Slow development of front limbs | (*29, 40*) |
| | *Group 5: Adult* | |
| *d* | White | (*29, 40, 42, 44, 45, 62*) |
| *a* | Albino | (*29, 33, 42, 63*) |
| *m* | Melanoid | (*29, 40, 42, 46*) |
| *ax* | Axanthic | (*29, 40, 43*) |

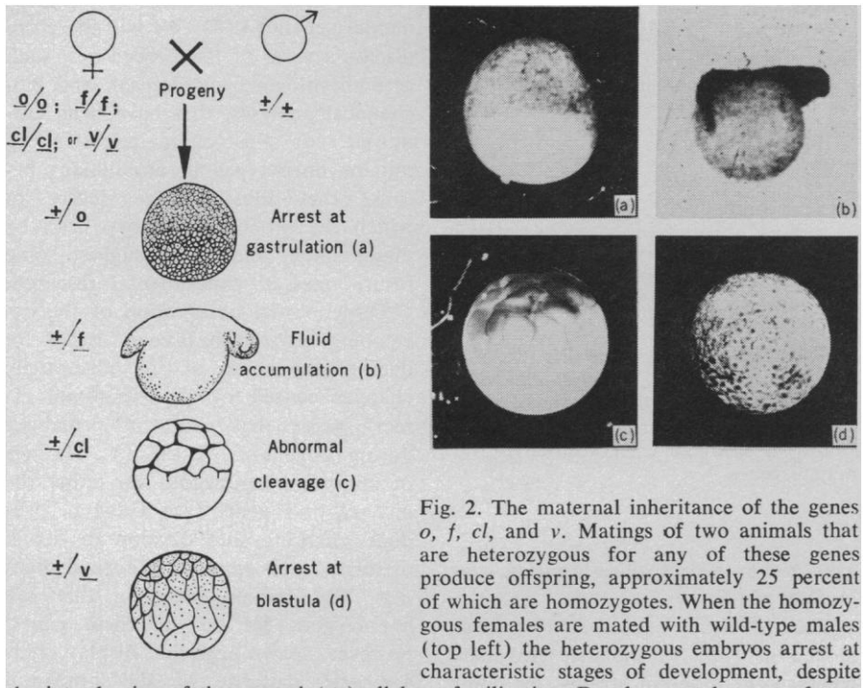
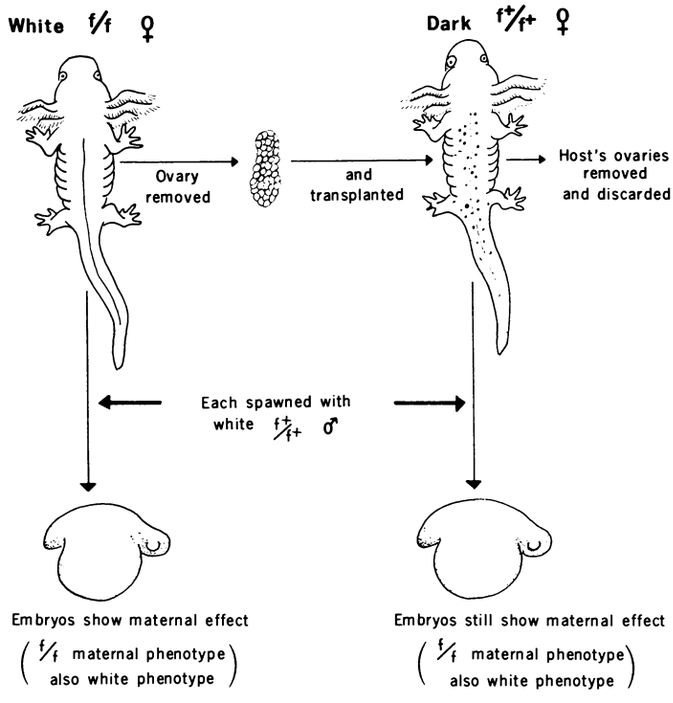



Fig. 2. The maternal inheritance of the genes *o*, *f*, *cl*, and *v*. Matings of two animals that are heterozygous for any of these genes produce offspring, approximately 25 percent of which are homozygotes. When the homozygous females are mated with wild-type males (top left) the heterozygous embryos arrest at characteristic stages of development, despite the introduction of the normal (+) allele at fertilization. Developmental arrest of eggs spawned by *o/o* animals (a), *f/f* animals (b), *cl/cl* animals (c), and *v/v* animals (d) are shown diagrammatically at left. Photographs of these embryos are displayed on the right.

served in the cells of animals which bear the nucleolar mutant genes.

Embryological experiments, in which mutant tissues are grafted or transplanted to normal animals, and in which animals suspected of having mutant genes are grown parabiotically with normal animals, have also provided information about mutant phenotypes. By tissue grafting it is often possible to determine the exact site in an embryo of an initial developmental lesion caused by a recessive mutant gene. Because genetic crossbreeding had revealed that the gene *f* was expressed as a maternal effect, a graft of embryonic ovary from *f/f* females into pigmented normal embryos was performed (*21*). Although the *f/f* oocytes developed through a normal vitellogenic cycle and were spawned in the usual manner by the host female, the abnormal phenotype illustrated in Fig. 2 persisted. The primary site of expression of the normal allele of the gene *f* in female animals is believed, therefore, to be the developing



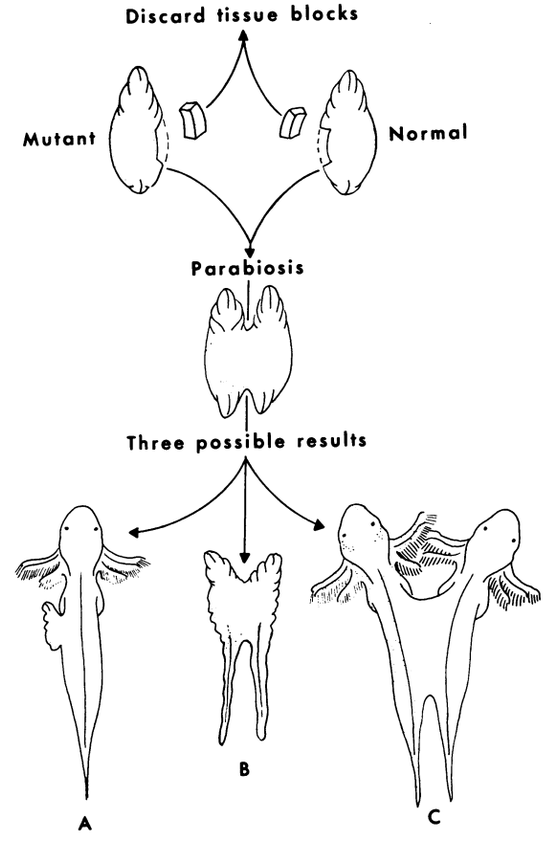




Fig. 3 (left). Grafting of ovary tissue from an *f/f* animal (white pigmentation) into a dark, wild-type (ovariectomized) female. The defect in the ovary tissue cannot be corrected by development in a normal host because the eggs spawned by the host female display the mutant phenotype. Fig. 4 (right). Parabiosis of normal embryos (white) with embryos homozygous for a mutant gene (stippled); ventral views. After the tissues at the site of the graft heal to permit a common circulation, development results in one of the following: (A) arrest and resorption of the mutant member, for example, genes *l*, *mi*, or *g*; (B) death of both members, for example, genes *st*, *x*, or *q*; or (C) normal development of both members, for example, genes *f* or *c*; dorsal views.

oocytes. The positive identification of the *f/f* ovary graft can often be greatly facilitated by using as recipients embryos from a pigmented strain of axolotl (Fig. 3).

Reciprocal grafts of tissues between normal and mutant axolotls have been useful for determining the site of action of the following mutations: *c*, defective myofibrils in the heart (*22, 23*); *o*, oöplasmic deficiency of a substance required for organogenesis (*24*); and *f*, defective oocytes which produce a defect in the endoderm of the embryo (*5, 21*).

Grafting techniques have also proved valuable for producing chimeric or mosaic embryos. Axolotls homozygous for the gene *e* are sterile as a result of an effect of the gene on the hypothalamus. Transplantation of posterior sections of animals which display defective eyes (*e/e* embryos) to anterior portions of normal embryos (+/*e* or +/+) has permitted the reconstitution of embryos that develop into chimeras. These chimeras, which contain gonads from the *e/e* animal and functional anterior structures from the normal animal, are fertile and provide a convenient source of eggs for experimentation, 100 percent of these eggs being homozygous for the gene *e* (*25*).

Parabiosis provides an alternative experimental approach. Useful information about either hormonal or circulatory effects can be obtained with this method. Two complete embryos can be united, at the tailbud stage of embryogenesis, so that they develop a common circulatory system. As development proceeds one of three results is observed: (i) the mutant member fails to show an improved development; (ii) both members in parabiosis display the lethal effects of the mutant member; or (iii) the mutant's developmental arrest pattern is corrected. These possibilities are illustrated in Fig. 4; several of the mutant genes listed in Table 1 were tested in this way (see legend to Fig. 4). Such experiments provide useful information but do not enable one to determine the initial site of action of the mutant gene.

The mutant genes of the Mexican axolotl that have been used most extensively in experimental embryology are those that are tissue or organ specific. Other mutant genes, including those that do not display improved developmental potential when employed in grafting or parabiosis experiments, appear to be cell lethals, that is, the genes (*l* or *y*, for example) in the homozygous condition appear to affect the development and the function of all the organs and tissues (*26*). It is possible, however, that after further investigation many of the mutant genes now categorized as cell lethals will be found to have more subtle cell or tissue specificities and to be of considerable value in embryological analyses.

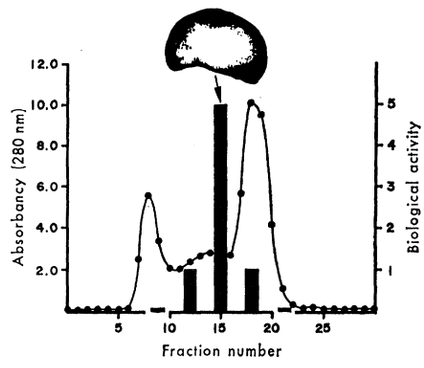



Fig. 5. Partial purification of the $o^+$ substance by Sepharose 6-B column chromatography. Crude homogenates of normal axolotl eggs were frozen, thawed, and centrifuged at 39,000*g*. The soluble extract was applied to the molecular sieve column and eluted with buffer. Protein concentration was monitored by the absorbancy at 280 nm The fractions were injected into eggs spawned by *o/o* females and the biological activity of each fraction was assayed by observing the extent to which normal development was promoted in the defective recipient eggs (vertical bars). Completely normal morphogenesis, shown by the photograph of the embryo in the tailbud stage, was promoted by some fractions.

## Present Applications of the Mutant Genes of the Axolotl

Many of the genes listed in Table 1 are now being used for studies of various aspects of embryogenesis. Some examples are as follows:

*Group 1, maternal effects.* The nature of the components of the egg cytoplasm which are responsible for the pattern of morphogenesis can be investigated with the maternal effect genes. It has been demonstrated by Briggs and Cassens (*24*), for example, that the effects of gene *o* can be corrected by microinjection of a small quantity of normal egg cytoplasm into eggs spawned by *o/o* animals. The active component has been tentatively identified as a protein. A bioassay provided by this technique is now being used as a guide in the purification of the $o^+$ substance (*27*) by differential sedimentation and molecular sieve column chromatography (Fig. 5).

In this laboratory we are attempting to correct the maternal effects of the other genes (*cl, f, v*) and are searching for additional maternal effects. In addition, by means of nuclear transplantations in the axolotl, we are examining the manner in which a specific cytoplasmic component (the $o^+$ substance) interacts with the nucleus. The $o^+$ substance produces a stable alteration in the blastula nucleus so that it can support normal development when transferred into a recipient egg spawned by an *o/o* female. These data suggest that the $o^+$ substance may actually be a regulatory molecule that is necessary for the activation of the embryonic nucleus during the blastula stage (*28*).

*Group 2, early development.* The appearance and function of the nucleolus during early development of the fertilized egg is being investigated with the aid of mutant genes that result in a change in the size of the nucleolus. Three mutant genes, $n^1$, $n^2$, and $n^3$, which act to reduce the size of the nucleolus have been described by Humphrey (*29*). Preliminary investigations reveal that these mutations might be of a regulatory type, in that the size of the nucleolus in the mutant strains is not the result of a reduction of the number of ribosomal genes (*30*). In the mutant gene $n^4$, however, a difference in the amount of ribosomal deoxyribonucleic acid (rDNA) has been observed. Animals of the pure Holtfreter strain have approximately one-third as many copies of rDNA per unit of whole-cell DNA as do animals of the Wistar, Dutch, or English strains. Cytological observations indicate that the length of the constriction of the nucleolar organizer region in pregastrular embryos is proportional to the number of copies of rDNA (determined by rRNA-DNA hybridizations) (*31*).

These mutations should provide a basis for the study of nucleolar function, because the behavior of the various alleles in heterozygous animals can now be analyzed. In addition, these altered nucleoli are potentially useful as cell markers in a variety of embryological experiments (*32*).

*Group 3, specific organs and tissues.* The eight recessive mutations that affect the development of certain organs and tissues may provide a basis for the

examination of inductive relationships during development. Two genes within this group have been demonstrated to exert specific effects, and may provide a system whereby the molecular basis of specific inductive processes can be examined.

Embryos that are homozygous for the cardiac mutation (*c*) do not develop a functional heart. Electron micrographs of the mutant and normal hearts show that in the mutant heart the myocardial cells fail to differentiate functional organized myofibrils (*33*). It is known that the development of a functional heart is dependent upon a specific inductive interaction between the mesoderm (reacting tissue) and the anterior endoderm (inducting tissue) (*34*). Humphrey has demonstrated by grafting experiments that the anterior endoderm is the site of action of this mutant (*22*). These results suggest that the mutant anterior endoderm lacks the ability to induce the formation of a functional heart, possibly by a failure to form one or more of the heart muscle proteins.

Embryos homozygous for the gene for eyelessness (*e*) fail to form optic vesicles (*35*). By means of reciprocal grafts between normal and mutant embryos of presumptive anterior chordamesoderm and presumptive anterior medullary plate, it has been demonstrated that the gene *e* acts directly upon the anterior medullary plate. The mutant gene affects specifically the ectodermal component of the inductive system for eye formation. In the mutant embryo it alters the competence of this ectodermal component to respond to the inductive stimulation of the mesoderm (*25*).

The gene for anemic (*an*), discovered by Humphrey, has been shown to affect the transition of hemoglobin from a larval to an adult type (*36*). The anemic condition of larvae homozygous for the gene *an* apparently results from a premature suppression of larval hemoglobin synthesis. This would suggest that the mutant gene may have a regulatory effect, in that it appears to modify the "switch mechanism" involved in the transition from larval to adult hemoglobins.

Two other genes of this class, short toes (*s*) and phocomelia (*ph*) affect the development of the skeletal system in different ways. The growth of the major long bones is delayed in the *ph/ph* larvae (*29*). In homozygous *s* larvae the gene acts to suppress completion of the growth of the Müllerian duct, produces renal failure, and causes a reduction in the number of phalanges in the toes. The rib formation in these homozygotes is also affected (*37*).

Larvae homozygous for the spastic gene (*sp*) exhibit altered behavioral patterns. They lack the normal righting reflexes and swim with erratic movements. It is thought that this mutation may act to affect neurophysiological interactions (*20, 38*).

The last gene of this group, the gene for microphthalmia (*micro*) produces varying degrees of reduction in the size of the eyes of larvae homozygous for this mutation (*39*).

*Group 4, cell and tissue function.* Thirteen recessive mutants of this group have been described (*29*). These can be divided into three general categories based upon the time of death and the feeding behavior of the larvae. In the first category, the mutant genes (*p*, premature death; *q*, quivering; and *st*, stasis) display lethal effects at about the time of hatching: the mutant larvae do not feed. In the second category (*g*, gill lethal; *t*, twisted gill; and *mi*, microphthalmic) the larvae feed rarely, if at all, and the mutant genes exhibit lethal effects during a period of 1 to 2 weeks after hatching.

In the third category (*r*, renal insufficiency; *b*, Brandon lethal; *l*, London lethal; *x*, "unknown" lethal; *u*, Utrecht lethal; *y*, "unknown" lethal; and *h*, "hand" abnormality) the larvae feed, but die within a period of several weeks after hatching.

The common feature of these lethal mutations is that the embryos cannot be rescued by parabiosis with normal embryos. Most of these mutant phenotypes have been examined by grafting or transplanting mutant tissues to normal embryos. Because grafts of mutant organ primordia (gill or limb) do not survive on normal recipients it is thought that the mutations in this group produce defects in cellular metabolic functions. Two alternative general explanations have been proposed to explain these lethal effects: (i) novel gene products (of the normal alleles) are required during this period of development (*40*); or (ii) substances produced and stored in the egg during oogenesis by a heterozygous female support development to hatching, and additional synthesis of these essential substances is required for further development (*29*).

*Group 5, adult.* "Wild-type" axolotls possess three types of pigment cells—melanophores (black or brown), iridophores (iridescent white), and xanthophores (yellow). The color pattern of the adult animal is dependent upon the relative numbers and distribution of these three types of cells (*41*). Four recessive mutations affecting either the types of pigment synthesized or the number and the distribution of the pigment cells have been described (*29*).

The albino (*a*) mutants fail to synthesize melanin, but do differentiate melanoblasts (*42*). The axanthic (*ax*) gene blocks the synthesis of pteridines which are responsible for the yellow color of the xanthophores. This mutant does, however, produce xanthoblasts (*43*).

Two genes affect the distribution and the numbers of pigment cells. The gene *d* produces a "white" phenotype by acting to restrict the number and the distribution of the melanophores and xanthophores (*44, 45*). In another mutation for melanoid phenotype (*m*) the iridophores are not present and the number of xanthophores is reduced. In these homozygotes the melanophores are increased both in number and in extent of distribution (*46*).

These pigment cells provide model systems for the study of cell differentiation and cell-cell interactions. They are useful for studies on melanogenesis and can be used as indirect genetic markers for the various embryological analyses described earlier in this article. In addition, the complete absence of melanin granules in the albino facilitates the examination of histological sections with various autoradiographic techniques (*47*).

## Concluding Remarks

Amphibians provide material of exceptional value for research in molecular biology, cell biology, embryology, reproductive biology, and developmental genetics. Because they are particularly useful for embryological studies genetic stocks of such amphibians as *Rana pipiens* (*48*), *Xenopus laevis* (*49*), *Pleurodeles walti* (*50*), and the Mexican axolotl have been developed. The largest array of mutant genes for a single species is now available in the Mexican axolotl (*29*), and by describing some of the ways in which these genes can be employed we hope to stimulate further research with the axolotl by other workers (*51*). The large numbers of eggs from single spawnings

and the type of mutants available make the Mexican axolotl a versatile experimental system suitable for the analysis of many research problems related to biology and medicine. Due mainly to the efforts of Humphrey and other workers new mutant genes are frequently being added to the present collection, and new stocks are continually being imported from Mexico. Continued inbreeding, and the backcrossing of new stocks with the highly inbred lines currently available is expected to yield more mutant genes and to increase the hybrid vigor of present stocks.

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51. Inquiries related to requests for experimental material or methods for the laboratory care of the axolotl are invited by A.J.B. An annotated list of mutant genes is available upon request.
52. Histocompatibility analyses of Holtfreter and Wistar strains maintained within a single colony suggest that the progeny segregate into three codominant factors, H-$1^{A,B,C}$. The Dutch stock segregate into two histocompatibility groups, designated $N^1$ and $N^2$, with rejection times similar to the H-1 groups. Studies on locus similarity among the Holtfreter, Wistar, and Dutch strains are not complete. Preliminary results with imported Mexican stock indicate that if only one locus is assumed, a minimum of eight alleles would be required to account for the observed histoincompatibilities. Now being analyzed is the difference in ability of H-$1^C$ of Wistar lineage and that of other origins to allow an H-$1^C$ strain-specific lymphosarcoma to progress or regress. Expression of histoincompatibility to lymphosarcoma in the Dutch and DeLanney strains begins to be expressed at about 3 months after spawning at 20°C [A. H. Meier and L. E. DeLanney, *Am. Zool.* **2**, 431 (1962); L. E. DeLanney and M. K. Blackler, in *Recent Advances in Cancer Research, Biology of Amphibian Tumors* (Springer-Verlag, New York, 1969); L. E. DeLanney, personal communication].
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