Alkylation treatment of the Mexican axolotl: an approach to the induction of new mutations

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To date, all the mutations discovered in the axolotl have either arisen spontaneously in laboratory stocks, or have been uncovered among the immediate progeny of animals imported from Mexico. The aim of this project has been to determine whether treatment with allylating agents is practical for the induction of new mutations.

Intraperitoneal injection of doses of methylmethanesulfonate (MMS) considerably higher than those lethal to a mouse or rat had no deleterious effects. Injection of labeled MMS showed that the label was rapidly excreted back into the medium. Lethal concentrations were finally established by exposing animals for several days to MMS or ethylmethanesulfonate (EMS) added directly to their water. Tracer studies indicated that the alkylating agents were taken up, but metabolic breakdown products could not be detected.

When mature females were treated with sub-lethal concentrations of EMS, spawnings proved difficult to obtain for several months. The animals appeared unable to ovulate in response either to direct stimulation of the ovaries by injected FSH, or to normal stimulation initiated by mating and insemination. Spawnings were obtained more readily 3-4 months after treatment, but fertility was still reduced, and there was a high percentage of abnormal embryos, few of which survived to hatching. When mature males were treated with EMS, spermatophores could be obtained within a week after treatment. However, fertility was very low, and none of the fertile embryos survived to hatching. Again, a period of several months was required for even partial recovery.

In an attempt to overcome the problem of probable side effects from treating whole animals, a series of experiments were carried out in which the sperm were treated directly and then used in an artificial insemination. Increasing concentrations of EMS reduced the ability of the sperm to fertilize the eggs. However, among the fertile embryos, abnormalities were no more frequent than among controls, suggesting that inactivation of the sperm may not be due to genetic damage.

Progeny from such artificial inseminations, and from spawnings where one of the parents had been treated, will be tested when they reach maturity to see if any new mutations can be uncovered. At present, the only positive result is a gold-colored variant obtained in a spawning from an MMS treated female.

Development of Pigment Patterns of Amphibians

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Pigmentary mutants in amphibians provide important vehicles for studying basic problems in development. Some of these mutants exert influences on the tissue environment in which the chromatophores differentiate and others involve the expression of pigment in specific types of pigment cells. Melanophores are the best known of all chromatophores, and albinism has been much studied. Genes controlling its expression may operate at different levels. Some are involved in the production of tyrosinase, while others affect the melanosomal matrix. In contrast, melanoid mutants are characterized by an overproduction of eumelanin, usually through the differentiation of an excessive number of melanophores.

Melanoid mutants of the axolotl also exhibit a great diminution in xanthophore and iridophore number, and the same seems to be true of some melanoid-like mutants of leopard frogs. The pteridine pigments of xanthophores and purines of iridophores are often products of xanthine dehydrogenase (XDH) activity, and it is suspected that the expression of the melanoid phenotype may result from a genetic defect involving this enzyme. This is supported by experiments involving the administration of an XDH inhibitor, allopurinol, to normal larvae. This inhibitor results in the production of partial phenocopies of the melanoid condition. Blue mutants involving either partial or complete diminution of xanthophore and iridophore pigments may also be based upon deficient XDH activity.

ANALYSIS OF THE o MUTATION, A MATERNAL EFFECT MUTANT OF *AMBYSTOMA MEXICANUM*

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The nature of the control of gene action during development is one of the central problems in Developmental Biology. Morphogenetic substances apparently are synthesized during oogenesis, stored in the egg cytoplasm and eventually arranged in a pattern which acts after fertilization to control the development of the zygote into the differentiated multicellular organism. This control must involve an interaction between the zygote nucleus and the components of the egg cytoplasm. The nature of these morphogenetic substances and the manner in which they function are unknown. Genes which exert maternal effects through modifications of the egg cytoplasm are therefore of special interest, since they provide a means of approaching the problem of how the egg cytoplasm acts in controlling early embryonic development.

A simple recessive maternal effect gene (o, for ova deficient), was discovered by Dr. R.R. Humphrey in the Mexican axolotl. Females homozygous for o produce eggs which always arrest at gastrulation regardless of whether or not the normal allele is introduced by the sperm at fertilization. This gastrular arrest is due to a cytoplasmic deficiency, and can be completely corrected by the injection of nucleoplasm from a normal oocyte nucleus (germinal vesicle) or cytoplasm from a normal mature egg. The active substance seems to be a protein(s) or to depend on protein(s) for its activity. This o+ substance is produced during oogenesis, and the presence of this substance appears to be essential for the normal activation during blastulation, of the nuclear genes required for gastrulation and organogenesis.

A test of the heritability of the nuclear activation was constructed by transplanting nuclei (which had been exposed to the o+ substance in the normal egg cytoplasm) from various stages of normal blastulae into enucleated mutant eggs (which lack the o+ substance). If the activation was not stable, then the recipient eggs would in all instances develop like typical mutant eggs and arrest at gastrulation. If the activation was stable and heritable, then the activated nuclei, but not the unactivated ones should promote normal development of the recipient enucleated mutant eggs. The results of such experiments demonstrate that the presence of the o+ substance in the egg cytoplasm is essential for the establishment of a stable nuclear activation during mid-blastula stage. The sensitive period for this activation is restricted to one point during embryonic development and is a mitotically heritable condition. The isolation and characterization of the o+ substance provides an opportunity to examine the nature and function of a morphogenetic substance.

Analysis Of The Eyeless Mutant In The Mexican Axolotl (Ambystoma mexicanum)

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The variety of experiments carried out by many investigators to analyze eye development have helped to formulate such basic concepts in embryology as induction, evocation, and competence (for review see Jacobson 1966, Spemann 1938, Twitty 1955). The results obtained by grafting eye-cups from early embryonic stages to abnormal positions in host embryos revealed that the grafted material had the capacity to cooperate with the epidermis to form perfectly shaped eyes. This ability of the eye-cup to induce a lens and a cornea in the epidermis was, however, not found in the material of the prospective eye-cup at the early neurula stage. This revealed that the inducing capacity of the optic-vessicle depended on an inductive stimulus from the mesoderm during neurulation. This important phenomenon, that inductive capacity might depend on induction at earlier stages of embryogenesis has been described as the "hierarchy of inducers" (for review see Hadorn 1972).

In the eyeless mutant of the axolotl found by Humphrey (1966), van Deusen (1973) investigated the problem of whether the mutation affects eye induction during neurulation. Van Deusen grafted mutant prospective mesoderm into wild type hosts at the early gastrula stage and found that this combination gave rise to normal eyes. Prospective ectoderm from mutant embryos grafted to wild type hosts however did not result in eye formation. These results clearly showed that the mutation had its effect on the ectoderm and that the mesodermal part was completely normal.

The concept of the experiments to be reported was to find out whether the defect in the mutant ectoderm is located in the neural ectoderm in the epidermis, or in both ectodermal derivatives. In order to answer this question, prospective eye vesicles of eyeless embryos were grafted under the epidermis of normal hosts at late neurula stages. This combination resulted in normal, heterotopic eye formation. The prospective eye-cup of mutant embryos is therefore able to work normally with wild type epidermis. Normal eye formation was also obtained by grafting normal epidermis on the prospective eye vessicle of mutant embryos.

These experiments show, that the defect caused by the e/e genotype does not reside in the neural ectoderm, but is restricted to the epidermis.

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ABSTRACT

IMMUNOGENETIC PROFILE OF THE AXOLOTL: 1977

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The axolotl has been grown in 7 strains partially inbred between 4 and 13 generations. A crucial question, in view of the phylogenetic position of the urodeles, is whether they possess a predominant histocompatibility locus that expresses more strongly than other loci or whether skin transplants are chronically rejected under the influence of cumulative effects of numerous "weak" loci. While the definitive experiment with these strains has not yet been productive, the data from skin graft rejection significantly support a position of the presence of a predominant locus. The relationship between this putatively predominant locus and the strong locus of animals such as mice, men, or dogs that reject allografts acutely has not been established.

The behavior of allografts of whole limbs does not precisely follow the behavior of allografts of pieces of skin in that the limbs show a higher incidence of seeming acceptances than do skin allografts. However, histologically such "accepted" allogeneic limbs are well-infiltrated with mononuclear leukocytes. This raises the question of the definition of tolerance in this amphibian.

Animals ranging between 10 and 14 years old and with traits usually associated with aging when allografted with skin from a common source did not show reduced immunocompetence, as usually expressed in endotherms. Instead, they rejected allografts significantly more rapidly than did hosts between 2 and 5 years of age. This may be indirect evidence for varying proportions of subpopulations of immunocompetent cells between the animals of the different age groups.

On the other hand, old animals show a variety of tumors (other than the lymphosarcoma carried on one strain of animals of this colony) that are not found on younger mature animals.

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A Discussion of the "Eyeless" mutant.

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Humphrey (1969) reported a mutant gene (e), "eyeless," in the Mexican axolotl, which had spontaneously appeared in the axolotl colony at Indiana University. When present as a homozygous recessive, gene (e) prevents the formation of optic vesicles in the embryo. In addition, e/e animals are sterile and possess an abnormal pigment pattern.

Van Deusen (1973) provided evidence that failure of the optic vesicle to form was due to an effect of the gene on presumptive forebrain ectoderm. Animals bearing reciprocal transplants of the ectoderm and mesoderm involved in vesicle formation produced eyes when e/e mesoderm was combined with E/-ectoderm but not when e/e ectoderm was combined with E/-mesoderm. Transplants of E/-hypothalamic primordia, also located within presumptive forebrain ectoderm, into e/e hosts resulted in fertile rather than sterile adults, again indicating the effect of the gene on the ectoderm.

In an electron microscopic study, Ulshafer and Hibbard (1976) described differences in the diencephalon of eyed and eyeless embryos. Among these differences is that the space between diencephalon wall and head ectoderm is filled with mesenchyme in eyeless embryos but is empty in eyed embryos. They propose an alternative hypothesis for the action of (e), that head mesenchyme may repress optic vesicle evagination.

Epp (1972) and Van Deusen (1973) have shown that the enhanced pigment pattern of the mutant is a secondary effect of the gene, caused by the lack of eyes. Reciprocal neural crest transplants reveal host pigment patterns in each case. Hypophysectomy of e/e embryos results in reduced pigmentation and parabiosis of e/e with E/-embryos leads to enhanced pigmentation in both partners, indicating that ciruclating MSH levels are responsible for the abnormal pigmentation of the mutant. Implanting an optic vesicle into proper position in e/e embryos allows the development of an eye which is often functional, and when so, associated with reduced, i.e. normal, pigmentation. Further, Hibbard and Ornberg (1976) have recorded neurophysiological responses from the tectal lobes of such eye-grafted animals. Schwenk and Hibbard (1977) have demonstrated that in normal axolotls, all optic fibers cross to the contralateral side of the brain, while in eye-grafted animals the retinal projections from the grafted eye pass to both the ipsilateral and contralateral tectal lobes.

Humphrey (unpublished observation) has noted that gene (r), "renal," as a homozygous recessive, acts in conjunction with (e) in heterozygous embryos (E/e) to produce abnormal eyes. Sections reveal that in this case induction and evagination of optic vesicles occur but spatial organization of the developing eye is lost. Eyes may be abnormal in size, symmetry or position and may lack one or more components, e.g. lens of cornea. Gene (x), an unknown lethal, enhances the effect of rr on E/e but apparentely does not itself lead to the development of such abnormalities when in combination with E/e.

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Approaches to problems of cell type determination and gene regulation in amphibians

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Crucial to an understanding of development is the elucidation of the mechanisms behind both cell type determination and gene regulation. I will discuss several approaches to these problems involving the use of the amphibian system.

One approach has been the injection of somatic cell nuclei into oocytes in order to determine how the oocyte cytoplasm affects gene expression in these nuclei. When nuclei from liver cells (synthesizing a liver specific enzyme alcohol dehydrogenase-ADH-and a ubiquitous enzyme lactate dehydrogenase-LDH) are transferred into oocytes(synthesizing LDH but not ADH) it is observed that the genes coding for LDH in the transferred nuclei continue to synthesize their product, while those coding for ADH are inactive. These results suggest that the oocyte cytoplasm is able to regulate the synthetic activity of the transferred somatic cell nuclei so as to conform to its own synthetic output. Thus, genes coding for differentiated products not normally found in oocytes are turned off in the transferred nuclei, while genes coding for ubiquitous or housekeeping functions remain active. I will report on further experiments aimed at examining the regulation of specific gene products in transferred somatic cell nuclei.

Another approach involves the use of interspecific hybrids between two amphibian species which exhibit electrophoretically distinguishable forms of the same enzyme.This enables one to determine the exact time of gene expression for these enzymes by observing when the maternal, paternal, and hybrid forms of the enzymes appear during development. This technique has been utilized in determining when genes for LDH, tetrazolium oxidase(TO), and ADH are expressed in hybrid combinations involving the axolotl(Ambystoma mexicanum) and Ambystoma texanum. It appears that the maternal allele for ADH (a differentiated product) is expressed prior to the paternal allele when either texanum or mexicanum is used as the maternal species, while maternal and paternal alleles for LDH and TO (products found in both the liver and oocytes) are expressed simultaneously. It has also been observed that in a cross between A. texanum with the wild type allele of ADH and an individual of the same species with a variant ADH both the maternal and paternal alleles are expressed simultaneously. These observations suggest that there may be a regulatory incompatability either at the transcriptional or post- transcriptional level between the two species with regards to regulation of ADH.

Amphibian neurogenesis: an overview

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An overview of neurogenesis is presented in which the specific and stereotyped diversity of neurons is viewed as graded, related to cell position, and based on unique combinations of (shared) neuronal properties. Such combinatorial coding extends to the locus specificities mediating selective synaptogenesis in retinotectal map assembly. A unifying phenomenology is traced backward from (i) the signalling functions which convey positional information to nascent ganglion cells during growth of the retina, to (ii) the establishment of retinal polarities, to (iii) anatomical pattern determination of the eyebud, to (iv) organization of retinal founder cells in neural plate. I suggest that a single master signalling apparatus, based on positional diversification, is used redundantly in space and reiteratively in time to evoke progressive determination steps in the emerging CNS: The response of the cells changes, by lineage-dependent differentiation, between successive iterations. The argument is supported by embryonic transplantation studies illustrating cross-reactivity of signalling systems and by studies relating signalling with 'polyclonal' lineages in genetic chimerae.

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The spastic gene and neurogenesis in the axolotl

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The analysis of neurological mutants has contributed to understanding how single genes influence neural function and ontogeny. The spastic mutation, discovered by R. R. Humphrey, induces swimming coordination and equilibrium deficiencies in the Mexican axolotl. Behavioral ontogeny studies determined that spastics fail to develop behavior trains of sinusoidal flexures necessary to mediate escape swimming at the time of onset of cerebellar function. Behavior analysis, after lesioning different cranial nerve roots and CNS areas in wild-type animals, confirmed the "behavioral focus" of the mutation to lie in the auricle or vestibulo-cerebellum.

Single unit recordings in the cerebellar auricle and adjacent brainstem vestibular zone (area acoustico-lateralis) of mutants revealed a full complement of vestibular unit types found in wild-type. However, the gene appears to alter the physical location of vestibular units in both areas, including a ventral "translocation" of auricular units responding to sustained ipsilateral tilt. Correlated with this unit translocation, mutant Purkinje cells and allied afferent tracts are malpositioned ventrally, i.e., "crowded" into an ectopic zone in the ventro-posterior auricle.

Studies on cerebellar structure at the time of onset of spasticity (early feeding stage) confirmed the ventral malpositioning of cerebellar cells and fiber tracts seen in adults. In conjunction with these studies, mutant larvae injected with tritiated thymidine during early cerebellogenesis and assayed at the early feeding stage revealed a medio-ventral malpositioning of labelled cells; in wild-type, labelled cells were positioned laterally. Thus, the spastic gene appears to alter the capacity of presumptive cerebellar cells to carry out movements crucial to the neurogenetic sequence.

IS THE CARDIAC LETHAL MUTANT REAL? Robert R. Kulikowski and Francis J. Manasek. Department of Anatomy, The University of Chicago, 1025 East 57th Street, Chicago, Illinois 60637

Homozygosity for gene c in Ambystoma mexicanum results in no detectable embryonic heartbeat in situ. Humphrey showed that heart rudiments from Harrison stage 29 c/c embryos developed normally when transplanted into normal hosts. Reciprocal transplants failed to develop. He suggested c/c embryos had either a defect in heart induction or produced an inhibitory substance that prevented normal heart differentiation. We tested this hypothesis directly by explanting hearts from c/c embryos to organ culture. When placed in such a neutral environment, hearts which had not beat in situ began to beat within minutes of exposure to Holtfreter's medium. Polarized light microscopy revealed organized birefringent structures in both normal and c/c embryos with no significant difference in spatial orientation within the myocardium Prominent striations were seen in hearts from normal embryos whereas none were observed at this level of resolution in hearts from c/c embryos. It is nonetheless obvious that a functional contractile apparatus is present in these hearts and the myocardium therefore contains differentiated muscle cells. We conclude that heart induction is essentially normal in c/c embryos but that cardiac function is inhibited in the intact animal. This is not necessarily meant to imply the synthesis of an inhibitory substance, but may be as simple as an altered cardiac environment due to homozygosity for gene c. In this light, this abnormality should be viewed as a secondary defect.

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Morphological, Biochemical and Immunohistochemical Studies on Heart Development in Cardiac Mutant Axolotl Embryos

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Humphrey (Develop. Biol., 27:365-375, 1972) reported studies on a naturally occurring genetic mutation, which he designated c for "cardiac lethal". Homozygous recessive embryos exhibit a total absence of heart contractions even though initial heart development appears normal. The mutants show normal swimming movements indicating that gene c does not affect skeletal muscle. When mutant (c/c) heart mesoderm is transplanted into normal (+/+;+/c) hosts, functional hearts are obtained. If reciprocal transplants are made, of donor heart primordia into mutant recipients, no heart beat is observed. These results strongly suggest that gene c exerts its effect by way of abnormal inductive processes from surrounding tissues. Further support for this conclusion is derived from detailed morphological studies of normal and mutant sibling anterior endoderm, a known primary heart inductor (L. Lemanski, B. Marx, C. Hill, Science, 196:894-896, 1977) It was determined that mutant endoderm cells differentiate more rapidly than normal. This finding could indicate that the mutant endoderm has progressed beyond the point at which it can induce precardiac mesoderm to become contracting heart tissue; mutant endoderm may even have an inhibitory effect of some kind.

Morphological, biochemical and immunofluorescent investigations have been performed on hearts of normal and mutant embryos from stage 34 (heart-beat stage) through stage 41 (when mutant embryos die). Electron microscope studies of normal hearts show some myofibrils to be present at stage 34; by stage 41, the normal myocytes have become highly differentiated muscle cells. Although some mutant heart cells contain a few thin actin and thick myosin filaments, sarcomere organization is absent. Instead, amorphous proteinaceous collections are prominent at the cell peripheries where myofibrils would be expected to first organize in normal cells. An analysis of the constituent proteins in normal and mutant hearts by SDS - polyacrylamide gel electrophoresis shows that actin (43,000 daltons) is present in almost normal amounts, myosin heavy chain (200,000 daltons) is reduced and tropomyosin (34,000 daltons) is virtually absent. Immunofluorescent studies extend these observations. Antimyosin sepcifically stains the A bands in normal hearts and reveals a progressive increase in myofibril organization with development. Mutant hearts show less staining for myosin than normal and localization is mainly in the amorphous collections at the cell peripheries. Anti-α-actinin stains the Z lines of myofibrils in normal myocytes. Mutant cells have significant staining for α-actinin at their peripheries which exhibits a periodicity similar to that of myofibrils in normal cells. Antitropomyosin stains the I bands of myofibrils in normal cells. There is almost no staining for tropomyosin in mutants; even as late as stage 41 the pattern in mutants is similar to pre-heart-beat normals. Heavy meromyosin (HMM) binding experiments show that the actin in mutant heart cells is contained within the amorphous collections in a non-filamentous state and the addition of HMM causes its polymerization

into filamentous form (F-actin) (L. Lemanski, M. Mooseker, L. Peachey, M. Iyengar. J. Cell Biol., 68:375-388, 1976). Since a close structure-function relationship of tropomyosin with actin in normally-organized myofibrils is well known, studies were undertaken to determine whether there might be a causal relationship between the absence of tropomyosin in mutants and a failure of the actin to form into filaments. The results indeed show that addition of tropomyosin to glycerinated mutant hearts or homogenates of mutant hearts results in the polymerization of amorphous actin into filaments.

Thus, this single gene mutation by way of abnormal inductive processes results in mutant cells having reduced, but significant, amounts of myosin and actin, even though non-filamentous, and substantial amounts of periodically arranged α-actinin. There is almost no tropomyosin. It is implied that the virtual absence of tropomyosin in mutant cells is someway related to the failure of normal myofilament formation, which in turn would seem to be an essential step in the normal organization of myofibrils.

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AN INTRODUCTION TO THE DEVELOPMENTAL GENETICS OF THE MEXICAN AXOLOTL

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The Mexican axolotl-although widely distributed among research laboratories around the world-is native to Lake Xochimilco near Mexico City. Its aquatic mode of life makes it particularly useful as a laboratory animal. Several highly inbred strains have, therefore, been developed in various laboratories for the purpose of having available a ready supply of genetically uniform experimental material. In addition, various mutant genes have been recognized-mainly by Dr. R. R. Humphrey--which are facilitating the gathering of important new information about various aspects of amphibian embryogenesis. These include nuclear-cytoplasmic interactions, cell differentiation and tissue and organ development. These mutant genes have been discovered during an extensive inbreeding program carried out by Humphrey. He has recognized approximately 3 dozen genes, which for the most part, exert specific effects on either one or another tissue or organ, or one developmental stage or another. The animals which bear those mutant genes are maintained in the Indiana University Axolotl Colony, and are made available to outside research and teaching laboratories.

Mutant genes in this collection are grouped in five different categories, based primarily on the developmental stage at which the mutant phenotype is first recognized. These groups include:

Group I: Oogenesis-mutant genes in this category all display true maternal effects.

Group II: Early development-several genes which affect the nucleus are included in this category.

Group III: Organogenesis-various genes which disrupt the normal development of specific organs (some of which will be subject of symposium talks) are listed in this group.

Group IV: Cell and tissue function-cell lethal genes make up this category.

Group V: Adult-a variety of very useful pigment markers are included in this category.

Representative mutant genes from each category will be the subject of many of the symposium presentations. One group, Group IV-cell and tissue function-will, however, be discussed in detail in this Introductory Paper. This group of genes makes up one of the largest categories, and includes over a dozen mutant genes. The common feature of these genes is that homozygous embryos cannot

be rescued by parabiosis. As well, grafts of mutant organ primordia do not survive on normal recipient hosts. These observations permit the speculation that mutations in this group produce defects in metabolic functions in virtually all cells of the mutant organism. Several of the mutant phenotypes will be briefly reviewed. Speculation will be put forth concerning the mechanism of action of the mutant genes.

Mutant genes are also available in other amphibian species, and they will be briefly mentioned. Several recent developments in the field of amphibian embryology will be reviewed, and opportunities for the further use of axolotls as experimental material will be discussed.

Aspects of Mauthner Cell Differentiation in the Axolotl

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In premetamorphic amphibians and fish, the Mauthner cells (M-cells), a single pair of large neurons, are present in the medulla at ear level. M-cells differentiate early, are easily recognized by morphological criteria, and in the axolotl, may be approached experimentally at all stages of development. Thus, the axolotl M-cell system is useful for the study of various aspects of neuronal differentiation and for the study of the role of cellular interactions in neuronal morphogenesis.

The withdrawal of a neuron from the cell division cycle is an early event in its differentiation. Gastrulae, neurulae and tailbud embryos were each given a single intra-archenteron injection of ^{3}H-thymidine and allowed to grow to early feeding stages. Radioautographs showed label over M-cell nuclei when injections were made prior to the termination of gastrulation, but not when injections were made at later stages. Thus, the cells that give rise to M-cells cease DNA synthesis during late gastrula stages.

Unilateral 180^{o} rotations of presumptive M-cells in embryos of different stages were performed to see when the anteroposterior and mediolateral axes are specified. In contrast to what has been reported for the frog, the axes of the axolotl M-cell have not yet been specified by late gastrula or mid-neurula stages: the rotated cell regulates and develops normally with respect to its polarity. Comparable studies with medullae of feeding larvae in which the rotations were performed at later stages are currently in progress so that the time at which the axes of the M-cell are irrevocably determined can be established.

A major source of sensory input to the M-cell is supplied by the ipsilateral vestibular system. To study the morphogenesis of the M-cell in terms of its interactions with groups of ingrowing axons, unilateral implants of otic vesicles were made either anterior to or posterior to the otic vesicle in host embryos. The implanted (extra) vestibular apparatus develops fairly normally. In the posterior implants, the ectopic vestibular axons travel anteriorly to enter the brain near where the normal vestibular input enters. In anterior implants, the converse is observed in that the vestibular axons travel posteriorly before entering the brain. The ectopic axons can be followed through the medulla in Bodian and horseradish peroxidase preparations to the region of the M-cell, but whether these axons actually form synaptic connections with the M-cell remains to be established.

Microtubule Proteins in Amphibian Development

Elizabeth C. Raff and Rudolf A. Raff

We have previously described a maternal effect mutation(nc) in the Mexican axolotl in which fertilized eggs become activated in the normal way but fail to initiate cleavage, apparently because the microtubule protein in the eggs fails to assemble into the mitotic apparatus (Raff, Brothers, and Raff, 1976. Nature 260,615). However, when microtubule fragments are injected into activated nc eggs, they undergo normal cleavage in a normal time sequence and continue cleavage until they reach a partial blastula stage.

In order to understand the role of tubulin in the developing axolotl egg and to define the nature of the nc mutant we have characterized tubulin from normal axolotl eggs and developing embryos by means of polyacrylamide gel electrophoresis and ^{3}H-colchicine binding (Raff, 1977. Devel. Biol. 58,56), and compared the tubulin from nc mutant eggs with normal egg tubulin. It was found that axolotl eggs and embryos contain authentic tubulin similar to other known tubulins but with several properties unique to this organism. We have concluded that neither the tubulin per se nor the tubulin pool in the nc egg is defective, and that the lesion in the nc mutation involves the function of nucleation sites for initiation of microtubule assembly, perhaps at a step in activation of the egg.

Tubulin accumulates in the growing axolotl oocyte throughout oogenesis. Mature oocytes and unfertilized eggs contain a tubulin pool of approximately 2µg/egg. This level declines by about 20% after initiation of cleavage and thereafter remains constant through development at least until early tailbud stage.

It was found that normal axolotl eggs differs in several properties from that prepared from albino axolotl eggs. Further, normal egg tubulin differs in electrophoretic properties on SDS-polyacrylamide gels, but not in colchicine binding properties, from tubulins prepared from either adult brain or testis. Further evidence that tubulins from egg and testis indeed differ was indicated by cleavage of egg and testis tubulins with chymotrypsin and a protease from Staph. aureus. Different spectra of peptides were obtained; heterogeneity was greater in peptides from the α than from the β subunit.

Experiments with some other species of amphibia also showed differences between oocyte tubulins and tubulins from adult tissues.

METAMORPHIC EVENTS IN THE AXOLOTL

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The Mexican axolotl, Ambystoma mexicanum, is normally neotenous, although it can be induced to undergo physical metamorphosis by exposure to thyroid hormone. However, during the growth of the neotenous larvae, a variety of morphological and biochemical changes occur which are usually associated with amphibian metamorphosis. The eyes become elevated from the surface of the head, urea excretion is substituted for ammonia excretion, and there are changes in serum proteins and hemoglobin species. The hemoglobins of the axolotl are different from those of most vertebrates in that they are larger. This is the result of larger globin chains. The larval globins are found exclusively in animals up until 70-100 days old, when the adult forms can be detected. After 150 days, only the adult forms are found. The change in the globin genes used in hemoglobin synthesis is controlled by thyroid hormones. The change is effected by a lower titer of thyroxine than that necessary to induce the events of physical metamorphosis.

The axolotl probably evolved from a metamorphosing species through genetic lesions lowering the output of hypothalamic thyroid release factor and/or pituitary thyroid stimulating hormone. It is possible that the sensitivity of biochemical metamorphic events to thyroxine was increased so that in the axolotl lower titers of thyroxine could accomplish the control of these events.

Analysis of the anemic gene of the axolotl has shown that the control of these biochemical metamorphic events are independently mutable. Animals homozygous for this recessive gene develop normally until they are about 100 days old. They then become severely anemic for about two weeks. Some affected animals die; others show reduced eye growth subsequent to the anemia. Those animals which survive recover normal hemoglobin titers. Both the larval and adult hemoglobins of an/an animals are normal in their electrophoretic characteristics. Prior to the anemia, only larval hemoglobins are present in an/an animals. Subsequent to the anemia, almost all of the hemoglobin is of the adult type. The period of anemia corresponds to the time in control animals when a mixture of nearly equal amounts of larval and adult hemoglobins are present. The entire anemic effect can be suppressed by immersing anemic animals in 5×10^{-8} M thyroxine. This causes a rapid transition from larval to adult hemoglobin synthesis.

These results suggest that the genic control mechanism for turning on adult hemoglobin synthesis has been mutated in the an/an animals to be less sensitive than normal to thyroxine. On the rising titer of thyroxine which drives the biochemical metamorphosis, larval hemoglobin synthesis is turned off normally in an/an animals, but the adult hemoglobin synthesis is delayed until a thyroxine titer sufficient to turn on the mutated control gene is reached.