Fish as Model Systems

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Fish represent the largest and most diverse group of vertebrates. Their evolutionary position relative to other vertebrates and their ability to adapt to a wide variety of environments make them ideal for studying both organismic and molecular evolution. A number of other characteristics make them excellent experimental models for studies in embryology, neurobiology, endocrinology, environmental biology, and other areas. In fact, they have played a critical role in the development of several of these disciplines. Research techniques that enable scientists to make isogenic lines in a single generation, create and maintain mutants, culture cells, and transfer cloned genes into embryos signal an increasing role for fish as experimental models.

DARWIN'S *THE ORIGIN OF SPECIES* EMPHASIZED THE IMportance of systematic and zoogeographic studies and, as a result, ichthyologists like David Starr Jordan became major figures in American evolutionary thought. At the turn of the century, Jacques Loeb, Thomas Hunt Morgan, and others felt that Louis Agassiz's dictum to "study nature, not books" should include experimental manipulation as well as natural history (*1*). In the next decades, as fish were used to probe the secrets of nature, a few species emerged as particularly useful models.

Fish models have been exploited by essentially every biological discipline (*2–4*). Because there are more than 20 disciplines and thousands of fish species, I have chosen a few representatives to communicate the flavor of this research. I will also point out some instances where fish have played particularly important roles in specific disciplines (for example, neurobiology) and enumerate some advantages of these model systems.

Advantages of Fish as Models

Fish are the oldest and most diverse vertebrates. They evolved around 500 million years ago, and today there are more fish species than all other vertebrates combined. Research with fishes provides a conceptual framework and evolutionary reference point for other vertebrate studies. They live in a wide variety of habitats that range from fresh to salt water, from cold polar seas to warm tropical reefs, and from shallow surface waters to the intense pressures of the ocean depths. Elucidating the evolutionary strategies and mechanisms that fish use to adapt to these diverse environments is one of the exciting challenges for modern biologists.

Many fish species are amenable to both field and laboratory experiments and are easily raised and bred under laboratory conditions. There is extensive animal husbandry information available from hundreds of years of practical experience by fish farmers, hobbyists, and aquaculturists. Many fish are much less expensive to buy and raise than their mammalian, avian, reptilian, or amphibian counterparts. They are generally the most fecund, some producing hundreds of eggs on a periodic basis, whereas others produce thousands. These eggs are usually large and externally fertilized, and, because some are transparent, embryonic development can be easily followed. Historically, these advantages and the economic importance of some fish have made them favored models for such studies and, as a consequence, the detailed embryology of many species has been carefully documented.

Fish are useful models for genetic manipulations. There are several highly homozygous strains, and general methods for obtaining new strains and mutants have been established. For example, inbred strains of medaka (*Oryzias latipes*), top minnows (*Poeciliopsis lucida*), and others have been produced by classical repetitive inbreeding. In addition, naturally occurring hermaphroditic (*Rivulus marmoratus*) and gynogenetically reproducing fish are available. Some scientists have imitated nature, successfully producing gynogenetic diploids in the laboratory. Streisinger and his colleagues (*5, 6*), for example, introduced methods for large-scale production of homozygous diploid zebrafish (*Brachydanio rerio*). With this simple technique, eggs were activated by sperm having DNA that had been inactivated by ultraviolet irradiation, then the maternal haploid genome was duplicated. The first cell division is then prevented by heat stress or hydrostatic pressure; however, subsequent cell divisions are allowed to proceed without intervention. Thus, the offspring is a diploid homozygote with the maternal genome. Because of the unusual sex-determination characteristics, the offspring are both males and females, so that normal breeding can continue in the next generation. This general approach has been applied to other fish, but some require hormones to produce males. A novel variation on Streisinger's theme used active sperm to initiate development of trout eggs with DNA that had been photoinactivated (*7*).

Mammalian models range from small species (for example, mice, rats, and guinea pigs) to large ones (for example, cows, sheep, and primates), each of which may be used to answer different types of scientific questions. Fish represent an even more diverse morphological group than mammals and, thus, the choice of a particular model depends on the question being addressed. Large fish, such as dogfish sharks (*Squalus acanthus*), the electric ray (*Torpedo californica*), winter flounder (*Pseudopleuronectes americanus*), rainbow trout (*Onchorhynchus mykiss*), and carp (*Cyprinus carpio*), tend to be used in studies where experimental manipulations are significantly facilitated by the larger size or distinct adaptation of the organ systems of the fish, for example, the historic advances with the fish kidney model. Although a number of these large fish have also been the focus of genetic analysis, the time required for sexual maturation and the cost of maintaining a large number of genetic stocks have made

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them less useful than for other studies. On the other hand, a number of small fish species with shorter life cycles, such as zebrafish (*Brachydanio rerio*), medaka (*Oryzias latipes*), killifish (*Fundulus heteroclitus*), guppies (*Lebistes reticulatus*), mollies (*Poecilia formosa*), platyfish (*Platypoecilus maculatus*), and swordtails (*Xiphophorus helleri*), may be used as models for even the most sophisticated genetic analyses.

Recently, a number of researchers have successfully microinjected cloned DNA into fertilized fish eggs and, in some cases, the transferred gene has been integrated into genomic DNA, the protein has been expressed, and the transferred gene inherited in a Mendelian manner (*8*). For example, studies with growth hormone constructs indicate significant enhancement of growth in transgenic fish. Because the first generation offspring of these transgenic fish are usually genetic mosaics, some of their F_1 offspring may carry and express the foreign gene, whereas others may not. This phenomenon is dramatically illustrated in Fig. 1.

Neurobiology

For decades, neurobiologists have found fish to be excellent models. In fact, most biologists and physicians over 40 years of age had their first exposure to vertebrate neuroanatomy when they dissected the brain and cranial nerves of the dogfish shark. Comparative neurobiology can provide insight about the human nervous system and its role in health. Fish models can lead to understanding of vertebrate neurology in general and provide perspective by their fundamental evolutionary relationship with other vertebrates. Our present concept of vertebrate color vision, for example, was significantly influenced by a series of classical studies on fish retina (*9*). Certain progressive neurological diseases are best understood in the context of the evolutionary states of the nervous system in which evolutionarily "higher" central nervous system functions are lost first, then sequentially "lower" evolutionary states, with the order being reversed during recovery (*10*). Bullock has provided several examples that are consistent with that hypothesis (*10*).

Prosser described neurobiology as the neuronal basis of animal behavior, determined by neural circuits that are controlled by cell-to-cell communications, including chemical coupling through neurotransmitters (*3*). Most neurotransmitters are amino acids, their derivatives, or peptides. Acetylcholine (ACh) is a combination of choline (from serine) and acetyl coenzyme A. Acetylcholine is perhaps the most universally recognized neurotransmitter among nonneurobiologists. It is widespread among most taxa and performs a variety of important functions. Research on the ACh receptor and the Na^+ channel of fish has played a critical role in the development of neurobiology.

Approximately one-third of vertebrate neurons respond to ACh. In the brain, there are at least two types of ACh or cholinergic receptors, muscarinic and nicotinic. The most important studies on the nicotinic receptor have used fish models. The electric organ of the ray, *Torpedo californica*, contains cholinergic neurons that innervate electrocytes that develop from myotubes but have 1000 times more ACh receptors than muscle cells (*11*). The abundant ACh receptor has been extensively characterized, including the cloning and sequencing of all four of its protein subunits (*12*). Moreover, this receptor has been reconstituted in artificial membranes, and its role has been elucidated in the disease myasthenia gravis (*13*). These studies on the ACh receptor are an important success in molecular neurobiology and a model approach.

Another example in which research on fish has paved the way for molecular approaches to neurobiology is provided by the studies on the voltage-controlled Na^+ channel. This integral membrane protein is responsible for the extremely rapid depolarization associated with propagated action potentials in nerve and muscle cells of animals from many phyla. Studies of Na^+ channels have guided research into other voltage-controlled channels, such as K^+ and Ca^{2+} channels, that are even more widespread and functionally diverse.

The Amazonian electric eel, *Electrophorus electricus*, like the electric ray, *Torpedo californica*, is well endowed with electroplax tissue for delivering strong electric shocks to both predators and prey. Extensive research during the 1950s and 1960s on the electric organ of this fish resolved a conflict between bioelectric and biochemical interpretations of the nervous system. Since that time, this fish has been a favorite model for other neurochemical studies. For example, because the electric organ is such a rich source of Na^+ channels, it was used as a tissue source for the purification of the first Na^+ channel (*14*). This Na^+ channel was also the first from which essentially normal functional activity was successfully reconstituted (*15*). Those studies on fish Na^+ channels have become the model for studies on other channels (*16*).

As was the case for the ACh receptor, a fish Na^+ channel was the first of such molecules to be cloned (*17*). The primary structure revealed four repeating homologous units, each of which contains a unique sequence in which five to seven positively charged lysine or arginine residues occur at every third position, with most of the intervening positions occupied by polar residues (Fig. 2). Because of the similarity between this sequence and the hypothetical structure of the voltage-dependent gating machinery for the channel (*18*), it was proposed that this macromolecular machine was a transmembrane structure that underlies the intermembrane charge movement that triggers opening of the channel, referred to as gating current. This concept is central to all proposed structural models that attempt to account for Na^+ channel function (*19*).

The unusual S4 sequence identified in the eel Na^+ channel is not unique. Nearly identical structures have now been identified in essentially every other voltage-controlled channel that has been cloned and sequenced: two more Na^+ channels from rat brain (*20*) and one from *Drosophila* (*21*), the *Shaker* K^+ channel from *Drosophila* (*22*), and the dihydropyridine receptor from rabbit skeletal muscle, a putative Ca^{2+} channel (*23*). The presence of an S4-like sequence has been used to identify genes coding for voltage-controlled ion channels (Fig. 2). Work on the electric eel has directly shaped our present understanding of the molecular structure and function of these and perhaps all channels gated by membrane voltage.

Endocrinology

Approximately 400 million years ago, the ancestors of modern bony fishes (teleosts) invaded fresh water and diversified. A few hundred million years later, new species returned to the ocean and proliferated; afterward, new teleosts reinvaded fresh water. Today, some fish are restricted to either fresh water or salt water and others spend part of their life in each environment. Freshwater fish are in a Na^+-poor environment and have evolved mechanisms to retain salt. Although they do not drink water and their skin is relatively impermeable, a significant influx of water occurs across the gills, which is eliminated as a dilute urine through the glomerular kidney. Salt lost in the feces and urine is replaced from food and by active Na^+ uptake at the gills. The opposite situation exists for saltwater fish, which must conserve water and exclude salt. They drink water and eliminate excess ions either at the gills or through feces; urine output is minimized. Fish that migrate between fresh and salt water must, therefore, regulate these mechanisms in order to survive (*24*).

Pickford and Phillips (*25*) showed that hypophysectomized killifish, *Fundulus heteroclitus*, failed to survive when transferred from salt

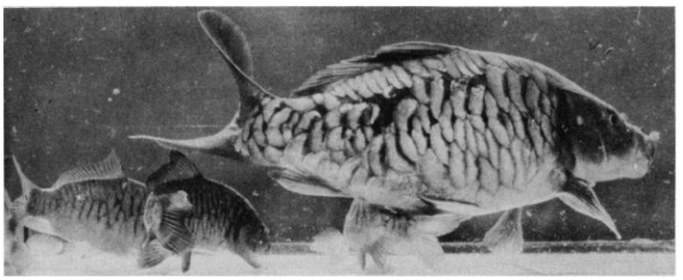

Fig. 1. The carp in this figure are the F_1 offspring of mosiac transgenic parents. The larger individual is transgenic and carries and expresses a mammalian GH gene whose expression is driven by a metallothionein promoter. The smaller individuals in the figure are the larger individual's nontransgenic siblings. This photograph was provided by Z. Zhu of the Institute of Hydrobiology, Academica Sinica in the People's Republic of China, and senior research scientist of the Center of Marine Biotechnology, University of Maryland.

water to fresh water but did survive when injected with prolactin (Prl). The rapid loss of Na^+ at the gills was essentially halted by Prl injections. This and other papers by Pickford and her colleagues set the stage for delineating of the role of Prl in the complex process of osmoregulation. Although all the details are not yet known, in at least a few fish species (*24*), Prl appears to stimulate active Na^+ uptake, inhibit Cl^- excretion, retain Ca^{2+}, enhance production of dilute urine by inhibiting water reabsorption, and facilitate Na^+ retention by suppression of a Na^+,K^+-ATPase (*24, 26–29*). In fact, when fish migrate from fresh water to salt water, the Na^+,K^+-ATPase can increase by as much as an order of magnitude (*29*).

Pickford also studied the growth-promoting effect of growth hormone in hypophysectomized killifish (*30*). Since growth hormone (GH) and Prl arose from a common evolutionary precursor (*31*), it should not be surprising that there were reports of overlapping biological activities (*32, 33*). However, it was eventually shown that Prl and GH from the same species had mutually exclusive effects (*33*). In trout (*33*), GH functions in a manner opposite to Prl in osmoregulation; it enhances Na^+,K^+-ATPase activity and Na^+ exclusion at the gills. When some fish migrate from salt water to fresh water, Prl increases, GH decreases, and Na^+ is retained by the reduction of Na^+,K^+-ATPase activity. When they migrate from fresh water to salt water, the opposite is true and growth is accelerated because of increased GH concentrations (*24*).

The detailed mechanism of this control and its general applicability to other species (*24*) is not yet known, but the availability of large quantities of biosynthetic GH and Prl from cloned cDNAs (*35*) should provide adequate material to study these mechanisms. Now that the fish GH gene structure is known (*31*) and the Prl gene structure is being elucidated, the mechanisms controlling gene regulation should follow. These molecular studies as well as similar efforts on other pituitary hormones (*36*), hormones from the hypothalamus (*37*), and other endocrine hormones, signal that a new phase in fish endocrinology has begun. Moreover, these tools can be used on other fish species to explore evolutionary variations on this theme of osmoregulation.

Developmental Biology

Modern developmental biology tends to focus on (i) embryonic pattern formation, including the movement and eventual fate of specific cells (*38*); (ii) the mechanisms responsible for developmental stability; (iii) the expression of specific genes during development, including their regulatory mechanisms; (iv) agents responsible for initiating new developmental programs and shifting the timing of developmental events; (v) sex determination; (vi) the mechanisms of cellular and tissue differentiation; and (vii) the mechanisms that control organ system development (*38*). Although many of these topics can be addressed in a variety of organisms, a vertebrate is, clearly, needed for questions relating to typical vertebrate development. For previously mentioned reasons, fish have been favorite models of embryogenesis for over a century. For example, the killifish, *Fundulus heteroclitus*, and the medaka, *Oryzias latipes*, played central roles in embryology in the United States and Japan, respectively. In a recent review of fish developmental genetics (*39*), the authors emphasize that gene regulation is intrinsically tied to evolutionary adaptation.

In the absence of an extensive array of laboratory mutants, researchers have taken advantage of interspecific fish hybrids, unisexual fish, and species derived from polyploid ancestors. The use of interspecific fish hybrids to study the fate of alleles and gene regulation is an in vivo analog to the in vitro somatic cell hybrid technique commonly used to study gene regulation in mammalian cell culture. The application of this approach has been reviewed by Whitt (*40*). He has shown that the greater the evolutionary distance between the parental stocks of hybrids, the greater the frequency of expression of abnormal characters; also maternal alleles are generally expressed at their normal time, whereas paternal alleles are delayed (*40*). With the recent advances in genetic techniques to manipulate fish genomes, the mechanisms responsible for these interesting observations may be examined.

Shifts in the timing of developmental events (heterochrony) have also been studied with fish models. These shifts can sometimes be traced to a single locus. For example, Kallman has shown that differences in the time required to reach sexual maturity in the swordtail, *Xiphophorus maculatus*, is a function of a single locus that regulates luteinizing hormone–releasing hormone (*41*).

Some investigators have shown that developmental rate and time required for hatching are correlated with specific enzyme–encoding loci that presumably play a role in the timing of developmental events. For example, DiMichele and Powers (*42*) showed that developmental rate and hatching in the killifish, *Fundulus heteroclitus*, was highly correlated with genetic variation of the "heart" locus of lactate dehydrogenase (*Ldh-B*). The homozygote for one allele, $Ldh\text{-}B^a$, consumed oxygen faster and hatched earlier than the homozygote with the other allele, $Ldh\text{-}B^b$. Recently, DiMichele (*43*) showed that oxygen consumption was altered in a predictable way by the type of lactate dehydrogenase microinjected into fertilized eggs, indicating that the enzyme had a direct effect on development. Development and hatching differences have been observed for other loci in *Fundulus* (*44*) and trout (*45, 46*).

One of the major drawbacks to the use of vertebrates for developmental studies has been the paucity of mutants. For example, because of the nature of mammalian development, the problems associated with identifying and isolating mutants have been both costly and difficult (*47*). On the other hand, the zebrafish has been an unusually successful model for generating and analyzing developmental mutants (*48*). The success of the zebrafish system is largely the result of methods that (i) allow developmental mutants to be identified in a single generation, (ii) engender completely isogenic stocks in a single generation, (iii) permit the cryopreservation of gametes, (iv) make possible the artificial creation of mutants, and (v) enable the formation of transgenic fish (*8, 49*).

The movement and eventual fate of cells during development can be studied by direct observation of unmarked or marked cells (*38*). The latter is usually accomplished by introducing one of several nontoxic tags that facilitate observation. Kimmel and his colleagues (*50*) have used direct observation of unmarked cells, of cells tagged with fluorescent dyes, and of genetic mosaics to study the lineages

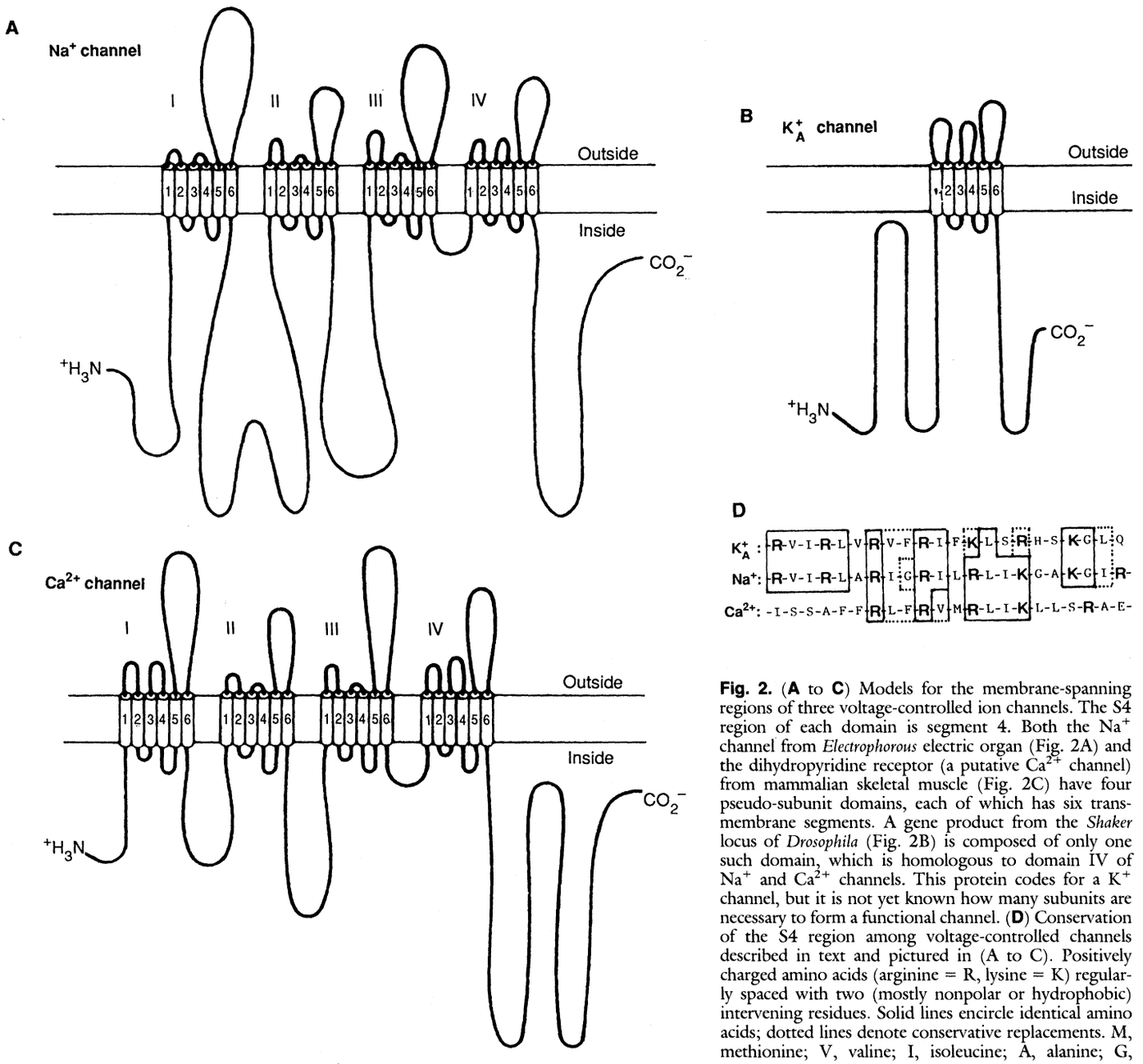

Fig. 2. (**A** to **C**) Models for the membrane-spanning regions of three voltage-controlled ion channels. The S4 region of each domain is segment 4. Both the Na^+ channel from *Electrophorous* electric organ (Fig. 2A) and the dihydropyridine receptor (a putative Ca^{2+} channel) from mammalian skeletal muscle (Fig. 2C) have four pseudo-subunit domains, each of which has six transmembrane segments. A gene product from the *Shaker* locus of *Drosophila* (Fig. 2B) is composed of only one such domain, which is homologous to domain IV of Na^+ and Ca^{2+} channels. This protein codes for a K^+ channel, but it is not yet known how many subunits are necessary to form a functional channel. (**D**) Conservation of the S4 region among voltage-controlled channels described in text and pictured in (A to C). Positively charged amino acids (arginine = R, lysine = K) regularly spaced with two (mostly nonpolar or hydrophobic) intervening residues. Solid lines encircle identical amino acids; dotted lines denote conservative replacements. M, methionine; V, valine; I, isoleucine; A, alanine; G, glycine; S, serine; T, threonine; Q, glutamine; N, asparagine; D, aspartate; E, glutamate. Adapted from (*19*).

and eventual fates of embryonic cells. They have shown that blastomere lineages are indeterminate but lineage restriction exists after gastrulation. However, it is not yet clear that postgastrula cells are irrevocably committed.

The studies of Grunwald and his collaborators illustrate the power of Streisinger's method for isolating and analyzing developmental mutants (*51*). One of Grunwald's mutants causes degeneration of later developing central nervous system components but does not affect early primary neural tissue. This and other studies on zebrafish mutants are providing insights about vertebrate development that could not have been as easily perceived with vertebrate models. Zebrafish are becoming a powerful tool to study vertebrate development, and recent molecular studies on fish neuropeptides (*36*), hormones (*31, 35, 37*), and homeobox genes (*52*), coupled with the ability to make transgenic zebrafish (*8*), promise an expanded role for zebrafish in developmental biology.

Aquatic Toxicology and Carcinogenesis Research

Fish models cannot replace mammals for research into mammalian physiology, but they can offer an inexpensive and, in some instances, more acceptable alternative for chemical carcinogen testing. Fish are particularly useful for the assessment of water-borne and sediment-deposited toxins where they may provide advanced warning of the potential danger of new chemicals and the possibility of environmental pollution. Recent public awareness of increasing contamination of the oceans and the potential associated health risks should encourage further research in aquatic toxicology.

Russel and Kotin (*53*) were the first to suggest a correlation between pollution and incidence of fish tumors. Those correlations were emphasized when an outbreak of liver cancers in cultured rainbow trout was traced to the presence of an aflatoxin in their food

(*54*). Recently, hepato-cellular carcinomas have been found to be significantly elevated in the winter flounder (*Pseudopleuronectes americanus*) from Boston Harbor (*55*) and in the English sole (*Pleuronectes vetulus*) from Puget Sound (*56*). The latter study suggested that the tumors might be the result of excess polycyclic aromatic hydrocarbons in the sediments. The elegant studies of Hendricks *et al.* (*57*) demonstrated that, indeed, the polycyclic aromatic hydrocarbon, benzo[*a*]pyrene, was capable of inducing hepatomas (*58*).

The ability to induce neoplastic lesions in fish tissues has inspired innovative methods for detecting chemical carcinogens. One particularly novel test involves the use of fish embryos. In this test, fertilized eggs from rainbow trout (*59*) or medaka (*60*) are exposed for a short period to a defined concentration of a toxin. After removal, the embryos are allowed to develop, and evidence of tumors is recorded. This is a highly sensitive test that, in some ways, is superior to the analogous test in rodents. The main advantages and disadvantages are discussed elsewhere (*59, 60*).

In addition to chemical carcinogens, viruses have been suggested as potential causes of fish neoplasms. For example, Papas and his colleagues suggested that lymphosarcomas in the northern pike were the result of a C-type virus (*61*). Kimura *et al.* (*62*) and Sano *et al.* (*63*) indicated that lesions or tumors in salmon could be induced by exposure to some herpes viruses.

The discovery that melanomas could be generated in hybrid crosses between swordtails (*Xiphophorus helleri*) and platyfish (*Platypoecilus maculatus*) opened a new area of melanoma research (*64*). After this discovery, a number of scientists analyzed the genetic basis of this interesting phenomenon (*65*). Platyfish have melanin pigment patterns that are coded by specific color genes. For example, one strain has a black pigmented spot or spots on the dorsal fin, the expression of which is controlled by an allele of a sex-linked locus. If a female is crossed with a melanin-lacking male swordtail, the offspring will have abnormal melanization that will often lead to melanomas. Anders *et al.* (*66*) described the color gene as an oncogene and suggested that it was correlated with c-*src*. This "color oncogene" is controlled by at least one regulatory locus and a host of environmental and physiological factors.

There have been other oncogenes studied in fish and some have been analyzed at the molecular level. The *ras* gene was cloned from goldfish liver DNA (*67*), and the c-*myc* gene was isolated and characterized from rainbow trout (*68*). Each of these showed a remarkable similarity to their mammalian and avian counterparts (*68*).

Schartl and Peter (*69*) demonstrated progressive growth of fish tumors when malignant melanotic melanoma tissue, from the swordtail fish, *Xiphophorous*, was transplanted into thymus-aplastic nude mice. Moreover, while the tumor adapted to the physiological conditions of the mammalian host, it retained its fish-specific morphology and biochemical specificity. In a recent study, winter flounder tissues that contained histopathological lesions were assayed for oncogenes by transfection of the DNA into mouse fibroblasts. The transfected fibroblasts induced subcutaneous sarcomas when transferred into nude mice, and there is some evidence that these sarcomas may contain fish c-K-*ras* oncogenes (*70*).

Biochemical and Genetic Adaptation

Comparative physiologists and biochemists are interested in the mechanisms that organisms use to adapt to environmental stress. Fish are particularly good models because they live in a variety of habitats and must adapt to environmental parameters, like temperature, pressure, oxygen, *p*H, and salinity, which are easily measured and controlled under laboratory conditions. Hochachka and Somero (*4*) pointed out many of the adaptive mechanisms and strategies of aquatic animals.

Temperature is a useful parameter to illustrate biochemical adaptation. Fish are cold-blooded organisms, and their success involves adaptations to changes in environmental temperature. This is accomplished by a host of metabolic, physiological, and behavioral changes (*3, 4*). Like many organisms, fish use heat shock genes in response to elevated temperature (*71*), but for extreme cold, some have evolved a novel set of antifreeze genes to encode proteins that keep their blood from freezing. DeVries (*72*), who discovered antifreeze proteins (AFP), found that polar fish express these genes all year, whereas temperate species, like winter flounder, express AFPs only in winter (*73*). This seasonal variation is an excellent system to study the role of environmentally regulated gene expression.

AFPs of Antarctic fish are composed of repeating units of Ala-Ala-Thr with a disaccharide, galactosyl-*n*-acetylgalactose amine, glycosidically linked to the threonine (*74*). Winter flounder AFPs differ in that they do not have disaccharides. The flounder express an alanine-rich helical protein whose primary, secondary, and tertiary structures have been determined (*74–76*), and the elegant mechanism by which these proteins bind micro–ice crystals and lower the freezing point of the blood has been formulated (*75*). Recently, the DNA sequences encoding these genes have been elucidated (*76*), and microinjection of the gene into other species has been successful (*8*). Now the tasks of understanding the regulation of this gene family and delineating the molecular mechanisms involved in the transfer of information from the environment to the target tissue remain.

The potential adaptive role of genetic variation at enzyme-synthesizing loci has been the subject of intense investigation for almost three decades. Indeed, few subjects in biology have been more debated than the evolutionary significance of protein polymorphisms (*77*). Most of the debate centered around two contrasting views: the "selectionist" and "neutralist." Proponents of the first idea assert that natural selection maintains protein polymorphisms, whereas those of the second school argue that the vast majority of such variation is selectively neutral. In 1974, Lewontin (*77*) summarized the failure of evolutionary biologists to resolve this important issue. Since that time, a number of researchers have addressed the controversy with renewed vigor and with the sophisticated tools of molecular biology (*78*).

A few in vitro studies of enzymes have suggested selection, however, there is little corroborating evidence at other levels of biological organization (*78*). My colleagues and I are addressing the significance of genetic variation in the killifish, *Fundulus heteroclitus*. Using temperature, *p*H, oxygen, and salinity as model environmental variables, we are investigating a series of allelic isozymes for evidence of natural selection at several levels of biological organization. We have analyzed enzyme kinetics, protein structure, gene sequences and their regulation, cell metabolism, oxygen transport, developmental heterochrony, swimming performance, population biology, zoogeography, and have carried out laboratory and field selection experiments (*44, 78, 79*). Based on the results of some of those studies, DiMichele predicted heterochrony and mortality differences between phenotypes as a function of temperature and salinity, and some of those predictions were realized as he found hatching time and mortality differences for both single and multilocus phenotypes at high temperatures (*43*). Moreover, those that survived the highest temperature regime were also the most common phenotypes at the warm southern extreme of the natural distribution of the species. Although this multidiscipline approach is just beginning to bear fruit, it already appears that some enzyme loci are maintained by natural selection while others are not.

Another example of this approach is the work of Vrijenhoek and

his colleagues, who have shown dramatic differences in survival among populations of the fish genus *Poeciliopsis* (*80, 81*). In addition, laboratory and field selection experiments reveal the action of natural selection on genetic variants marked by four enzyme-encoding loci. Using acute cold, heat, and hypoxia, variables that mimic seasonal environmental stress, they demonstrated that allozyme diversity and survival were intrinsically linked (*81*). It will be exciting to explore the biochemical and molecular basis for this phenomenon in the future.

Elucidating the array of mechanisms that animals use to adapt to diverse environments is one of the exciting challenges for modern biology; fish provide excellent models to meet that challenge.

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Research Articles

Stabilization of Z DNA in Vivo by Localized Supercoiling

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Biological processes such as transcription may generate domains of supercoiling on a circular DNA. The existence of these domains in *Escherichia coli* was investigated by the ability of different lengths of (CG) tracts, cloned upstream or downstream from the tetracycline resistance gene (*tet*) of pBR322, to adopt the Z structure in vivo. Segments as short as 12 base pairs adopt the Z form when cloned upstream from the *tet* gene (Eco RI site), whereas no Z DNA was detected when this sequence was cloned downstream (Sty I site), even with a 74–base pair (CG) tract that requires less supercoiling than shorter tracts for the B-Z transition. Hence the localized supercoil density in pBR322 can be as high as −0.038 and as low as −0.021 at different loci. These data demonstrate the existence of the Z structure for commonly found natural sequences and support the notion of domains of negative supercoiling in vivo.

The polymorphic nature and flexibility of DNA in response to local environmental conditions has been well documented (*1*). Left-handed Z DNA as well as other non–B DNA structures, such as cruciforms and triplexes, are induced by negative supercoiling in recombinant plasmids. These structures have been extensively investigated in vitro (*1*).

Recent studies demonstrated the existence of Z DNA in living cells (*2, 3*). Furthermore, the B-to-Z structural transition causes deletions (*4*). These discoveries contributed substantially to the long-standing hypothesis that DNA structural microheterogeneity plays a key role in cellular processes. However, Z DNA was found in vivo only in alternating CG [(CG)] inserts longer than 40 to 45 bp (*2, 3*), which are uncommon in naturally occurring sequences (*5*).

Transcription can be a major contributor to the level of DNA supercoiling in bacteria (*6–8*) and yeast (*9, 10*). The actual local supercoiling in vivo may be highly positive, highly negative, or negligible, depending on the position of promoters, the rate of transcription, and the efficiency of supercoil removal by topoisomerases (*8, 11*). The waves of negative supercoiling generated behind the transcription machinery can be sufficient to transiently induce non–B DNA structural transitions (*11*).

In this article we describe a more sensitive assay that detects shorter regions of left-handed Z DNA in vivo. Osmium tetroxide (OsO_4) was used to probe the structural distortions at the B-Z or Z-Z junctions at the base pair level, directly inside *Escherichia coli* HB101. Tracts of (CG) as short as 12 bp adopt the Z conformation in vivo when located upstream from the pBR322 *tet* promoter but not when cloned downstream from the *tet* gene. This discovery of short, naturally occurring segments undergoing the B-to-Z transition has significant biological implications. Indeed, long (CG) stretches, which are permanently in the Z form, would not be regulated, whereas the existence of small Z helices, which are more sensitive to slight changes in available free energy, would offer greater opportunities for their involvement in biological regulatory

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