MINIREVIEW

A Perspective on the Value of Aquatic Models in Biomedical Research

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For at least 150 years, biological scientists have congregated at marine laboratories, located at the edge of the sea, to explore aquatic life. The purpose of this minireview is to offer a brief perspective on the relevance of this activity to our knowledge of human physiology and disease, drawing heavily on the experience of the authors and without attempting to offer a comprehensive history of the many contributions of marine models to biomedical research. Exp Biol Med 230:1–7, 2005

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The historical development of aquatic biological laboratories is best understood as a nineteenth-century phenomenon. These institutions began as a natural outgrowth of the intense interest in natural history that characterized high society in the eighteenth and nineteenth centuries.

The Swedish scientist Karl von Linne (Carolus Linnaeus, 1707–1778), who invented the binomial system of classifying plants and animals by genus and species that we still use today, personified the idea of "cataloging the whole creation" that possessed naturalists of the 1700s and 1800s. Spurred by the explorations of Africa, South America, and the Pacific, the prime occupation of the "natural historian" involved the precise description and classification of the anatomical details of newly discovered and previously known species, including fossils. Linnaeus raised money from kings and princes abroad as well as in Sweden and attracted scores of students whom he sent all over the world. At that time, a modest familiarity with what was called natural history was felt to be part of the equipment of an educated man. Heads of state commissioned Linnaean catalogs of their colonies and summoned noted scientists to serve in their courts.

Alexander Humboldt (Baron von, 1769–1859), who advised the King of Prussia and was the contemporary and confidant of Galvani and Gay-Lussac, was commissioned at the age of 30 years by the King of Spain to report on the geology and natural history of Spanish possessions in South America. His 30-volume report, completed in 4 years, enormously excited scientific circles and the general public. Darwin's voyage on HMS *Beagle*, sponsored by the British admiralty, was in part an outgrowth of the intense interest in South America generated by Humboldt. Darwin's book *On the Origin of Species*, published in 1859, greatly intensified the enormous popular and scientific interest in the beginning of life (presumably in the ocean) and in its evolution from primitive to higher forms.

Among the foremost proponents of the new marine biology was Jean Louis Rodolphe (Louis) Agassiz (1807–1874), the Swiss-American naturalist, geologist, and Harvard professor who was an intellectual descendant of the Humboldt tradition and a lifelong opponent of Charles Darwin's theory that species evolved through natural selection. Agassiz, a specialist in Linnaean classification, tended to view the idea of the mutability of species as heretical. In his mind, each species was "a thought of God." Nevertheless, he preached that the creed of the naturalist should be "Study nature, not books!" In the last year of his life, he organized a marine biology course in an old barn at

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Penikese, an uninhabited island in Buzzards Bay off the coast of Massachusetts. The "Laboratory at Penikese" served as a model that others imitated. The example of Agassiz and his injunction to "Study nature, not books!" helped to stimulate, in the last quarter of the nineteenth century, the establishment of a handful of marine biology stations around the world that exerted an extraordinary influence on the development of the biological sciences. These included the laboratories at Plymouth, England, and Naples, Italy; the biological laboratory at Woods Hole, Massachusetts; Stanford University's Hopkins Marine Station; the laboratory at Friday Harbor on the Pacific coast; and the Mt. Desert Island Biological Laboratory of Maine. The insights that life began in the sea and that all living creatures are related generated the notion that at least some life processes in "higher" forms of life might be clarified by studying them in "lower" forms (1).

For the biologist interested in function, there are several practical advantages to the study of vital processes in aquatic forms rather than in warm-blooded mammals. First, the ocean is an almost inexhaustible medium, relatively constant in composition, temperature, and oxygen content, providing fresh specimens of living creatures that are readily available. Second, because most aquatic animals are poikilothermic, there is no need to maintain their cells and organs at 37°C when studying them at the bench. Third, perhaps because of this second item, cells and organs from marine creatures tend to be sturdier than those of warm-blooded mammals when studied in isolation at low temperatures. Fourth, cells of aquatic animals are sometimes larger than those of their mammalian counterparts and are therefore easier to puncture and manipulate. Fifth, because the development and maturation of many aquatic forms take place outside the body and are so rapid, they can be observed to unfold literally under the bioscientist's eye. The effects of genetic manipulation can be easily and quickly observed. Finally, because of the multiplicity of species living in the ocean, advantage can be taken of "Nature's experiments" in which a critical physiological or anatomical function has been either atrophied or exaggerated.

Perhaps the most famous example of a seminal contribution to human physiology by a marine biological model is found in the experiments of A.L. Hodgkin and his collaborators, Huxley, Keynes, and Katz. These investigators, working in the Marine Biology Station of Plymouth, England, from 1939 to 1955, first inserted an electrode into the interior of a giant axon of the squid to study the mechanism of the electrical impulse that causes the squid's mantle to contract rhythmically, providing a jet of water that propels the animal from one place to another (2). The squid's giant axon is about 0.5 mm in diameter, more than 25 times the width of nerve fibers in mammals and amphibians. Because the squid axon is so large, it was possible not only to record the electrical potential of the axoplasm in relation to the bathing medium but also to measure the flux of sodium and potassium across the plasma membrane, using radioactive isotopes (3). The principles of neurotransmission that were derived involved rapid sequential alterations in ionic permeability of the plasma membrane. These principles apply to nerve fibers in all animal species, including man.

Equally basic to modern concepts of membrane physiology was the description in 1957 of the enzyme in nerve cells that was responsible for transmuting the potential energy stored in adenosine triphosphate (ATP) into the movement of sodium ions across the cell membrane, against an opposing physico-chemical gradient, from the interior to the exterior of the nerve cell (4). Because the enzyme required both sodium ions and potassium ions to be activated, Jens Skou, a Danish physiologist, surmised that the splitting of ATP could provide the energy needed for the active transport of sodium and potassium ions in opposite directions. Such a process was postulated by Hodgkin and Keynes to be necessary to restore the low internal concentration of sodium that is disturbed by the passage of a nerve impulse.

Skou conceived the idea of searching for a lipoprotein ATPase that would catalyze this process while he was on a working vacation at the Marine Biological Laboratory at Woods Hole. Here, following the lead of Hodgkin and Huxley, the squid axon was being extensively investigated. Because squid were not available in the cold waters of the Baltic Sea surrounding Denmark, Skou, when he returned to his home university in Aarhus, tested the action of sodium ions on homogenates of the leg nerve of shore crabs, found in large numbers on Danish beaches. He found that the hydrolysis of ATP (in the presence of Mg^{++}) was enormously accelerated by sodium ions. Six months later, he found that the enzyme was also stimulated by potassium ions (5). The results ignited an explosion of interest in the membrane-bound Na-K-ATPase, which was soon identified as the sodium pump present in all animal cells and responsible for the difference in cationic composition between intracellular and extracellular fluids. Inhibition of the pump by digitalis-related steroids was thought to be responsible for the beneficial action of these drugs in heart failure, which had been known empirically for centuries (6).

The importance of the Na-K-ATPase in unidirectional transport of sodium across epithelial cell layers as well as into and out of individual cells was further elucidated by experiments with aquatic species that made use of "experiments of Nature." Here, the principle enunciated by the Nobel winning biochemist Hans Krebs was invoked: "When the traffic over a enzymatic pathway is greatly increased, the activity of the rate-limiting enzyme rises." Seawater teleosts (bony fish) drink constantly and excrete sodium chloride across their gills. The gill-mediated efflux of sodium in teleosts adapted to seawater is orders of magnitude higher than that of similar fish in freshwater. Sure enough, the activity of Na-K-ATPase in the gills of fish adapted to ocean dwelling was several times that of fish in freshwater lakes and streams (7, 8). The concentration of the

"sodium-pump enzyme" is especially high in the specialized glands developed by marine birds or by elasmobranch fishes to secrete NaCl. With this in mind, the first isolation, purification, and characterization of Na-K-ATPase was performed by Lowell Hokin, starting with the rectal glands of spiny dogfish, which are known to be rich in Na-K-ATPase, obtained at the Mt. Desert Island Biological Laboratory in Maine (9).

Because the task of the kidney to preserve a constant *milieu interieur* is so varied and demanding among the vertebrates of land and sea, the insights provided into renal function by comparative marine physiologists are particularly noteworthy. The molecular basis of literally all of the diuretic drugs in clinical use today has been clarified by experiments in aquatic species. The action of "loop-diuretics" (furosemide and its derivatives) to inhibit secondary active transport of chloride in the thick ascending loop of Henle in mammals was suggested by furosemide inhibition of secondary active transport of chloride in cell membranes of the shark rectal gland (10) (Fig. 1). The effect of using thiazide diuretics to block NaCl cotransport by distal tubules of mammalian kidney was elucidated by identifying the NaCl cotransporter in the flounder urinary bladder (11). The action of acetazolamide (Diamox) to inhibit carbonic anhydrase and thereby produce a proximal tubular diuresis in human patients was clarified by T.H. Maren in work with marine species (12).

Studies of the kidneys of fish have also thrown light on the fundamental mechanism of urine formation, the nature of which had preoccupied physiologists for more than 150 years. Following the anatomical description of renal glomeruli by Bowman early in the nineteenth century, it was held by the great German physiologist Ludwig that urine was primarily formed by filtration of fluid through the glomerular sieve, propelled by the hydrostatic pressure inside the glomerular capillary. His contemporary, Heidenhain, in contrast, maintained that urine was secreted by glomerular cells via an active, "vital," cellular process. In 1914, Cushny proposed that the urine was indeed initially formed by the physical process of filtration through glomerular membranes but that active reabsorptive transport by tubular cells resulted in the return of an "ideal" fluid to the body before the final urine-containing constituents could be safely rejected and could be excreted.

Formation of urine by aglomerular fish (of which there are about 40 species) constituted indubitable proof that the initial formation of urine did not require glomeruli (13). Tubular secretion must be responsible for urine formation in at least these aglomerular vertebrates. Through the work of Klaus Beyenbach, it is now apparent that the secretion of an isotonic fluid containing sodium and chloride into the lumen of kidney tubules plays a major role in the formation of urine not only in aglomerular teleosts but also in the kidneys of elasmobranch fishes (which do have glomeruli) and even in the glomerular kidneys of freshwater bony fish. Active secretion of sodium and chloride has now been detected in

	Extracellular	Intracellular	Duct Lumen
Na^+	280	47	450
K^+	5	155	10
Cl^-	270	57	460
mV	0	-83	-15

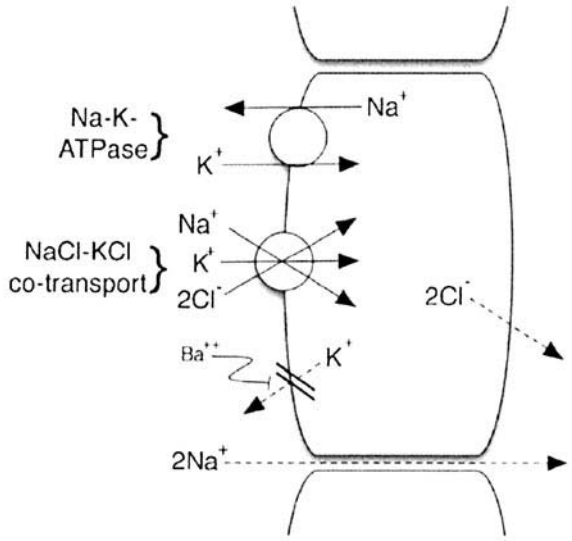

Figure 1. Model of secondary active chloride secretion in shark rectal gland. The motive power for the transcellular movement of Cl across the rectal gland epithelium is supplied by the Na-K-ATPase pump, which pumps Na^+ out of the cell into the blood. Na^+ moves into the cell across the basilateral cell membrane down the electrochemical gradient, through the $Na^+K^+\pm 2Cl^-$ cotransporter, dragging Cl and K^+ with it. Intracellular Cl concentration therefore exceeds that predicted by the Nernst equilibrium equation. When the gland is stimulated to secrete, Cl channels open in the luminal membrane (controlled by the cystic fibrosis transmembrane regulator protein) and chloride exits the cell into the duct lumen. Na^+ moves passively down its electrochemical gradient through paracellular pathways into the duct lumen.

tubules of mammalian kidneys and is thought to be primarily responsible for the slow enlargement of cysts that characterizes the inherited condition of polycystic kidney disease. Agents that inhibit salt secretion by excretory tubules in fish might therefore be used some day to slow the indolent but inexorable swelling of cysts and the progression of renal failure in human patients with polycystic kidneys.

The Utility of Aquatic Species in the Genomic and Postgenomic Eras

Among the most powerful tools of modern genetics is the use of small organisms such at the nematode (*Caenorhabditis elegans*) and fruit fly (*Drosophila melanogaster*) to study the effects of specific genetic mutations during development and in the adult. These organisms are useful because they can be housed in large quantities, they rapidly reproduce, and single genes can be randomly affected by exposure to mutagens followed by large-scale phenotypic evaluation. However, a significant drawback of these animal models is the evolutionary distance between

these organisms and man and the inability to study many aspects of relevant physiology in these distantly related species. In this regard, aquatic species have provided an important compromise allowing for genetic analysis and mutational screens in vertebrates and for a new era of comparative physiology derived from comparative genomics.

The most well-established example involves the use of zebrafish (*Danio rerio*). This small freshwater fish, commonly found in household aquariums, has been used for large-scale genetic mutagenesis screens in laboratories around the world (14), thus expanding the scientific use of aquatic species far beyond the domain of seaside marine biology stations. The first large-scale screens were performed beginning around 1993 using ethyl nitrosourea as a mutagen in the laboratory of Nobel laureate Dr. Christiane Nusslein-Volhard in Tubingen, Germany (14), and independently by Dr. Mark Fishman at Harvard (15, 16). Ethyl nitrosourea causes single base-pair mutations in DNA that can result in altered protein function. These large-scale approaches, and many subsequent targeted screens, have produced hundreds of independent phenotypes that are reproducibly passed to offspring (17). Increasingly complete coverage of the zebrafish genome sequence, led by the *Danio rerio* sequencing project at the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk), and an increasingly dense physical map of the genome are making the identification of mutant genes more straightforward than was previously the case, allowing for the identification of specific genes that cause each disorder and for genotype–phenotype correlations.

Zebrafish reproduce relatively quickly (having about a 3-month generation time) and produce large batches ("clutches") of eggs every few days, year round. They can be easily housed so that large numbers of unique fish can be bred in a relatively small laboratory. The developing embryos mature outside of the mother, making manipulations more accessible and developmental defects more apparent. In addition, the eggs are large and can be easily microinjected with DNA or RNA constructs to cause overexpression or underexpression of proteins. In recent years, the advent of reproducible and convincing data using morpholino antisense nucleotides to "knock down" expression of endogenous genes has become a common technique for assessing the function of a specific gene during embryonic zebrafish development (18, 19). Increasingly sophisticated tools for genetic manipulation are also becoming available, including "knockout" technologies that allow directed deletion or mutagenesis of specific genes, using homologous recombination in embryonic stem cells (20).

In many important ways, organ function and basic aspects of physiology are conserved between fish and human. For example, development and function of the cardiovascular system in zebrafish resemble aspects of these events in man (21). Despite the fact that zebrafish have a two chambered heart and lack lungs and a pulmonary circulation, many genetic programs governing heart and vascular formation are conserved.

The transparency of the developing zebrafish embryo allows for easy observation of morphogenetic events and for evaluation of cardiac function. Recently, transgenic approaches have permitted the labeling of vascular endothelial cells and other structures with green fluorescent protein, making visualization of the vasculature even easier and increasing the sensitivity for detection of mutant phenotypes (Fig. 2) (22). This powerful approach has allowed for the detection of previously unappreciated molecular pathways in angiogenesis and vascular patterning. For example, abnormal patterning of the intersomitic blood vessels was observed in a line of fish after ethyl nitroso urea mutagenesis (23). This mutant was called *out of bounds* because blood vessels crossed intersomitic boundaries. The mutated gene has recently been identified as a member of the semaphorin family of signaling molecules, previously investigated for their role in axonal patterning in the central nervous system (24). This observation suggests that molecular similarities exist between axon guidance and blood vessel patterning. A mammalian homolog of the *out of bounds* gene is expressed by endothelial cells, and inactivation of this gene in mice leads to blood vessel patterning defects and congenital cardiovascular disease (25). Thus, this gene and related family members are implicated as candidates for the cause of adult and congenital cardiovascular disorders.

A growing number of zebrafish mutants serve as models of other forms of congenital and adult cardiovascular disease and offer tractable systems for the elucidation of molecular pathways responsible for pathogenesis. In some cases candidate genes for human disease have been suggested by phenotypic evaluation of mutant lines of fish. For example, the *gridlock* mutant displays a coarctation of the descending aorta and is caused by mutation of a transcription factor gene, *Hey2* (26, 27), which has homologues in man. The *van Gogh* mutant (which displays abnormal development of the otocyst accounting for its colorful name) is caused by mutation of the *Tbx1* gene (28), which is implicated as a cause of DiGeorge syndrome in man (29–31). Another member of the T-box family of transcription factor genes, *Tbx5*, is mutated in the zebrafish *heartstrings* mutant that displays heart and fin defects (32). The human homologue of this gene is responsible for the Holt-Oram syndrome characterized by heart and limb abnormalities (33). These and other examples serve further to legitimize the use of fish to model human disease.

Zebrafish have been especially useful for modeling human disorders of hematopoiesis (34). Transparency of the zebrafish embryo allows for visualization of circulating blood and relatively easy identification of many disorders. As in humans, there is an early primitive wave of hematopoiesis, followed by a more definitive stage. This definitive stage initiates in the dorsal aorta, as it does in mammals. However, in the adult fish, definitive hema-

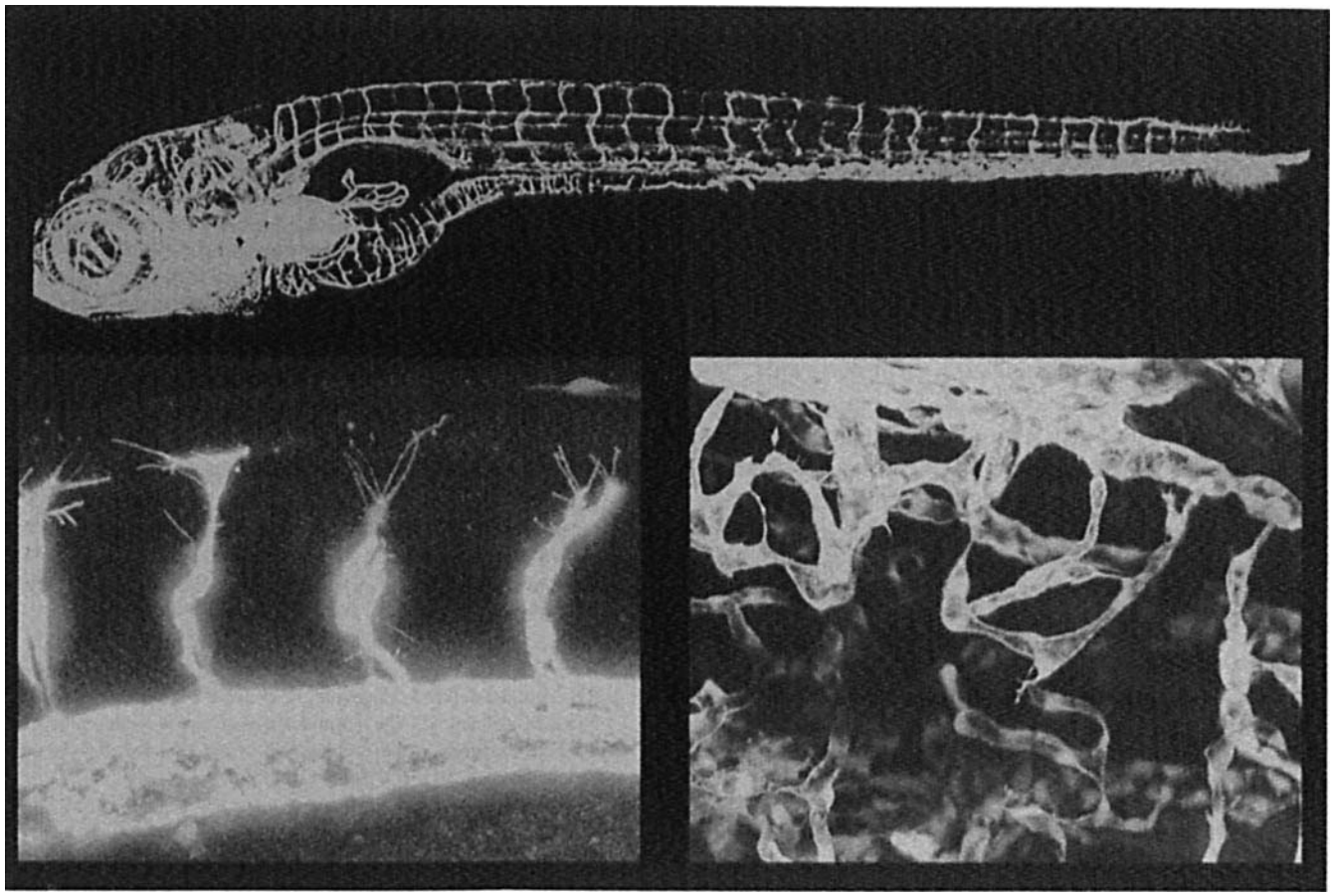

Figure 2. Endothelial cells of the zebrafish vasculature are labeled with green fluorescent protein in transgenic fishes in which the Fli1 promoter directs green fluorescent protein expression. The top panel shows a lateral view of a transgenic fish 7 days postfertilization (dpf). The bottom right shows growing trunk intersegmental vessels at approximately 1.25 dpf (lateral view), and the bottom right shows remodeling vessels in the hindbrain (dorsal view) at about 2 dpf. Images courtesy of Dr. Brant Weinstein.

topoiesis takes place in the kidney as opposed to the bone marrow (35). Large-scale mutagenesis screens in fish have revealed at least 25 distinctive phenotypes affecting hematopoiesis, with individual mutants mimicking human disorders such as hemolytic anemia, porphyria, and leukemia. These models will allow for the identification of molecular pathways affecting hematopoiesis and for the identification of potentially novel therapeutic targets. In addition, there is increasing interest within the private sector for using zebrafish models of human disease for small molecule screens and proof of principle studies relevant to the development of new pharmacologic therapies (36, 37).

Comparative Genomics

The human genome project has taught us that only a small percentage of the DNA sequences that make up our chromosomes encode proteins. The vast majority of the DNA sequences represent regulatory regions, control loci, repeat sequences, and vast regions of undefined or unnecessary sequence. An important task in the postgenomic era is to decipher the meaning not only of coding DNA but of this vast noncoding region.

A lesson that has emerged from comparative biology in the postgenomic era has been the dramatic conservation of protein sequences and structure across evolutionary millennia. Regulatory proteins responsible for orchestrating eye development in the fly are conserved almost in their entirety in humans, where mutations cause blindness (38). This conservation of protein sequence, structure, and function has further validated the use of small animals and diverse organisms as models of human disease. Likewise, more recent analysis indicates that functionally important regions of noncoding DNA are also conserved across evolution. Hence, in the noncoding genomic DNA sequence surrounding the coding exons of many genes, short sequences of DNA (several hundred to several thousand base pairs in length) are highly conserved across diverse species, whereas the intervening sequence bears no resemblance. These conserved regions of noncoding DNA have, in many instances, been demonstrated to function as enhancer sequences regulating the temporal and special expression of nearby genes (39). The pioneering work of Nobel Laureate Sydney Brenner has taken advantage of the genomic structure of pufferfish (*Fugu rubripes*) to provide insight into the functional significance of noncoding DNA (40).

The *Fugu* genome is composed of about 500 million base pairs of DNA, making it about one-eighth the size of the human genome. Nevertheless, both genomes encode approximately the same number of proteins. In the noncoding regions, however, there are marked differences. The human genome is littered with large numbers of repeat sequences that probably arose by viral infection and duplication over the millennia. Amazingly, these sequences comprise between 35% and 45% of mammalian genomes. Repeat sequences are rare in *Fugu* (making up less than 5%–15% of the genome). Hence, the compact *Fugu* genome is dense with important regulatory elements, while "nonsense" or unnecessary sequences are rare. Comparative genomics indicates that noncoding regulatory sequences such as tissue-specific transcriptional enhancer elements are conserved between *Fugu* and man, although in many cases they have been dispersed in the larger human gene loci. The *Fugu* genomic sequence was the first vertebrate sequence made publicly available after the human genome and the ability to identify conserved noncoding regions by comparative analysis has provided a rapid method for identification of critical regulatory sequences amidst the sea of noncoding DNA in the human genome. The zebrafish and medaka (*Oryzias latipes*) genomes are as divergent from human as is *Fugu*, although the genomes of zebrafish and medaka are not as compact. However, technical limitations related to ease of breeding have allowed for the development of transgenic techniques for the *in vivo* analysis of regulatory elements in zebrafish and medaka (41), emphasizing the relative advantages of each aquatic species.

Analysis of the *Fugu* genome has also suggested the existence of previously unrecognized human proteins. The compact and more straightforward genomic structure of *Fugu* has allowed for the prediction of about 31,000 gene loci (similar to the number predicted in the human genome) (40). However, nearly 1000 of the predicted peptides encoded by the *Fugu* coding regions have no identifiable homology to predicted human proteins, although homology at the genomic level can be identified. Careful analysis of teleost fish at the genomic level has predicted novel aspects of human genetics that are likely to lead to productive investigations.

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