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Commentary

Molecular biology of ion motive proteins in comparative models

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Summary

This article will review the utility of comparative animal models in understanding the molecular biology of ion transport. Due to the breadth of this field some 'disclaimers' need to be established up front. 'Comparative' will be defined as non-mammalian. 'Genetic species' will be defined as organisms that have been selected as models for genetic studies and for which the genome has been largely sequenced. 'Non-genetic species' will include other non-mammalian organisms. The review will be limited to ions that play a major role in extracellular (EC) ionoregulation (Na/K/Ca/Cl) and not to micronutrients (Fe) or heavy metals (Cd, Zn). The review will focus only on ion motive proteins that have been associated with vectorial transfer at epithelial tissues. The review is therefore intended as a guidepost to researchers new to the field as well as to inform biologists of the power of comparative genomics.

Key words: ion, transport, channel, pump, cotransporter, antiporter.

Introduction

Readers are directed to any introductory cell biology text (Cooper, 2000) to review the basic cellular processes of ion translocation and the molecular architecture of the key transmembrane proteins. Very briefly, ion regulation in eukaryotes is achieved primarily at transporting epithelial tissues (gill, kidney, gut, skin etc). Epithelial cell membranes are polarized into apical (outer, facing the exterior or lumen) and basolateral domains [inner, facing the extracellular (EC) fluid]. Movement of ions can occur either passively (down a concentration gradient) through facilitated diffusion *via* channels, or actively (against the concentration gradient), driven by ATP hydrolysis or ion gradients. Active transport driven by ATP hydrolysis is exhibited by ion pumps. Active transport against a concentration gradient can also be driven by ion gradients; cotransporters move ions in the same direction whereas antiporters (exchangers) move ions in opposing directions. Ion motive proteins that have been sequenced in non-mammalian species can be identified through searching for key phrases using the available internet resources listed in Table 1. Taxon-specific databases are also provided, since many of the genes for genetic species have not yet been annotated in GenBank. The taxonomy browser of NCBI enables readers to examine the phylogenetic distribution of known genes in particular gene families. Another resource, Interpro, shows sequence number phylogenetically, and Gene Ontology offers a controlled vocabulary for gene function. In order to write this article 160 published sequences with confirmed identities as epithelial ion motive proteins in non-mammalian species were reviewed. This represents a subset of the available sequences for epithelial ion motive proteins in non-mammalian species, since many await annotation and curation.

Channels

Channels are open pores that allow free passage of the ion, based on appropriate charge; they are not permanently open but are gated, typically by ligands causing an allosteric change in the molecules that result in opening. Proteins in this category include the epithelial Na^+ channel (ENaC), the epithelial Ca^{2+} channel (ECaC), the Cl^- channel (ClC) and the cystic fibrosis transmembrane conductance regulator (CFTR, an apical, c-AMP regulated, low conductance Cl^- channel).

Pumps

Proteins in this category include Na^+/K^+-ATPase (the Na^+ pump), H^+/K^+-ATPase, the vacuolar H^+-ATPase (V-ATPase) and a range of Ca^{2+}-ATPases on both plasma (PMCA) and sarcoplasmic/endoplasmic reticulum (SR/ER) membranes (SERCA). The well-studied basolateral Na^+/K^+-ATPase transports and maintains an unequal distribution of Na^+ and K^+ (3 Na^+ out and 2 K^+ in per ATP molecule hydrolyzed) across the plasma membrane of nearly all eukaryotic cells, serving an essential role in maintenance of osmotic balance and membrane potential. It is worth mentioning that this protein, arguably the best studied of all ion motive proteins, was originally described in nerves of the shore crab *Carcinus*

Table 1. *A sample of internet resources to support comparative genomics*

Internet resources	Host	Utility/Purpose
General genomic resources		
www.ncbi.nlm.nih.gov	National Center for Biotechnology Information (NCBI), National Institutes of Health (NIH) USA	A resource for molecular biology information (GenBank). The Taxonomy browser has direct links to model organisms
www.geneontology.org	Gene Ontology Consortium	To enable a controlled vocabulary for gene function
www.ebi.ac.uk/interpro	European Bioinformatics Institute (EBI)	Can monitor progress on sequencing projects
www.ensembl.org	European Molecular Biology Laboratory (EMBL)-EBI and Sanger Institute (Wellcome Trust)	Maintains automatic annotation on metazoan genomes
www.sanger.ac.uk/projects	Sanger Institute (Wellcome Trust)	Monitor progess on sequencing projects
Taxon specific resources		
www.wormbase.org	NIH, USA; Medical Research Council (MRC) UK	*Caenorhabditis* sp.
www.flybase.org	NIH, USA; MRC, UK	*Drosophila* sp.
www.fruitfly.org	Berkeley Drosophila Genome Project	*Drosophila* sp.
www.ensembl.org/Anopheles	EBI-Sanger	*Anopheles gambiae*
www.bioinformatic.ksu.edu/Beetlebase/	Kansas State Univ (NIH)	*Tribolium castanum*
www.ab.a.u-tokyoac.jp/silkbase	Japanese Society for Promotion of Science	*Bombyx mori*
genome.jgi-psf.org/ciona4/ciona4.info	Joint Genomics Institute (JGI) USA Department of Energy (DOE)	*Ciona intestinalis*
genome.jgi-psf.org/fugu/	JGI; DOE	*Fugu rubripes*
www.fugu-sg.org/	Institute of Molecular and Cell Biology (ICMB)	*Fugu rubripes*
www.zfin.org	NIH	*Danio rerio*
genome.jgi-psf.org/xenopus/	JGI	*Xenopus tropicalis*

(Skou, 1957). Since then its three subunits (α, catalytic; β, targeting; γ, regulatory) have been characterized in a range of species. The apical V-ATPase was initially discovered in insect vacuolar membranes; subsequently its role was broadened phylogenetically and it is now recognized as an important energizer of apical plasma membranes in the same way that the Na^+ pump energizes the basolateral membrane in transporting epithelia (Wieczorek et al., 1999). V-ATPases are known to acidify/alkalinize EC spaces of polarized epithelia. In terms of Ca^{2+} pumps, sequence analysis has focused on the SERCA pump, initially because of its abundance in muscle. Subsequently it has been shown that SERCA plays an important role in regulation of intracellular (IC) Ca^{2+} (sequestration in SR/ER), allowing vectorial bulk flow through epithelial cells without toxic effect. The basolateral PMCA regulates cytosolic Ca^{2+} levels by transporting it against its electrochemical gradient using energy from hydrolysis of ATP. The H^+/K^+-ATPase transports K^+ inward and H^+ outward, leading to IC alkalinization and EC acidification.

Cotransporters

Cation–chloride cotransporters perform a variety of physiological functions including ion and cell volume regulation (Mount et al., 1998). The electroneutral Na^+/Cl^- cotransporter (NCC) is associated with Cl^- absorption. The $Na^+/K^+/2Cl^-$ cotransporter (NKCC) effects net inward salt transport in response to cell shrinkage. The K^+/Cl^- cotransporter (KCC) couples movement of K^+ and Cl^-. It is normally a net efflux pathway using the favorable K^+ chemical gradient maintained by the Na^+ pump to drive Cl^- out of the cell. The Na^+/HCO_3^- cotransporter (NBC) effects acid–base regulation.

Antiporters/exchangers

The antiporters reviewed for this article include the Na^+/H^+ exchanger (NHE), the Na^+/Ca^{2+} exchanger (NCX), the K^+-dependent Na^+/Ca^{2+} exchangers (NCKX), the Cl^-/HCO_3^- exchanger and the Na^+-dependent anion exchanger (NDAE). The NHE is a reversible electroneutral antiporter that functions in pH homeostasis, cell volume regulation and transepithelial Na^+ transport. The NCX regulates IC Ca^{2+} at or below 100 nmol l^{-1} using the transmembrane Na^+ gradient as an energy source. NCXs are divided into two families (NCX and NCKX), both of which are reversible. They usually couple high external Na^+ (NCX, $1Ca^{2+}$ for $3Na^+$, electrogenic) or high external Na^+ and high internal K^+ (NCKX, $1K^+ + 1Ca^{2+}$ out for $4Na^+$ in) to transport Ca^{2+} out of the cell. Anion exchangers include $Cl^-/OH^-/HCO_3^-$ (pendrin), and the NDAE.

Unifying characteristics of ion motive proteins: diversity, versatility and interdependence

Ion motive proteins exhibit diversity, versatility in function and, in many cases, interdependence. Some can transport alternative ions. An example would be the invertebrate electrogenic brush border $2Na^+$-$1H^+$ antiporter that can transfer

both monovalent and divalent cations as needed (Ahearn, 1996). Vectorial transfer of an ion typically involves coordination of ion motive proteins on different cellular domains. For example, net Na^+ uptake will involve apical NHE, ENaC and NKCC in series with the basal Na^+ pump. $Cl^–$ secretion will involve basal $Cl^–$ entry by NKCC and $Cl^–/HCO^–_3$, followed by exit through apical $Cl^–$ channels. Regulating IC Ca^{2+} involves coordination of ECaC, SERCA and PMCA. Cotransporters/antiporters that transport multiple species coordinate larger constituencies of ions. For example the NKCC routinely operates in conjunction with the Na^+ pump, K^+ channel and $Cl^–$ channel. Similarly the NDAE mediates the transport of $Cl^–$, Na^+, H^+ and $HCO_3^–$.

Certain ion motive proteins are ostensibly more important in the cellular hierarchy than others. For example, the Na^+ pump establishes the electrochemical gradients used by apical and basolateral mechanisms such as NHE, NKCC and various ion channels. Likewise the apical V-ATPase, through establishing an electrochemical H^+ gradient, drives Na^+ uptake through apical ENaC or NHE. Ion motive proteins often accomplish physiological functions other than ion regulation. For example ENaC, in addition to mediating apical Na^+ uptake, may serve in mechanotransduction (Goodman and Schwartz, 2003). In addition to mediating transepithelial $Cl^–$ transport, $Cl^–$ channels are involved in cell volume regulation, stabilization of membrane potential, endocytosis and charge compensation necessary for acidification of IC endosomes. In addition to being intimately linked to acid–base balance, V-ATPases can energize fluid secretion.

Most ion motive proteins have multiple isoforms, often targeted to different cellular domains and with different function. For example there are typically two NKCC isoforms in vertebrates, one located basolaterally that is involved in volume regulation and NaCl secretion, and a second located apically and involved in NaCl uptake. A second example would be vertebrate NHE, where one isoform (NHE1) is located on the basolateral membrane and serves in IC pH regulation and volume regulation, whereas the apically located isoforms (NHE2/3) are involved in Na^+ reabsorption and proton secretion.

Comparative physiology of ion regulation

The August Krogh principle (Krogh, 1929) has historically provided the best rationale for the field of comparative biology, namely that nature has already invented an ideal natural system/species for the study of physiological processes. Homeostasis has been explored by examining organisms that have adapted to extreme environments through possession of unique tissues, or exaggerated responses. In other cases comparative models have been selected because they resemble a human process.

Adaptation to environment

Comparative models have historically informed the field of ion regulation because non-mammalian organisms occupy a diversity of ionic environments ranging from the Dead Sea (salinities several-fold that of seawater, SW) to inland freshwaters (FW) and, in so doing, encounter unusually large ionic gradients. The gills of aquatic species (particularly fish and crustaceans) have become popular models for ion exchange due to issues of experimental accessibility and simplicity. Teleost and elasmobranch fish have received particular attention primarily because they are the oldest group of vertebrates (500 million years, MY). Comparisons between related aquatic species that inhabit environmental extremes can yield important mechanistic information (FW crayfish *vs* SW lobster; FW *vs* SW teleost fish). Euryhaline species possessing the ability to move between different ionic environments (the euryhaline killifish *Fundulus heteroclitus* or the portunid crab *Callinectes sapidus*) have engendered significant interest, particularly those that routinely migrate as part of their catadromous life history (for example migratory *vs* non-migratory eels).

Mimic biomedicine

Other non-mammalian models have emerged due to unique anatomical structures or processes that could be easily extrapolated to a given human process. Examples of this would be: the urinary bladder of the toad serving as a model for the mammalian renal collecting duct; the shark rectal gland and the avian salt secreting gland that both mimic mammalian salt secreting epithelia; and the killifish skin and gill epithelium that both mimic the mammalian airway epithelium.

Extreme responses/anatomy

Other models have gained popularity based on some extreme response or anatomical feature. In our laboratory, the moulting cycle of the aquatic crustacean has emerged as a model to study Ca^{2+} transporting proteins and the genes that encode them. The postmoult stage of the FW crayfish *Procambarus clarkii* has presented a unique temporal ‘light switch’ for up/downregulating the Ca^{2+} motive proteins involved in transepithelial Ca^{2+} fluxes required for rapid cuticular remineralization (Wheatly, 1997). Other laboratories have extended the model to terrestrial isopods (*Porcellio scaber*; Zeigler, 2002) that, in an effort to conserve Ca^{2+} in terrestrial environments, moult the posterior and anterior cuticle sequentially, necessitating both temporal and spatial regulation of Ca^{2+} transport. In other cases the model has been built upon some unique cellular or tissue attribute. For example the *Xenopus laevis* oocyte has emerged as a model system for the heterologous expression of a range of ion channels and transporters, primarily because the cell diameter (1 mm) is accessible for micropipettes/electrodes. The simplified anatomy of the teleost renal tubule enables *in vivo* studies of intact tubules due to the direct access to the renal portal system. Other comparative models have been structured around a high abundance of a particular ion motive protein (for example, the Na^+ pump and NKCC in shark rectal gland), which have led to the discovery of orthologues in humans.

Not surprisingly, many of the comparative species for which

sequence data are available are organisms that have emerged historically as models for ion transport. In some cases the molecular sequence of the ion motive protein was first cloned in a comparative model. An example would be the electroneutral NCC, which was first cloned and sequenced in the urinary bladder of the winter flounder (Gamba et al., 1993). In other cases sequences have emerged almost serendipitously. For example, SERCA was initially selected for study by fish researchers as a way to differentiate muscle fiber types rather than for any intrinsic interest in Ca^{2+} regulation (Tullis and Block, 1996). Researchers using *Artemia* as a model to study regulation of gene expression during embryonic development also selected SERCA because the gene was abundant in the only accessible tissue in such a small organism (Escalante and Sastre, 1994). Not surprisingly, many ion motive proteins were first cloned in mammalian models (ECaC, PMCA, SERCA, NHE), largely reflecting the size of the research community and the application to human health.

Comparative genomics of ion regulation: utility of genetic model organisms

As rapidly, highly accurate sequencing techniques have become readily available, the genomes of several species have been completely sequenced within the space of a few years. A genome sequencing project typically involves multiple sequencing centers in several countries. Many of the available sequences for epithelial ion motive proteins originate from these large-scale sequencing projects. Genetic model species are typically those that are genomically defined (i.e. the genome has been sequenced) and genetically tractable (i.e. transgenic technology is available). At the time of writing, the non-mammalian species for which complete (or draft) genomes are available or in progress would include the nematodes, *Caenorhabditis elegans* and *C. briggsiae*; the fruit fly, *Drosophila melanogaster;* the mosquito *Anopheles gambiae;* the ascidian *Ciona intestinalis*, the zebrafish *Danio rerio* and the puffer fish *Fugu rubripes*. Taxon-specific internet databases typically support a community of researchers (see Table 1). These species, and not mouse, have emerged as organisms of choice for genetic studies due to their small size, short generation time, high reproductive rate and cost-effective husbandry.

The soil nematode *C. elegans* genome (97 Mb genomic sequence, encoding over 19 000 genes) was the first to be completely sequenced and published (The *C. elegans* Sequencing Consortium, 1998) as a collaboration between the Sanger Center (Wellcome Trust, UK) and the Genome Sequencing Center at Washington University School of Medicine, USA. In the 1960s Sydney Brenner recognized that this primitive species could provide a useful model for the genetics of development and neurobiology in humans. The worm is conceived as a single cell, which undergoes embryonic cleavage, morphogenesis, development, nerve function, behaviour and aging – in a nutshell encompassing all the mysteries of modern biology. In the case of *C. elegans*, developmental studies are simplified because the identity, position, lineage and fate of every somatic cell (959 in total) are known through embryogenesis and larval development. Not only are genetic and reverse genetic screens possible, but techniques such as double-stranded RNA-mediated gene interference also allow researchers to phenocopy a null allele in the space of days. This metazoan is unique in that it can be grown and genetically manipulated with the speed and ease of a microorganism, yet it offers many features common to higher organisms.

The *C. elegans* sequence was followed within a year by the completed genome of the fruitfly, *Drosophila melanogaster*, a species that had been employed as a genetic model for almost a century. The *D. melanogaster* genome was sequenced as a collaboration between Celera and Berkeley Drosophila Genome Project and was first published in 2000 (Adams et al., 2000). As with *C. elegans*, *Drosophila* has a genetic system that is easy to manipulate, can be maintained at relatively low cost, and affords biological complexity that is believed to parallel mammals.

By comparison, the *Anopheles* genome project attributes its origins less to an existing community of genetics researchers and more to the role this species plays as vector in the worldwide spread of malaria. The need to control the spread of this disease and to develop improved antimalarial drugs and vaccines has fuelled this project. The genome sequence was a collaboration between several sequencing centers and was supported by the World Health Organization (Holt et al., 2002).

The sea squirt *Ciona intestinales* initially appears to be a most unlikely candidate for genomics; however it transpires that it has the smallest genome (estimated size 155 Mb) of any manipulable chordate and provides the opportunity to explore the evolutionary origins of the chordates (over 550 MY) through easily visualized cells and transient transgene expression. A draft of the genome was published by Joint Genomics Institute, US Department of Energy (Dehal et al., 2002).

Likewise the Japanese puffer fish *Fugu* (or *Takifugu*) *rubripes* has emerged as a model genetic species since it possesses one of the smallest genomes of all vertebrates (400 Mb, or one eighth the size of the human genome), attributable to a compactness of introns, intergenic distances and marked reduction of repetitive DNA sequences. A preliminary analysis of the genome is reported in *Science* headed by the Joint Genomics Institute (Aparicio et al., 2002). It was the first vertebrate genome available following the sequencing of the human genome in 2001. Sequence comparison will enable interpretation of the human genome, assist drug discovery and inform the evolution of vertebrate genomes over the 450 MY since the species diverged from a common ancestor.

A powerful combination of genetics and embryology has established the zebrafish *Danio rerio* as an important model organism for the analysis of vertebrate development, physiology and behavior. The transparency and external

development of the embryo allow exquisite manipulations such as dye-labelling, transplantation and *in vivo* time-lapse imaging that illuminate the function of mutated genes at the cellular level.

The zebrafish sequencing project was commenced in 2001 at the Sanger Institute and the completed genome sequence is anticipated at the end of 2005. With a genome that is half the size of mammalian models (1.6–1.7 Gb), it promises to serve as a model for human biology and disease.

In addition to the large-scale genomic sequencing projects, EST (expressed sequence tags) and cDNA sequencing projects are becoming available for a growing number of additional species. Within the Arthropoda this would include several insect species that are associated with disease transmission [such as the mosquitos (*A. aegypti*, Dengue fever; *A. albopictus*, Dengue fever; *A. triseriatus*, encephalitis; *Culex pipiens*, West Nile virus) the tick, *Amblyomma americanum* and the tsetse fly *Glossina morsitans*, sleeping sickness] or have agricultural importance (such as the honey bee *Apis mellifera*, the silkworm *Bombyx mori* and the beetle *Triboleum castanum*). Additionally two frog species are being studied as models of vertebrate embryonic development (*Xenopus laevis* and *X. tropicalis*). Just like the large scale genomics projects, these also support emerging internet resources (Table 1).

As genomes of additional model species are sequenced (at the predicted future rate of several each year), around several thousand putative 'transport' proteins (as much as one fourth of the genome) are deposited into the genomics databases at one time. While the genomics data are expanding exponentially, annotation is following at a snail's pace. Genes are often assigned to gene families on genome-specific databases where they await confirmation of gene identities for inclusion in the GenBank. Even so the available sequences offer great opportunities for homology-based cloning, especially for researchers who study either arthropods or chordates.

In most cases the nucleotide sequences of the ion motive proteins of genetic species have resulted as by-products of the genome sequencing project and less for any intrinsic value in furthering the understanding of ion transport. A good example of this would be the early cloning of SERCA in *Drosophila* that was rapidly followed by its gene mapping to the right arm of chromosome 2 at band 60A-B (Magyar and Varadi, 1990). Subsequently the gene was characterized based on accessibility of genomic libraries (Magyar et al., 1995). On the other hand, since these genome projects are spread widely through the animal kingdom, it has facilitated the work on non-genetic species since it is now possible to identify candidate cDNAs in the closest genetic organism. In some cases sequences from genetic models have led to the mammalian equivalents. A good example would be the NDAE, which was first discovered in *Drosophila* (Romero et al., 2000) and may assist in the molecular identification of other cation and anion-coupled HCO_3^- transporters. Its discovery in *Drosophila* has enabled genetic manipulation, suggesting that disruption is lethal.

Role of genetic organisms in the post-genomics era: closing the phenotype gap

Genome projects have focused initially on high-throughput sequencing and on large-scale target gene selection (chip technologies). Functional genomics can be defined as the functional analysis of individual candidate genes in the context of an organism's genome. The way this is most expediently done is through reverse genetics (deduction of gene function by analysis of corresponding mutant phenotypes). It relies on the presence of a homologous gene in a model organism in which a knock-out can be created. In the mouse gene knock-outs are often lethal so early in development that they cannot contribute to a functional analysis of candidate genes. The genetic models will continue to be the organisms of choice as integrative physiologists attempt to interpret how gene products function in cells, tissues or organisms, a process referred to as 'closing the phenotype gap'.

An excellent review by Dow and Davies (2003) illustrates how the extensive genomic resources of *Drosophila* have already informed the structure, function and control of epithelial ion transport in the insect Malpighian tubule, a tissue with functional similarities to the mammalian kidney. For example, they describe how reverse genetics in *Drosophila* illuminated the role of V-ATPase through a knock-out that enabled characterization of expression patterns in tissues and cell types. Further, they illustrate how enhancer trapping (ET) technology can be used to detect tubule-specific enhancers in the genome that can provide insight into the spatial organization of this tissue. Put simply, an ET element is a transposable DNA element (usually a P element) that inserts at scattered sites throughout the genome close to an endogenous enhancer of gene expression. For enhancer trapping, a P element is used in which the transposase gene has been replaced by three elements: a visible marker enabling new insertions to be tracked through crossing schemes (white+, for example, confers red eye color in a white-genetic background), a reporter gene (such as *lacZ*) whose expression is readily visualized, and an *E. coli* origin of replication to facilitate plasmid-rescue of the flanking sequences. Without a source of transposase the element is trapped within the genome; this enzyme can be provided by crossing flies carrying the P-element to another line in which it has been inactivated. 'Jump-starters' are flies (typically males) that carry both the ET element and a source of transposase. By breeding true from thousands of progeny of such a male, it is possible to look for interesting patterns of expression of the reporter gene in the tissue of interest. Those authors screened 1500 lines with the P{GATB} transposon and discovered 20 lines that were subsequently used to identify genes of interest in the Malpighian tubule (much akin to a differential cDNA library screen). The technique has subsequently illuminated the regional specialization and multiple cell types in this important epithelial tissue.

A second example from *Drosophila* would be the parallels between the insect tracheal system and the mammalian airways. Both consist of branching networks of tubular epithelia

delivering oxygen to respiring cells, which must convert during development from liquid- to air-filled systems through the common mechanism of active salt absorption using ENaC. As such, the genetic model *Drosophila* has served as an excellent non-mammalian species to better understand the role of ENaC during the water-to-air transition (Liu et al., 2003).

Two examples can be used to illustrate how *C. elegans* research has informed the physiology of ion motive proteins, specifically ClC and NHE. One of the advantages of *C. elegans* as a model system is that both genetic and reverse genetic screens are accessible. Researchers can rapidly generate cell-specific antisense inhibition of a given ClC isoform or employ RNAi knockdown of message levels along with *in situ* patch clamp of the worm, enabling them to answer very specific questions about the role of ClC such as expression patterns and properties of the individual isoforms (Schriever et al., 1999; Nehrke et al., 2000). *C. elegans* has also contributed to the identification and understanding of NHE homologues (Nehrke and Melvin, 2002), specifically whether certain isoforms are expressed at the cell surface or on membranes of IC organelles and whether the particular characteristics of an isoform may be tailored to the functions exclusive to a single cell type.

Role of non-genetic organisms in the post-genomics era: comparative, environmental and evolutionary genomics

Over the years the comparative physiology community has been somewhat marginalized due to the misconception that 'primitive' animal models lacked relevancy to human health and disease due to lack of conservation of body plan. However, recent advances in genomics have verified that the human genome is remarkably similar to those of evolutionarily ancient organisms. Importantly, comparative sequences from evolutionarily distant species now have the potential to build a conceptual framework for interpretation of the mammalian sequences. This leads several authors to conclude that the comparative approach will be increasingly valued by the biomedical community in the postgenomic era (Ballatori and Villalobos, 2002; Dow and Davies, 2003). Comparative genomics of non-genetic species will be used to inform the function of gene products as well as their evolution.

Aligning differences in function with differences in sequence

In situations where the mammalian sequence is already known, cloning and sequencing the same ion motive protein in an evolutionarily distant species can provide valuable information, particularly concerning differences in functional characteristics or sequence homology. A good case study to illustrate this point would be the NCX (He et al., 1998). NCX was first physiologically characterized in squid giant axon. In due course the canine cardiac NCX was sequenced (Nicoll et al., 1990). What is the value in now cloning the NCX from squid axon? While some of the basic properties of the squid and canine cardiac NCX are conserved [stoichiometry, Na^+-dependent inactivation, secondary activation by cytoplasmic Ca^{2+}, and deregulation by chymotrypsin, stimulation by ATP and phosphatidylinositol (4,5)-bisphosphate (PIP_2)], there are important differences between the two. In squid the ATP dependence involves phosphorylation by protein kinase whereas in canine cardiac NCX it reflects generation of PIP_2 from phosphatidylinositol. Further ATPγS activates the squid NCX but not canine cardiac NCX. Cationic agents that bind anionic lipids inhibit the canine cardiac NCX but agents like pentalysine do not inhibit the squid NCX. The squid NCX is regulated by a phosphoarginine-dependent process that may involve protein kinases unique to invertebrates whereas it has no effect on canine cardiac NCX. In squid the Ca–Ca exchange operation of the NCX is voltage dependent while the Na–Na exchange is not. In sum, there are interesting functional differences between the NCX proteins of these two species.

When the squid NCX was sequenced it exhibited about 58% identity with canine cardiac NCX and regions determined to be of functional importance were well conserved [specific acidic residues within the binding site for regulatory Ca^{2+}, endogenous exchanger inhibitory peptide (XIP) region involved in Na^+-dependent inactivation, predicted topology]. Sequence conservation was highest in the proposed transmembrane segments 2, 3, 8, 9 (the α repeats) consistent with a catalytic role of the hydrophobic domains in ion translocation. However there was a noticeable deletion of 47 residues in the large IC loop. In mammalian NCX this region displays extensive alternative splicing. Transmembrane region (TM) 11 is the least conserved domain, suggesting that the C terminus plays a lesser role in exchanger function. Also the N terminus, which represents a signal peptide region, is poorly conserved among NCX proteins. The molecular basis of differences in the voltage dependence of canine cardiac NCX and squid NCX can now be pursued by the combined methods of molecular biology and electrophysiology.

Tracing the evolution of important gene families

Comparative genomics of species separated over evolutionary time can also assist in reconstructing the history of gene families. An example from our own work would be the evolution of SERCA pumps (Wheatly et al., 2001). In vertebrates there are three homologous alternatively spliced genes that encode five isoforms. By comparison in invertebrates a single gene transcript has been identified in *C. elegans* (Cho et al., 2000), *Drosophila* (Varadi et al., 1989; Magyar et al., 1995), *Artemia* (Palmero and Sastre, 1989; Escalante and Sastre, 1993) and *Procambarus* (Zhang et al., 2000; Chen et al., 2002). In nematode, brine shrimp and crayfish, two isoforms originate from this transcript. In *C. elegans* both isoforms appear to be essential for embryonic development and post-embryonic growth and survival. In brine shrimp and crayfish the two isoforms are homologous to vertebrate SERCA2a and SERCA2b, and originate by the same alternative splicing mechanism. Put simply, the final six amino acids of one isoform are replaced in the other isoform by an extended C terminus of 30 residues, possessing hydrophobic properties that could potentially form an additional transmembrane domain. There is no evidence to show that the carboxyl terminus of a Ca^{2+} pump

is vital to function and yet diverse species have conserved alternative SERCA2a and b termini through millions of years, suggesting that there is some selective advantage to retaining multiple isoforms. Before the ancestors of crustaceans and vertebrates diverged 600 MY ago, they probably shared the same ancestral SERCA gene with a charged C terminus that gave rise to three homologues (SERCA1, 2, 3) in vertebrates; the same alternative splicing mechanism was retained in the invertebrate gene and in the SERCA2 vertebrate gene while it was lost in the other two homologues (SERCA1 and SERCA3). The vertebrate genes for SERCA1 and 2 have unique promoters for transcription of the two isoforms encoded by each gene. The generation of one or other isoform is dependent upon the processing of the last exons of each gene that has been shown to be tissue-specific for SERCA2. In *Artemia* the expression of the two protein isoforms is regulated at the transcription initiation step (Escalante and Sastre, 1995). There are two different promoters that independently regulate the expression of each isoform. A second mechanism is the differential processing of the last two exons of the gene, which is also tissue specific, and this mechanism has obviously been conserved between *Artemia* and vertebrates.

The reverse is also true, namely that the analysis of ancient protein families can aid our understanding of phylogeny. To continue with the case of the SERCA pumps, an analysis by Hagedorn et al. (2003) confirmed that the sequences for the malacostracans *Porcellio* and *Procambarus* are more closely related to insects than to the branchiopod crustacean *Artemia*. The further study of other sequences from arthropods may help to resolve the controversy about the monophyletic origin of Crustacea. Some hypotheses have suggested that the Branchiopoda branched off the main stem of Crustacea before the insects.

The phylogenetic analysis of ion motive proteins can be useful for biomedicine. An example of this would be cystic fibrosis, which is a common lethal autosomal recessive disease caused by mutations of the CFTR gene. Sequence comparison can illumine the structure–function of the protein, which will facilitate interpretation of the identified mutations in the gene. A recent analysis by Chen et al. (2001) of 16 full-length CFTR amino acid sequences from a wide range of vertebrate species representing up to 420 MY of evolution, has enabled a functional R domain to be defined (phosphorylation of this domain regulates channel activity), redefined the boundaries of the two nucleotide-binding domains and the C-terminal tail, provided insights into the differential roles of the two halves of CFTR, and identified several well-conserved motifs that may be involved in inter- or intramolecular interactions. Such phylogenetic analysis will enable appropriate animal models to be selected for further in-depth analysis of complex CFTR defects, possibly leading to a better treatment of the disease.

Environmental genomics: aligning molecular biology with environmental need

The application of the Krogh principle will persist into the postgenomics era as researchers seek to learn how the molecular biology of an ion motive protein has evolved to fit environmental need. As in the past, organisms that are suited for life in hostile or unusual environments will inform the general understanding of these important proteins.

An example of this would be the ability of rainbow trout *Oncorhynchus mykiss* to maintain heart contractility under hypothermic conditions (4–15°C) that would be cardioplegic to mammals. Differential temperature dependencies in the mammalian *vs* teleost NCX are due to important differences in the primary structure of the isoforms (Xue et al., 1999). Knowledge of the sequence and biophysical properties of trout heart NCX can contribute to the understanding of the evolution of the NCX over 400 MY.

Comparisons would not necessarily be restricted to organisms separated so widely in evolutionary history. Closely related organisms that have ion motive proteins with different functional characteristics can be equally informative. An example would be the comparison between SERCA sequences in a freeze-tolerant frog, *Rana sylvatica*, *vs* a related cold-intolerant species, *Rana clamitans*. Muscle contractility at low environmental temperatures in *R. sylvatica* has been attributed both to functional (exhibits double the activity at 0°C and a lower activation energy below 20°C) and structural differences in SERCA1 (Dode et al., 2001). Sequence analysis revealed that *R. sylvatica* has two amino acids in SERCA1 that are unique, three of which are located in the ATP binding domain. These differences may shed light on the temperature dependence of ATP hydrolysis, kinetics with ATP, Ca^{2+} cooperativity and protein–protein interactions.

Regions of sequence difference cannot always explain the functional differences between molecules operating in different environments. For example CFTR sequence analysis has benefited from a comparison of amphibians and mammals (Price et al., 1996). In amphibians, which are adapted to living in FW, CFTR is located in the apical membrane of skin and urinary tract. Anion selectivity and pharmacological profile are both different to that of human CFTR. The R domain is the most divergent region between the two sequences, although phosphorylation sites responsible for protein kinase (PKA)-dependent regulation are conserved within the R domains. Much of the R domain can be deleted without disrupting channel activity. In this case differences in sequence obviously do not prevent correct folding and association between different domains.

In other cases sequence differences may reflect a combination of evolutionary history and/or environmental adaptation. For example the low sequence homology of the ray Na^+ pump α subunit with those of teleost fish, amphibians, birds and mammals may be due either to evolutionary age of the elasmobranch class of fish or, alternatively, to structural adaptation of these proteins to function in the presence of high circulating urea concentration (Cutler et al., 1995).

Providing technical advantage in spatial and temporal expression analysis

To come full circle, just as comparative models have aided

the early understanding of ion regulation, they will continue to provide technical advantages in understanding expression patterns of gene products, largely because of experimental accessibility.

A good example would be the study of ClC, whose functional characteristics are poorly understood. Chloride channels (ClC5) in both *Xenopus* and human cells have similar functional properties. Amphibian renal A6 cells have proved useful in studying trafficking and functional regulation of ClC-5. Mo et al. (1999) compared ClC-5 in amphibian renal A6 cells with human cells using isogenic constructs consisting of an open reading frame subcloned into an optimized *Xenopus* expression vector. The *Xenopus* clone resulted in strong rectifying outward currents that were not affected by Cl^- channel blockers. The anion conductivity sequence was NO_3^->Cl^->I^->HCO_3^-. A reduction in EC pH inhibited outward currents. Since ClC are often colocalized with H^+-ATPase in IC vesicles below the brush border, it has been suggested that these channels facilitate the acidification of endosomes (route for Cl^- influx as a counterion to H^+ transport).

Some unique expression property of a given tissue/cell type may dictate the need for a comparative model system in gaining insight into functional genomics. For example, the urothelium of *Bufo* can coexpress different Na^+ pump β subunit isoforms (Jaisser et al., 1992), only one of which is regulated by aldosterone. This model can assist in dissecting out the physiological relevance of different isoforms. A second example would be the existence of two populations of intercalated cells in turtle bladder. The α cells (acidifying) express apical V-ATPase that drives H^+ excretion and HCO_3^- reabsorption *via* a Cl^-/HCO_3^- exchanger; the β cells (alkalinizing) express basolateral V-ATPase involved in HCO_3^- excretion and Cl^- uptake *via* an apical Cl^-/HCO_3^- exchanger (Stetson and Steinmetz, 1985).

Expression of many ion motive proteins is being advanced through continued examination of aquatic species inhabiting a range of ionic environments. The simplicity of the gill can enable experimental approaches not afforded by 'higher' organisms. For example, a range of fishes with differing abilities to ionoregulate (*Myxine glutinosa*, *Raja erinacea*, *Fundulus heteroclitus*) are being used to further explore NHE expression trends (Edwards et al., 2001; Choe et al., 2002). Similarly a spectrum of crustaceans with different ionoregulatory abilities are being used to better understand the regulation of expression of V-ATPase (Weihrauch et al., 2001) and Na^+/K^+-ATPase (Towle et al., 2001).

NHE has been selected to illustrate how comparative species continue to advance our understanding of physiological function, location of isoforms and mechanisms of regulation. Claiborne et al. (1999) has utilized a range of teleost species to explore the competing demands placed on NHE for Na^+ transport and acid–base balance, depending upon the location of the isoform (apical NHE2 *vs* basal NHE1). So, for example, metabolic acidosis caused a decrease in expression of basal NHE1 in sculpin to enhance net H^+ transfer to the water *via* the apical NHE2 isoform. While Na^+ uptake across the gills may appear inappropriate when in SW, it constitutes only a portion of total Na^+ influx and may be worth the additional energetic costs to maintain acid–base balance. A third isoform, β-NHE, is involved in IC housekeeping (pH homeostasis and volume regulation). This isoform is activated by cAMP, which inhibits activity of apical NHE isoforms and does not affect those on the basolateral membrane. These differences in regulatory mechanisms can be explained either by the existence of different isoforms or the fact that there is one form of the exchanger and that differences in regulation are dictated by the cell type and different signaling networks. Parallel studies in crustaceans have revealed an NHE3-like isoform in gills of the crabs *Carcinus* and *Callinectes* (Towle et al., 1997) that may equate to the electrogenic exchanger ($2Na^+/1H^+$) documented in physiological studies. In the case of crustaceans there is evidence for both electrogenic and electroneutral NHE on both the apical and basolateral membranes.

Colocalization in comparative species can also shed light on the interdependence of the ion motive proteins. For example the V-ATPase and ENaC were colocalized on apical membranes of pavement and chloride cells in FW tilapia *Oreochromis mossambicus*, suggesting that they may be linked in function (Wilson et al., 2000). In the same vein, separation of localization can restrict function to different cell populations. In stingrays (*Dasyatis sabina*; Piermarini and Evans, 2001) V-ATPase abundance was greatest in FW-acclimated animals associated with increased NaCl uptake and H^+/HCO_3^- extrusion. Localization occurred diffusely throughout the cytoplasm and was associated with the basolateral membrane of large mitochondria-rich cells. V-ATPase and Na^+/K^+-ATPase were located in different populations of cells, suggesting that Cl^- uptake/HCO_3^- excretion occurs in V-ATPase-rich cells while Na^+ uptake/H^+ excretion occur in Na^+/K^+-ATPase rich cells. In a subsequent paper (Piermarini et al., 2002) the same authors established that the Cl^-/HCO_3^- exchanger (pendrin) immunoreactivity occurred at the apical region of V-ATPase-rich cells and not in the Na^+ pump-rich cells.

Comparison of expression patterns in response to environmental change can further our understanding of the regulation of the genes that encode these proteins. The α subunit of Na^+/K^+-ATPase in *Callinectes* exhibits greater expression in posterior gills than in anterior gills (Towle et al., 2001). When these euryhaline crabs are exposed to external dilution, their ability to hyperosmoregulate is associated with enhanced Na^+ pump activity in posterior gills. However, the documented increase in enzymatic activity is not reflected in increased expression of the mRNA. This suggests that post-translational mechanisms are responsible for the increased activity (such as subunit assembly, membrane trafficking or cell signaling) and not synthesis of new protein.

Certain comparative models avail themselves to the analysis of temporal regulation of expression of ion motive proteins. In our own laboratory the crayfish moulting model has shown that Ca^{2+}-ATPases on internal (SERCA) and external (PMCA) membranes appear to be inversely regulated. SERCA

expression is greatest in intermoult and decreases in postmoult whereas PMCA expression is lower in intermoult and increases in postmoult (Zhang et al., 2000). This agrees with the pattern observed in mammalian cells (Liu et al., 1996) where SERCA expression was downregulated when PMCA was functionally overexpressed in rat aortic endothelial cells. In the terrestrial isopod model, *Porcellio scaber* (Ziegler et al., 2002), SERCA expression showed the opposing trend. Messenger RNA abundance was increased during the change from non-transporting (early premoult) to Ca^{2+} transporting stages of the moulting cycle (late premoult to intramoult). Expression of SERCA in the anterior sternal epithelium (ASE) was different from the posterior sternal epithelium (PSE), suggesting that there is tissue specific regulation.

Summary

The relative contributions over time made by non-genetic and genetic species to our understanding of ion regulation are illustrated in Fig. 1. In the pre-genomics era non-genetic organisms were used extensively to supplement our basic understanding of the physiological mechanisms of ion regulation. With the advent of genomics, both non-genetic and genetic organisms have contributed sequences for ion motive proteins (channels, pumps, cotransporters and antiporters) to the genomic databases. The non-genetic species have contributed the earliest sequences. The contribution of the genetic species should not be underestimated, however, since the annotated sequenced genomes of genetic model organisms have accelerated the homology-based cloning of sequences from other comparative models. In the post-genomics era, both groups of organisms will continue to advance our understanding of the function of gene products. The genetic organisms will predominate in the area of functional genomics, simply because they afford the genetic resources to close the phenotype gap. For the non-genetic organisms, the role will be primarily in explaining the evolution of gene products (evolutionary genomics) and the environmental fit (environmental genomics). Collectively, genomics of comparative species will address the diversity in protein structure and function as well as complexity in spatial and temporal distribution. Clearly non-mammalian species will continue to play a major role in extrapolating the molecular biology of ion motive proteins to biomedicine and human health.

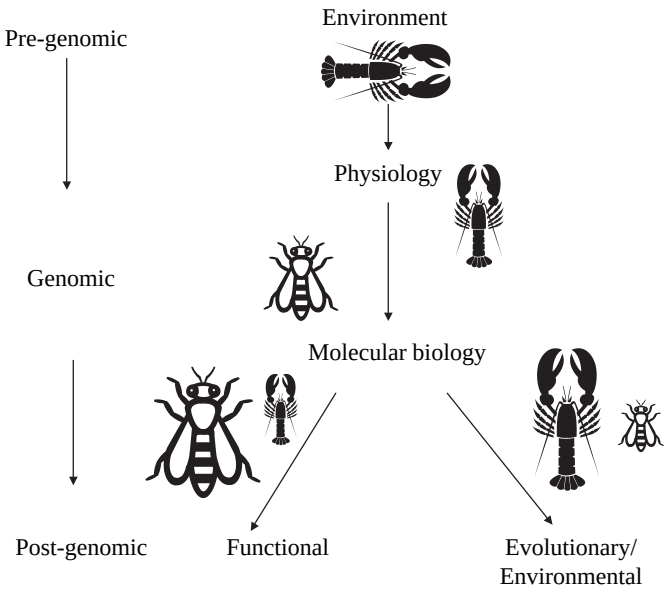

Fig. 1. Relative contributions over time (moving down the page) made by non-genetic (crayfish symbol) and genetic species (fruit fly symbol) to the understanding of ionoregulation. The size of the symbol is intended to represent the magnitude of the contribution.

List of abbreviations

ASE	anterior sternal epithelium
CFTR	cystic fibrosis transmembrane conductance regulator
ClC	Cl^- channel
EC	extracellular
EcaC	epithelial Ca^{2+} channel
EnaC	epithelial Na^+ channel
ER	endoplasmic reticulum
EST	expressed sequence tags
ET	enhancer trapping
FW	freshwater
IC	intracelleular
KCC	K^+/Cl^- cotransporter
MY	million years
NBC	Na^+/HCO_3^- cotransporter
NCC	electroneutral Na^+/Cl^- cotransporter
NCKX	K^+-dependent Na^+/Ca^{2+} exchangers
NCX	Na^+/Ca^{2+} exchanger
NDAE	Na^+-dependent anion exchanger
NHE	Na^+/H^+ exchanger
NKCC	$Na^+/K^+/2Cl^-$ cotransporter
PKA	protein kinase A
PMCA	Ca^{2+}-ATPases on plasma membrane
PSE	posterior sternal epithelium
RNAi	RNA-mediated gene interference
SERCA	Ca^{2+}-ATPases on SR/ER membranes
SR	sarcoplasmic reticulum
SW	seawater
TM	transmembrane region
V-ATPase	vacuolar H^+-ATPase
XIP	exchanger inhibitory peptide

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