Integrative Physiology and Functional Genomics of Epithelial Function in a Genetic Model Organism

JULIAN A. T. DOW AND SHIREEN A. DAVIES

Division of Molecular Genetics, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, United Kingdom

I. What Is Integrative Physiology? 688
II. Which Transgenic Model? 689
   A. Choosing your model organism is a trade-off 689
   B. Real physiology in model organisms 690
III. What Is Functional Genomics? 692
IV. Why Genome Projects Need Model Systems 692
V. What Is Reverse Genetics? 692
   A. Reverse genetics demands a genetic model 693
   B. Even human genomics requires simple models 693
   C. Other routes to functional analysis 693
VI. The “Phenotype Gap” 693
VII. The *Drosophila melanogaster* Malpighian Tubule 694
   A. History of Malpighian tubule physiology 694
   B. How is the *Drosophila* Malpighian tubule organized? 694
VIII. Physiology and Molecular Genetics of Ion Transport in Renal Function: V-ATPases 701
   A. *Drosophila vha55* is single copy and located at 87C 702
   B. The 87C region has been subjected to intense genetic analysis 702
   C. P-element *LacZ* reporter reveals V-ATPase expression patterns 702
   D. Correlating functional and genetic maps: V-ATPase expression levels 703
   E. V-ATPases are often found in specialized cell types 703
   F. The SzA locus provides multiple alleles of *vha55* 704
   G. A human renal and auditory phenotype is associated with a plasma-membrane V-ATPase 705
IX. How Do Chloride and Water Cross the Tubules? 706
   A. Leucokinin selectively increase chloride conductance 706
   B. Patch clamp reveals maxi-chloride channels in tubules 706
   C. Self-referencing electrode analysis shows that chloride moves through stellate cells 706
   D. Tubules contain CLC chloride channels 707
   E. Bartter syndrome type III 707
   F. How does water cross the tubule? 708
   G. Cation and anion transport are spatially separated 708
X. Pharmacology and Cell Signaling Mechanisms 709
   A. Cell-specific calcium signaling 709
   B. Neuropeptide-stimulated calcium signaling 710
   C. Calcium channels 712
   D. NO/cGMP signaling 713
   E. Cross-talk between NO/cGMP and calcium signaling 714
   F. New targets for drug discovery 715
   G. Insect tubules as targets for selective insecticides 715
XI. In Silico Mapping of Metabolic Pathways 715
   A. Human, mouse, and gout 715
   B. *Drosophila*, mice, humans, and gout 715
   C. Disorders of purine metabolism 716
   D. Does *Drosophila* have a xanthine oxidase mutant? 716
   E. Virtual metabolomics: an index of possibilities 716
   F. Eye color mutants have interesting causes 717
XII. *Drosophila* as a Model for Human Disease 718
   A. Can *Drosophila* cure human disease? 719
   B. How different can you be and still be the same? 719
Classically, biologists try to understand their complex systems by simplifying them to a level where the problem is tractable, typically moving from whole animal and organ-level biology to the immensely powerful “cellular” and “molecular” approaches. However, the limitations of this reductionist approach are becoming apparent, leading to calls for a new, “integrative” physiology. Rather than use the term as a rallying cry for classical organismal physiology, we have defined it as the study of how gene products integrate into the function of whole tissues and intact organisms. From this viewpoint, the convergence between integrative physiology and functional genomics becomes clear; both seek to understand gene function in an organismal context, and both draw heavily on transgenics and genetics in genetic models to achieve their goal. This convergence between historically divergent fields provides powerful leverage to those physiologists who can phrase their research questions in a particular way. In particular, the use of appropriate genetic model organisms provides a wealth of technologies (of which microarrays and knock-outs are but two) that allow a new precision in physiological analysis. We illustrate this approach with an epithelial model system, the Malpighian (renal) tubule of Drosophila melanogaster. With the use of the beautiful genetic tools and extensive genomic resources characteristic of this genetic model, it has been possible to gain unique insights into the structure, function, and control of epithelia.

I. WHAT IS INTEGRATIVE PHYSIOLOGY?

Physiology, like most life sciences, is analytical; that is, a seemingly intractable problem (how an animal works) is broken down into progressively simpler subproblems. Typically, this means moving from whole animal to isolated tissue (classical physiology). After this, one of two experimental paradigms is usually employed: cellular physiology, in which a cell line is derived from the tissue, in the hope that it expresses at least some differentiated property of interest; and molecular physiology, in which a gene is cloned, then heterologously expressed in an experimentally innocuous background (like the Xenopus oocyte) so that, with luck, there are no complicating factors (Fig. 1).

These are enormously powerful techniques. However, there is a set of problems that these techniques cannot address, simply because they have taken the system under study away from the physiological context in which the question was first posed. For example, in the context of epithelial biology, how does an epithelium develop? How is the epithelium polarized so that different transport proteins are found on opposite sides? How are heterogeneous cell types specified in the tissue, and how do they interact? What signaling processes allow the tissue to coordinate its function?
The realization that analytical techniques, and in particular molecular biology, cannot give all the answers has led to a call for a new, “integrative” physiology that seeks to move from single molecule back to the whole organism (164). However, rather than throw the baby out with the bathwater, modern genetics provides tools that let us combine the strengths of the reductionist approach with the relevance of organism-level studies. Ideally, we would like to be able to manipulate specific genes in defined cells in particular tissues at specific life stages in the intact organism, as easily as cells can be transfected with DNA in vitro. Once the problem is phrased in this way, it is clear that a transgenic approach is needed.

II. WHICH TRANSGENIC MODEL?

A. Choosing Your Model Organism Is a Trade-off

There are fairly few organisms in which transgenesis is routine (Table 1). These tend to be termed “genetic model organisms,” and in addition to transgenesis, there are frequently genome project resources associated. For biomedical research, it might seem that only the mouse is a realistic candidate for transgenics. It is a vertebrate and has a recognizable body plan, and reasonably well conserved physiology, compared with humans. However, its high biomedical relevance is offset by its very low genetic power. Although targeted mutagenesis by homologous recombination is possible, it is slow and very expensive, taking perhaps as much as 3 years. This makes it hard to both generate and analyze a transgenic line in a single grant cycle.

Although the mouse is, rightly, the biomedical model of choice, its genetic limitations are manifest. In contrast, *Drosophila* represents an ideal trade-off between human biomedical relevance and genetic power. It is no coincidence that Celera chose *Drosophila* as the warm-up genome to practice their techniques for humans. In fact, even simpler models have their place; just as nearly all we know about developmental genes was first established in *Drosophila*, so the bulk of our knowledge about the cell cycle was derived from yeast, and our understanding of DNA replication and gene regulation was pioneered by studies in the humble bacterium *Escherichia coli*. The relative merits of model organisms are nicely reviewed elsewhere (17). It is thus clear that good science is facilitated by a wise choice of model organism that is relevant to the question being posed, and a thrust of this article will be that the mouse is not necessarily the automatic choice for postgenomic physiology.

What is meant by genetic power? Targeted mutagenesis is certainly part of the equation, but there are other aspects, such as the facility with which forward genetics can be undertaken. Although mouse is the smallest and

<table>
<thead>
<tr>
<th>Organism</th>
<th>Biomedical Relevance</th>
<th>Genetic Power</th>
<th>Sequenced Genome</th>
<th>Targeted Mutagenesis</th>
<th>Classical Genetics</th>
<th>Genetic Screens</th>
<th>Classical Genetics</th>
<th>Targeted Mutagenesis</th>
<th>Genetic Screens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human (<em>Homo sapiens</em>)</td>
<td>Yes</td>
<td>No!</td>
<td>Serendipitous</td>
<td>No!</td>
<td>No!</td>
<td>No!</td>
<td>No!</td>
<td>No!</td>
<td>No!</td>
</tr>
<tr>
<td>Rat (<em>Rattus rattus</em>)</td>
<td>Partial</td>
<td>Yes</td>
<td>Some</td>
<td>Very hard</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Mouse (<em>Mus musculus</em>)</td>
<td>Partial</td>
<td>Yes</td>
<td>Some</td>
<td>Very hard</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Zebra fish (<em>Danio rerio</em>)</td>
<td>Partial</td>
<td>No</td>
<td>Some</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Fly (<em>Drosophila melanogaster</em>)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Worm (<em>Caenorhabditis elegans</em>)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Yeast (<em>Saccharomyces cerevisiae</em>)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Bug (<em>Escherichia coli</em>)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
cheapest vertebrate to keep, the costs are far too high, and life cycle far too long, to imagine setting up genetic screens routinely. However, the pay-offs can be large. For example, an industrial/academic collaboration has produced a systematic ENU mutagenesis of mouse, with a view to uncovering genes involved in human neurological deficits (6, 16, 108, 117, 167, 213). An alternative approach is to draw on the many strains of laboratory rat that have defined vascular defects and to map (painstakingly) a variety of blood pressure, osmoregulatory, and renal phenotypes through huge back-crosses, to identify loci by a quantitative trait locus (QTL) approach (113, 230). It is not types through huge back-crosses, to identify loci by a variety of blood pressure, osmoregulatory, and renal phenotypes through huge back-crosses, to identify loci by a quantitative trait locus (QTL) approach (113, 230). It is highly likely that loci identified in hypertensive rats will be directly relevant to our understanding of hypertensive disease in humans. However, these high-profile exceptions tend to prove the rule that rodent forward genetics is so expensive and time-consuming that it can only exceptionally be undertaken. Should this lack of genetics be of concern to a physiologist? Perhaps surprisingly, there are whole swathes of physiological endeavor with roots in forward genetic screens of “simple” model organisms. The next section reviews some areas in which our understanding of basic physiology has been advanced by classical genetic studies in Drosophila melanogaster.

B. Real Physiology in Model Organisms

1. Shaker

In the mid 1980s, the first voltage-gated sodium channels were cloned contemporaneously from rat (165), fly (200), and eel (166). There was massive excitement that all known ion channels matched the same structural template, with a six transmembrane motif repeated four times, presumably by two ancient gene duplication events (202). This argument was dramatically vindicated by the discovery of an “ancestral,” six-transmembrane potassium channel in Drosophila (41). The route to this discovery was unconventional: whereas the original sodium channels had been cloned by molecular biochemical means (cDNA libraries were screened with degenerate probes derived from peptide microsequence of sodium channels purified by tetrodotoxin affinity), the Shaker channel was identified by forward genetics.

A serendipitous behavioral mutation, in which flies’ legs shook under ether anesthesia (41), was analyzed physiologically and shown to be caused by an underactive A-type potassium current in muscle (201). The locus was identified by positional cloning (118) and shown to encode a quarter-sized channel, with six transmembrane domains. The identity of the locus was confirmed by showing that the gene was indeed mutated in Shaker mutants. Although the naïve interpretation would have been that flies, being primitive, carried an ancestral channel, this would have been far from the truth: Shaker homologs were rapidly identified in mammals (199). The beneficial interplay between physiology and genetics is nicely illustrated again; although Shaker encoded a potassium channel, A-type potassium currents remained in Drosophila Shaker mutants, suggesting that further related genes might exist (39). This led to a conventional library-screening search for related genes, identifying prototypes of three further Shaker subfamilies: Shal, Shab, and Shaw (39). These genes, in turn, were shown to have human counterparts (199).

In this case, there is no doubt that the Sh, Shab, Shal, and Shaw family of channels would have been discovered in time. However, the use of a genetic model organism advanced the field by at least a decade. Genetics and physiology can thus be potent fellows (202).

2. Learning and memory

To most scientists, the conspicuous success of comprehensive forward screens in Drosophila has been the revolution in our understanding of developmental biology, in recognition of which Edward B. Lewis, Christiane Nüsslein-Volhard, and Eric F. Wieschaus were awarded the 1995 Nobel Prize in Medicine. However, there are rare cases where elegant forward genetic screens have advanced our understanding of physiology. Here are two examples: learning and memory as well as circadian rhythms.

Although Drosophila is a relatively simple organism (10^4 neurons vs. 10^12 in humans), it displays recognizable behaviors, such as learning and memory. With a foresight that rivaled the great developmental screens, a systematic olfactory learning screen was employed to demonstrate a role for cAMP in learning and memory.

The original screen, much copied since, identified a mutation in a locus (dunce) that was defective in a range of simple associative learning tasks, but essentially normal in other aspects of behavior (74). Parallel work on adenosine metabolism showed that dunce mutants had lower levels of cAMP phosphodiesterase (40, 45). Dunce was subsequently found to encode a cAMP phosphodiesterase that was expressed predominantly in those brain regions thought to encompass learning behavior (163). Support for cAMP as a major messenger in learning and memory was gained from the discovery that rutabaga, another learning mutant, showed aberrant adenylate cyclase activity (75), and the gene was subsequently shown to encode an adenylate cyclase (137).

In parallel with the Drosophila work, cAMP was shown to play a role in a simple habituation response, the gill withdrawal reflex, in the sea hare Aplysia (119). The Aplysia result both extended the phylogenetic scope of cAMP as a mediator in learning and memory and nicely illustrates the limitations of work in a nongenetic model, as it was hard to take the work forward rapidly in Aply-
sia. In Drosophila, it was possible to extend the results of the learning screen by crossing dunce to rutabaga and showing that in the double mutant, both learning behavior and overall cAMP levels were almost normal (78). Thus a defect in adenylate cyclase could be compensated for by a defect in the cognate phosphodiesterase. The principle of investigating genetic interactions has been extended in subsequent work. Another feature of a genetic model organism is the availability of transgenics, and this has also been brought to bear. Overexpression of a dominant negative pseudosubstrate inhibitor of cAMP-dependent protein kinase (cAK) has been shown to disrupt learning, implicating the kinase in the learning and memory pathways (71). In Drosophila, it is relatively easy to target expression of transgenes, and this has allowed further physiological analysis: by overexpression of wild-type rutabaga adenylate cyclase in different brain regions, it was possible to show that the critical regions for learning and memory were the ventral ganglion, antennal lobes, and median bundle (255). It is hard to imagine how such functional insights could be obtained by other means.

3. Circadian rhythms

Another major screen for genes underlying basic behavior was that for mutants in circadian clock function. The archetypal clock gene, period, was first found in Drosophila (131). Although the structure of its encoded peptide was novel, it is the prototype of a large family since implicated in circadian function in mammals, and even humans (224).

The essence of the method is the design of the screen: only with a robust paradigm can a graduate student be expected to screen the 100,000 flies that might prove necessary to identify likely mutants. (The scale required also neatly illustrates the unsuitability of mouse for such screens.) In this case of Drosophila clock genes, the screen was suggested by a long history of classical work on molting and clock genes in insects; adult flies tend to emerge from the pupa (eclose) around subjective dawn (49). Therefore, if larvae are reared under a specific photoperiod then transferred to constant conditions, those with aberrant clocks will tend to emerge at unusual times. An experiment like this can be performed on a large scale with ease, using automated collection equipment (49): flies selected by the screen are collected and bred, and a small fraction of them can be expected to have a definable genetic lesion in a clock gene. This screen, or similar, was used to identify many of the major components of the circadian clock, for example, period (131) and timeless (207).

There is another lesson to be learned from this example. Although there is a massive literature on circadian clocks in humans, the human period-1 gene, on chromosome 17, was identified only in 1997 (220, 224), 10 years after the Drosophila sequence had been published (48). So, progress in human physiology might sometimes be accelerated by close attention to progress in simpler models.

4. The inositol trisphosphate receptor

Calcium signaling is a fundamental process, and in most cells, calcium is released from internal stores in the endoplasmic reticulum (ER) in response to hormone signaling, through the action of inositol 1,4,5-trisphosphate (IP$_3$) on its receptor (IP$_3$R), a large ER calcium release channel (24, 25). While most studies of calcium signaling have been performed on cultured cells, because of the experimental tractability, there have been some genetic experiments. In mouse, both the natural opisthotonos mutant and targeted knock-outs of the IP$_3$R show multiple neurological defects and die soon after birth, confirming that, despite the existence of alternative ER release channels, the IP$_3$R is essential for life. In Drosophila, the lethal phenotype is reproduced; however, here it has been possible to produce a range of alleles of differing severity (an “allelic series”), that includes severe hypomorphs, and lethal alleles that make viable, but hypomorphic, transheterozygotes. It is also relatively straightforward to construct chimeras so that lethal mutations can be studied in clones of homozygous cells in an otherwise viable heterozygote. In this way, slightly more informative results can be obtained; the key defect in hypomorphic alleles seems to be delayed larval development and adult emergence (101, 233). This implies a critical role for IP$_3$R in the endocrine control of growth and development, probably connected to the release of the molting hormone ecdysone (233, 234). Intriguingly, although IP$_3$R is single copy in the Drosophila genome, it does not seem to be necessary for visual phototransduction, a classical phospholipase C-mediated pathway (2, 182).

Such insights are not confined to studies in Drosophila. Similar analysis of IP$_3$R function in C. elegans was taken in a slightly different direction by the generation of green fluorescent protein (GFP)-tagged IP$_3$Rs, and by overexpressing the IP$_3$ binding domain, to produce a dominant negative effect (19). The former technique provides a dynamic means of following levels and distributions of gene products in living animals and showed that, although IP$_3$R is presumably pretty ubiquitously expressed, it is found preferentially, not just in the nervous system, but in the pharynx, gonad, intestine, and excretory cell (19). From here, it was relatively straightforward to map the gene’s control regions and correlate them with the observed expression levels (90). The latter, dominant negative approach (together with RNA interference) showed a role for IP$_3$R in two behaviors at opposite ends of the alimentary canal: feeding and defecation (235). The use of a heat-shock promoter for the dominant negative con-
struct allowed intervention in the adult, avoiding the confounding effects of disrupting IP$_3$R during development. These interventions would be difficult in a larger animal. It has also been shown in a yeast two-hybrid screen that IP$_3$R interacts directly with myosin; the availability of a simple model allowed these results to be tested directly in C. elegans, confirming the functional significance of the interaction in vivo (236).

From the examples above, it can be seen that harnessing the power of even a simple genetic model can produce fundamental insights in mainstream (i.e., de facto mammalian) physiology. It is of interest to note, though, that the three examples shown above are all of the "brain and behavior" phenotypic class. There have been very few successful attempts to exploit Drosophila or other simple models in other areas of physiology. Later, this article will address perhaps the most developed such phenotype for epithelial function, ion transport, and cell signaling, the Drosophila Malpighian (renal) tubule. This will illustrate both that real physiology is possible and that it can provide useful general insights.

III. WHAT IS FUNCTIONAL GENOMICS?

"Functional genomics" is a fashionable bandwagon, which has been hijacked to a number of laudable purposes. Originally, it was seen as a (necessarily) high-throughput route to assigning functions to new genes. As a first step into the postgenomics era of functional genomics, a range of ingenious, large-scale "chip" technologies have been devised that offer the promise of identifying candidate genes based on their differential expression in two tissues or in disease states. This is extremely attractive, particularly to the drug industry, because it allows the rapid identification and patenting of interesting genes, particularly to the drug industry, because it allows the rapid identification and patenting of interesting genes without necessarily knowing their function (204). It is now becoming increasingly clear that, although high-throughput technologies provide valuable lists of candidate genes, they can provide only caricatures of function. Ultimately, then, it is necessary to roll up one’s sleeves and work up the function of individual genes and gene families. This activity is as deserving of the epithet functional genomics as any high-throughput technology. It is thus perhaps useful to consider functional genomics as any elucidation of function in the context of an organism’s genome. In the next section, we will make the case that this functional genomics requires exactly the same approaches and reverse genetic technologies as integrative physiology, to the extent that the two areas can be considered to have merged.

IV. WHY GENOME PROJECTS NEED MODEL SYSTEMS

At first, it might seem self-evident that genome projects are a good thing. In particular, now that the human genome has been sequenced completely, the nucleotide sequence of every gene involved in any disease process must have been deduced. Unfortunately, connection between the two is not automatic. Even after coding sequences have been identified in humans, it remains to correlate each of them to their physiological function. There are essentially three ways in which this will be possible (Fig. 2).

1) A substantial fraction of genes will already be known, and their physiological roles will be effectively elucidated. This will include a number of the genes responsible for major genetic diseases, which are being directly sought in parallel with the main human genome project.

2) For a significant further fraction of the remaining genes, it will be possible to infer function either by similarity to other known human genes or to well-known genes characterized in other organisms. Additional information will become available from expression patterns or from the chromosomal localization of the gene mapping close to a known and plausible genetic disease.

3) However, a significant fraction of genes (from most genome projects, estimates are around one-third) will be genuinely new, with no structural similarities that could suggest likely avenues of research. It is for this class of gene that reverse genetics will be particularly important.

V. WHAT IS REVERSE GENETICS?

Reverse genetics is the deduction of gene function by analysis of corresponding mutant phenotypes (139, 176, 196, 197). This is acknowledged as the quickest and most promising way of inferring function for a new gene (Fig. 3). It relies on the presence of a homologous (or at least analogous) gene, in a model organism in which a gene knock-out can be created (135, 141, 179). The success of reverse genetics at the genome project level thus depends on the range of model organisms available; a wider range maximizes the probability that a homologous gene can be identified.

![Fig. 2. Genome projects require reverse genetics.](http://physrev.physiology.org/Downloadedfromhttp://physrev.physiology.org/ by 10.220.33.2 on June 19, 2017)
Perhaps less obviously, it further relies on the availability of a phenotype that can be studied for effects of the induced mutation. In other words, there is no point in mutagenizing a gene in a model organism in which there is no assay for effects of the mutation! For example, it would be a waste of effort to mutagenize a neural-specific gene in an organism too small to allow neurophysiological analysis. The choice of model is thus a trade-off between biomedical relevance, genetic power, and amenability to physiological analysis, exactly as we have outlined for integrative physiology above.

A. Reverse Genetics Demands a Genetic Model

Reverse genetics demands at the least some means of targeted mutagenesis of a gene of interest. Although generic approaches are being developed [RNAi (109) is an example], targeted mutagenesis has generally only been developed in organisms in which there is a considerable volume of classical genetics. Typically, this means that there is also a large body of literature covering known genes, visible markers, and recombination or physical maps. Such organisms have also invariably been selected as early subjects for genome projects. This confluence of classical and reverse genetics, together with genomics, provides a working definition of “genetic model” organism. Such organisms form a short but distinguished list and have impacted on nearly every field of biology to date, except physiology.

B. Even Human Genomics Requires Simple Models

Because reverse genetics will be essential to human functional genomics, it follows that model organisms are also essential. This explains the apparent contradiction that human genomics, in particular, and biomedical science in general, has hugely cross-subsidized fundamental research in genetic models. However, as described in Table 1, the choice of transgenic model is not automatic. There is a trade-off between biomedical relevance (with humans top, and mammals next), cost, and convenience of life cycle (which favors smaller model organisms), ethical desirability (which favors smaller model organisms), and power of the genetic tools available (which favors smaller model organisms).

At first, mouse would seem to be ideal, as it is the only mammalian model for which detailed genetic intervention is routine. It is possible to inactivate genes with single base-pair precision by homologous recombination and to introduce transgenes. However, gene knock-outs are often lethal sufficiently early in development as to be uninformative. In mouse, the ability to introduce more subtle defects (for example, to inactivate gene function only in adults, or only in a particular tissue) is highly limited and must be set up on a case-by-case basis, compared with other models, like Drosophila or C. elegans, where generic technologies are available. Accordingly, for the foreseeable future, these organisms are likely to be ideal models for integrative physiology.

C. Other Routes to Functional Analysis

Although it is generally accepted that reverse genetics is the most powerful method of elucidating function of novel genes, there are other special cases that are nonetheless worth noting. For example, cluster analysis of microarray data tends to gather together functionally related genes. So if a single novel gene clusters with known genes that are all implicated in a single process (like amino acid metabolism), then a clear functional hypothesis presents itself (76). Similar routes can be plotted at the proteome level, although there are more potent ways of identifying functional complexes. By systematically fishing out the partners of epitope-tagged proteins in yeast, it has proven possible to identify the constituents of multiple hetero-multimeric complexes (86, 105). Similarly, more advanced computational techniques may allow hypothetical proteins to be classified based on structural similarities more subtle than conventional BLAST searching at present (77, 111, 175). However, these exceptions also illustrate the rule: they all generate hypotheses that must be tested experimentally. So reverse genetics is still a required part of the transition from gene to function, and physiologists are essential participants in the endeavor.

VI. THE “PHENOTYPE GAP”

Unfortunately, exactly the qualities that make for a good genetic model militate against physiology. For rea-
sonable genetics, small size, low cost, rapid life cycle organisms are needed. This means that historically there is little depth of physiology available. This mismatch has been recognized by the genome project community and has been termed the “phenotype gap” (37, 38).

It is easy to demonstrate the phenotype gap objectively. For example, most rodent physiologists have concentrated on rats, guinea pigs, and rabbits rather than the smaller genetic model, the mouse. This difference can be marked: a literature search of Science Citation Index publications shows that for almost any keyword or tissue of choice, the recent literature on rats is about fivefold larger than on mouse (Table 2).

Although rat genetics and genomics will eventually rival those of the mouse in power, there is a pressing need to translate rat physiological methodology into mouse, and so provide the widest range of physiological assays possible. This will maximize the chances that mutation of any particular gene will have a detectable and informative physiological phenotype.

Genome projects acknowledge the need for physiologists to help close this phenotype gap, and this provides some of the most exciting openings for physiologists into the 21st century. Exactly how to ride this wave is discussed later.

In the next section, we will give an example that draws together the strands of the article: that combining physiology and genetics in a simple genetic model provides an ideal system for truly integrative physiology, and that by doing so physiologists can contribute to post-genomics by closing the phenotype gap.

VII. THE DROSOPHILA MELANOGASTER MALPIGHIAN TUBULE

Insect Malpighian tubules perform excretory and osmoregulatory roles analogous to vertebrate renal tubules and have been studied extensively in a literature that dates back over half a century. However, most studies have focused on far larger insects. Drosophila tubules are among the smallest ever studied, measuring ~2 mm long by 35 μm in diameter, and are comprised of ~150 cells. We have recently found that the Drosophila renal tubule can be used for physiological studies of fluid secretion (69) and that it shows a rich pharmacological repertoire that includes the cAMP, nitric oxide (NO)/cGMP, and Ca2+ signaling pathways (63, 66). This means that, for a very wide range of developmental, transport, and signaling genes, the tubule is probably the most robust Drosophila phenotype presently available.

A. History of Malpighian Tubule Physiology

This has been reviewed briefly elsewhere (63). Marcelo Malpighi (1628–1694), physician to the Pope, is well known as an early adopter of the microscope, contemporaneously with Hooke, and followed the discoveries of the great William Harvey on the nature of circulation. However, he was a comparative anatomist and also turned his microscope to insects, discovering the eponymous insect Malpighian (renal) tubule. However, it was not until the 20th century that insect physiology became feasible. Wigglesworth, then his pupil Ramsay (183), was able to demonstrate that insect tubules indeed produced urine, and Maddrell (150) was able to demonstrate endocrine control, using one of the more remarkable epithelial model systems, the tubule of the bloodsucking bug Rhodnius prolixus. As this bug feeds on huge blood meals perhaps once in 6 mo, and must then fit into cracks in walls and floorboards to escape discovery, it has a potent control of its diuresis and can accelerate urine production by more than a 1,000-fold after feeding (153). Since then, there has been a large literature on insect tubules from many species (for reviews, see Refs. 50, 62, 63, 66, 150, 152, 153, 173, 177, 231), and it is accepted that the insect tubule is both a useful epithelial system and a valid target for insecticide development. This classical literature suggested that the Malpighian tubule of Drosophila melanogaster might be amenable to physiological analysis, although it was smaller than any previously studied.

B. How Is the Drosophila Malpighian Tubule Organized?

1. The classical view

Insect Malpighian tubules are simple epithelia, free-floating in the insect’s hemocoel (there is no vascular circulatory system beyond a rudimentary tubular heart) (43, 247). Similarly to birds, and for similar reasons of water economy, the tubules do not open directly to the outside, but join to the alimentary canal at the junction of the endodermal midgut and the ectodermal hindgut (Fig. 4). The relative experimental accessibility of the insect
tubule compared with the vertebrate kidney tubule is an advantage; they are similar in overall dimensions. In the case of *Drosophila*, there are four tubules, joined in pairs through short common ureters to the alimentary canal. Interestingly, the right-hand pair are longer, with a prominent white initial segment, and are always placed anteriorly in the body cavity. In contrast, the left-hand pair was thought to lack initial or transitional segments, and always sit posteriorly, within the abdomen (242). (Incidentally, this could provide a useful screen for mutants of sinistrality, an area hardly explored in *fly*). There are two major cell types: the larger type I (or principal) cell and smaller intercalated type II (or stellate) cell (242).

2. **Enhancer trapping as a tool to understanding organization**

A fundamental part of a physiological study is to analyze the structural organization, or functional morphology, of the tissue of interest. In a nonmodel organism, our understanding of a tissue is based on the experimental techniques available and the training of the scientist. It is thus only possible to obtain an experimenter’s view of the tissue organization. In certain genetic model organisms, in contrast, a technique known as enhancer trapping can provide an insight into how the organism organizes a target tissue.

3. **The principle of enhancer trapping**

Enhancer trapping has been widely used in the context of development, for which it was originally developed (23). It makes use of an engineered transposon, in *Drosophila* usually the P-element (193). Transposons are a class of semi-autonomous mobile DNA elements (loosely similar to retroviruses), found widely in all organisms, that encode an enzyme (transposase) that can recognize the stereotyped ends of the transposon, and catalyze its excision and reinsertion in the genome (187). In most organisms, this parasitic process is largely benign, although occasionally a cell can be killed or transformed by disruption of a key gene (“insertional mutagenesis”). In *Drosophila*, one transposon (the P-element) has been engineered to carry a variety of genetic constructs that permit a remarkable range of genetic manipulations in the intact organism (192). For enhancer trapping, a P-element is used in which the transposase gene has been replaced by three elements: 1) a *white* marker gene, which gives red eyes in flies carrying the transposon and so allows it to be tracked through crossing schemes; 2) a reporter gene (such as *lacZ*), coupled to a weak, permissive promoter [expression of the *lacZ* gene is then sensitive to its genomic context (i.e., where the transposon is sitting within the genome, relative to other genes and their natural promoters and enhancers)]; and 3) a linearized plasmid, which allows the site of insertion to be determined by a technique known as plasmid rescue.

Without a transposase gene, this element is capable of mobilization but cannot transpose itself. It is thus trapped within the genome unless provided with a source of transposase. This enzyme can be supplied by crossing flies carrying the P-element to another line in which a P-element has been serendipitously inactivated by an imprecise excision event in the past. In the Δ2,3 line, a functional transposase is produced only in the fly’s germ cells, and as the element is missing one of its ends, it cannot be mobilized by its own enzyme (187). In an enhancer trap screen (Fig. 5), flies carrying both the enhancer trap element and a source of transposase are called jump-starters. In each of their sex cells (usually sperm: males are used by preference because there is no male meiotic recombination), there is the potential for the enhancer trap element to mobilize and reinsert somewhere else in the genome. By segregating and breeding...
true from hundreds or thousands of progeny of such jump-starter males (and by ensuring that the \( \Delta 2.3 \) transposase source is quickly crossed out of each line), a range of new, stable, insertion sites will be obtained. In some of these, the enhancer detector will have landed near some unknown gene with patterned expression in the tissue of interest, so by staining representatives of each line for the reporter gene, the 10% or so of genes of interest to a particular experimenter can easily be determined. (Of course, the remaining lines may be of interest to another experimenter, and it is common practice to exchange panels of lines within the *Drosophila* community.)

This description may sound esoteric in a physiological context, but its execution is straightforward, requiring only a series of crosses over months, and the physiological payback is huge, because the technique reveals the organism’s, rather than the experimenter’s, view of the organization of the tissue. In contrast to the methodology, the results are visually striking and easily grasped.

4. **Enhancer trapping can detect tubule-specific genes**

As part of a 1,500-line screen using the P(GATB) transposon (252), 750 lines were screened for tubule-specific patterns of expression in larvae and adults (214). Of these, ~10% were informative, and of these, ~20 lines were used to delineate boundaries of expression in the tubule. In most studies, enhancer trapping is used as a rapid means to identify genes of interest, and so generate data similar to the results of a differential cDNA library screen. However, the patterns themselves are informative, as they reflect the actions of combinations of cell-specific transcription factors on the enhancer detector. In this way, they genuinely reveal aspects of the tissue’s spatial and temporal organization.

5. **Enhancer trapping reveals regional specialization**

Classically, the anterior tubules were considered to have three domains and the posterior tubules one. We found lines that reflected these domains of expression, consistent with the previous studies (Fig. 6, A–D); however, we also found new aspects of organization. The lines that marked out initial and transitional segments in the anterior tubule also identified miniature domains in the posterior tubule (Fig. 6, A and B). The anterior and posterior tubules thus differ quantitatively in the extent of these regions, rather than qualitatively in the nature of their specification. The main segment of the tubule (Fig. 6C) can be divided into two, with a prominent lower tubule domain (Fig. 6D) that is in turn separable into three domains (214). Critically, then, there are six regions in both anterior and posterior tubules, and several of them had been refractory to experimental identification in the absence of enhancer trap studies.

6. **Enhancer trapping reveals multiple cell types**

The principle of these studies can be extended to cell types (Fig. 6, E–H). Surprisingly, few lines marked out all the principal cells in the tubule: the normal pattern was for only a subset of principal cells to be marked (Fig. 6E). This is significant because it means that morphologically indistinguishable cells are expressing different genes, and thus presumably performing different functions.

The other major cell type, the stellate cell, is also clearly labeled by this technique (Fig. 6F). Interestingly, the two lines that marked stellate cells had the most restricted pattern of expression in the rest of the fly of any studied. This may imply that their function is unique and
that they express relatively unusual transcripts. The distribution of stellate cells also helps to cross-validate the domains of expression outlined earlier: stellate cells are not found in the lower tubule domain, and their shape changes from stellate in the main segment to bar-shaped in the initial and transitional segments (Fig. 6G). The bar-shaped cells can in turn be distinguished from stellate cells by a single line that labels only the former.

There are other, minor cell types, such as tracheal cells, and tiny myoendocrine cells found only in the lower tubule region (Fig. 6H). Taken together then, there are six cell types and six regions in this tiny tissue.

7. Genetic domains can be quantified by counting nuclei

At this stage, it would be traditional to clone the flanking genomic regions by plasmid rescue and seek to identify the genes responsible for the patterns of expression that had been observed. However, we tried to quantify the sizes of different domains and to compare the sizes and stability of domains reported by different lines to see whether they were reporting the same boundaries. This would render the description of tubule structure at a quantitative rather than anecdotal level. In fact, it was
straightforward to label tubule nuclei with ethidium bromide and to count the nuclei in each domain (Fig. 7A).

Remarkably, it was possible to show that the positions of cell boundaries, and the numbers of cells in each region, were fixed to near single-cell precision (Fig. 7B). This means that every cell in the tubule has a precise view of its positional identity, and the development of the tubule is extremely robust and deterministic. Although all the insertions studied were homozygous viable, some are nonetheless disruptants of the genes in which they were inserted (see below). Despite this, cell numbers did not vary between lines, in any of those we studied, although, of course, there are some developmental mutants in which tubule development is perturbed (106). In this simple, one-dimensional system, it may be possible to undertake identified-cell epithelial physiology, for almost the first time. Although this is already technically possible for morphologically distinguishable cells (for example, Refs. 30, 95), it is clear that morphologically indistinguishable cells can express different genes (Fig. 6), and so have different functions (151, 171, 186). Enhancer trapping, and particularly the vital labeling of domains of gene expression, neatly provides a solution to this issue.

8. The GAL4/UAS system

The level of sophistication revealed by enhancer trapping might seem daunting, but the same enhancer trap technology provides the tool to intervene genetically in any population of cells that can be defined by an enhancer trap line.

In the second generation, or binary, enhancer trap system (Fig. 8), the reporter gene is the yeast transcription factor GAL4. This appears to be almost perfectly inert within the Drosophila genome, and so normally has no effect on the host organism. However, it is capable of driving transgenes under control of the yeast UASG promoter. Thus, given a panel of informative GAL4 lines, as described above, any genetic construct of choice (not just lacZ) can be directed to any of the lines. The GAL4/UAS system additionally acts as a switch, in that high levels of driven expression contrast with very low background levels. The binary nature of the system means that a new transgenic line can be generated in ~3 mo, without having to repeat the enhancer trap search for GAL4 drivers. This compares very favorably, in both time and cost, with the effort to make knock-in constructs for mouse and to generate and test the transgenics. There is another key advantage of the system: if a deleterious gene product (for example, a gene in the apoptosis pathway) is to be expressed, then the UAS stock can be kept safely without serious loss of fitness. The deleterious construct is only expressed in the progeny of the cross between the parent lines.

This GAL4/UAS binary system opens possibilities to the physiologist that are mouth-watering. At present, it is a technique specific to Drosophila, and a potent reason why experimenters should relish, rather than dread, the opportunity to work in this organism.

9. Correlating functional and genetic maps

Enhancer trapping suggested a clear, reproducible structure for the Malpighian tubule. However, it is critical to establish whether this genetic map has any relevance to physiology or is merely some genetic curiosity. To accomplish this, the genetic map was tested with a battery of functional assays, and in each case functional and expression domains were found to coincide perfectly. This section reviews evidence for the congruence between genetic and functional maps.

A) Fluid secretion. The key property of the Malpighian tubule is its ability to produce an isotonic fluid. Indeed, for most insects, this is the only property that has been measured. It is possible to adopt the classical Ramsay assay for fluid secretion to this tiny tissue, and the Drosophila tubule is actually very robust ex vivo, maintaining...
stable secretion rates of 0.5–1 nl/min for several hours in appropriate medium (69). It is possible to map secretion rates as a function of length along the tubule, although the spatial resolution of the assay is not as good as that of enhancer trapping. However, it was possible to show that the main segment of the tubule secretes fluid, while the lower tubule reabsorbs it (Fig. 9) (171).

B) ALKALINE PHOSPHATASE. A powerful product of the enhancer trap technology is the rapid identification of the genomic location of the insertion and thus frequently the gene near which the P-element is inserted. The lower tubule is delineated by two lines, which represent independent insertions in the same gene, encoding an alkaline phosphatase (Aph4) with greatest similarity to the human liver/bone/kidney (ALPL) type (253). In humans, ALPL is an ecto-enzyme that metabolizes phosphoethanolamine (PEA) and pyridoxal-5’-phosphate (PLP). In Drosophila, Aph4 is expressed only in the lower domain of the Malpighian tubule, and in a small group of cells, the ellipsoid body in the brain (253). It also appears to be an ecto-enzyme, as histochemistry for alkaline phosphatase activity labels only the apical surface of the lower tubule.

Remarkably, although the genome project has annotated 13 alkaline phosphatase genes in Drosophila, the expression patterns of the Aph4 enhancer trap insertions perfectly match the alkaline phosphatase histochemistry. This implies either that the other 12 alkaline phosphatase genes are not expressed significantly in the adult or that the nitro blue tetrazolium-based histochemical stain does not detect all the alkaline phosphatase activities in Drosophila.

In vertebrates, the roles of alkaline phosphatase are not entirely clear. In humans, mutations in ALPL are associated with hypophosphatasia (241). In mouse, the homologous tissue nonspecific (TNAP) isoform, when mutated, causes fatal seizures 2 wk after birth; these can be survived if pyridoxal is administered. There is also some evidence of hypomineralization in teeth (240). At least one human case has been reported with similar symptoms (244). However, the enigmatic renal role of
alkaline phosphatase might be addressed in *Drosophila*, as the P-element insertions in *Aph4* both disrupt expression and cause a transport phenotype (Fig. 10).

10. Phylogenetic scope of enhancer trapping technology

It is clear from the above that enhancer trapping delivers results that are highly relevant to the physiologist at several levels. First, it provides a screen for tissue-specific genes with added informational content by virtue of the expression pattern reported. Second, enhancer traps themselves report pattern in a tissue at a level that could probably never be deduced by explicit experimentation, so delivering new understanding of tissue organization. Third, second and later generation enhancer traps provide generic technologies for conditional expression, overexpression, and gene tagging. Fourth, enhancer trap insertions have the potential to be mutagenic, either directly by virtue of their insertion site or after imprecise excision of the P-element. They are thus vital components of a functional genomics strategy (22).

Obviously, these experiments can never be attempted in humans, so it is necessary for human physiologists to resort to model organisms. Enhancer trapping is at its most developed in the fly, with multiple classes of P-elements available, including the second generation GAL4/UAS binary system, together with several ES and other gene-trap vectors (22, 189).

In *Arabidopsis*, enhancer trapping and gene trapping have both been used, usually with the maize *Ds* transposon and GUS as the reporter gene (129, 221). However,
the technology does not appear to have been used to its full potential (22).

In zebrafish, a Rous-sarcoma virus LaCZ reporter injected into embryos showed patterned expression, although in only one line was it heritable (18). This is a fairly laborious method, as each line is derived from a separate injected embryo; there is no remobilization of a pre-existing integrated reporter gene.

In C. elegans, transposable elements are not easily tamed, because of the lack of clean germ line-specific transposase expression (22). However, it is relatively easy to introduce heterologous DNA into worms, and this has been used for a form of promoter trapping in which random genomic fragments are cloned upstream of a LaCZ reporter, then transformed into worms (145). If this technique is performed systematically on a large enough scale, it can emulate some of the functionality of enhancer trapping.

Enhancer trapping is technically possible in mouse, although the large genome size means that fewer insertions are likely to be informative. Additionally, the effort in generating and maintaining sufficient stocks (the limiting factor in Drosophila screens) makes a rigorous enhancer trap screen unlikely. However, promoter and gene-trap techniques have been applied widely (191). Such studies usually rely on a splice acceptor site within a retrovirus vector, producing a LaCZ fusion protein with the initial exons of the mouse gene. Such insertions are both rarer and much more mutagenic than enhancer traps, because they are guaranteed to disrupt the protein. Rather than try to jump the vector within the mouse, random insertions are generated within clones of embryonic stem (ES) cells. These can then be grown up into whole mice as desired. This can produce a valuable, high-throughput screen, as insertions can be screened for expression, or the insertion site sequenced, within ES cells; transgenic mouse lines are then only generated from cell lines of interest (116). At one level, this has less appeal than Drosophila enhancer trapping, because it implies preselection by the experimenter, and so reintroduces experimenter bias into the paradigm. However, at another level, it provides a potentially valuable route to reverse genetics of defined genes, perhaps those identified from high-throughput methodologies. Such an approach has been taken to a highly commercial level, with experimenters able to purchase ES clones with defined insertions from a bank of over 200,000 frozen lines (254) and produce their own transgenic mice (http://www.lexgen.com/ omnibank/omnibank.htm). There are obvious cost and intellectual property issues in such an approach.

Elements of the GAL4/UAS system are now used in mouse, in order specifically to provide switched expression (138). However, in mouse, GAL4 cannot be used as an enhancer trap element, because of the scale of the experiments implied. It is expressed downstream of a specific promoter, which must be characterized painstak-ingly first. There is no equivalent for panels of enhancer-trap insertions into anonymous genes that can be used for expression studies.

11. Emulating enhancer trapping in other organisms

Even if the GAL4/UAS system cannot be deployed in a target species, it would be highly desirable to obtain insight about the spatial organization of tissues of any species of interest. Is this achievable without true enhancer trapping? We propose two alternatives for studies in organisms where enhancer trapping is not feasible: comparative in situ and random in situ.

For the comparative technique, enhancer trapping of the analogous tissue in a suitable model will lead to a number of candidate genes. These can be used as probes for comparative in situ in the target tissue. For example, the mosquitoes Anopheles gambiae and Aedes aegypti are major vectors of disease (with completed genome projects), but transgenesis is still at too early a stage for routine enhancer trapping (3). However, some of the genes identified in an enhancer trap screen of midgut in Drosophila might be expected to delineate patterns in the Anopheles midgut, and so enhance our understanding of the target tissue through which parasites must migrate as part of their maturation. Such cross-species hybridizations are, of course, technically demanding, so an alternative approach might be to clone target species homologs of the genes identified in the genetic model. Another approach would be to use knowledge of the genes to identify functional assays (such as histochemistry) that can be used across species. This approach will be illustrated in section VIII.B1A, using our knowledge that alkaline phosphatase marks outer the tubule domain (see sect. VIII.A9b).

For random in situ, we propose large-scale in situ hybridization with random cDNAs from the tissue of interest. This approach could go hand-in-hand with an expressed sequence tag (EST) project, an accepted means of discovery in a tissue of interest. The power of the approach would be greatly increased by normalizing the library, a technique that selectively enriches the abundance of rare transcripts to nearly the same level as abundant transcripts within the library (212). The random in situ approach has the advantage, common to classical enhancer trapping, that domains of functional expression are mapped impartially, without introducing experimenter bias. Furthermore, if in situ could be performed systematically in a 96-well format, then relatively few plates could generate, in a few weeks, information similar to that obtained by our two-year enhancer trap study.

VIII. PHYSIOLOGY AND MOLECULAR GENETICS OF ION TRANSPORT IN RENAL FUNCTION: V-ATPASES

A critical test of the enhancer trap model would be to map the expression of the major transport processes that
energize fluid secretion, because this is known to be patterned according to the genetic domain model. In fact, these studies provided both a validation of the existing model and some more general insights into the partitioning of transport roles between cell types.

Historically, transport across most insect epithelia was thought to be energized by a primary, electrogenic, chemiosmotic K⁺-ATPase (98). The most accessible and spectacular example of this transport could be found in the larval lepidopteran midgut, which can develop lumens over 100 mV, drive currents of over 1 mA/cm² (96, 98, 100), and in some species generate a luminal pH in excess of 12, the highest in biology (58, 70). Within the midgut, the apical membrane of a specialized goblet cell was decorated with an array of 10-nm spheres (5), termed “portasomes” (97), and it was on this membrane that active K⁺ transport was shown to take place (67). However, painstaking biochemical purifications of the goblet cell apical membrane of lepidopteran midgut, based on purification of the portasomes (47), identified not a P-type ATPase similar to K⁺-H⁺-ATPases of vertebrates, but a V-type H⁺-ATPase (205, 246). This was the first demonstration that V-ATPases, considered important for vacuolar and endosomal acidification in eukaryotes, could also play a plasma membrane transport role. Contemporaneous with this discovery, V-ATPases were shown to reside on the plasma membrane of intercalated cells of the vertebrate kidney collecting duct (35). The plasma membrane role for V-ATPase was thus not an insect “special case” but was phylogenetically widespread: insects diverged from mammals ~400 million years ago. Since then, at least 200 plasma membrane manifestations of V-ATPase have been documented (99, 245) and are invariably associated with arrays of portasomes at the transporting membrane.

Given the two very different roles played by V-ATPases, it is thus of great interest to establish whether the plasma membrane and endomembrane V-ATPases are the same or different. The classical route to characterizing a transport process is through physiology, pharmacology, and membrane biochemistry. However, reverse genetics can be a potent adjunct to such an approach. Here, such an approach is described in Drosophila, and the insights it gives into V-ATPase function are outlined.

A. Drosophila vha55 Is Single Copy and Located at 87C

The first Drosophila V-ATPase subunit to be cloned was vha55, encoding the 55-kDa cytoplasmic B subunit (54). Most V-ATPase subunits show very tight sequence conservation at the amino acid level and so can readily be cloned by homology PCR with degenerate primers (54). Vha55 was shown to exist in single copy, but to have multiple transcripts. It thus seemed an ideal object of study for a genetic dissection of plasma membrane and endomembrane roles.

B. The 87C Region Has Been Subjected to Intense Genetic Analysis

Once a gene has been identified in a model organism, it is important to map it cytogenetically. In this way, it may be possible to reconcile a novel gene with a known genetic locus previously identified by forward genetics. If this is the case, a phenotype may already have been described for the locus. This applies a fortiori in Drosophila, as ever since the pioneering days of Morgan and Bridges, mutant loci have been mapped by recombination, and stocks kept alive at stock centers, in case they proved useful in the future. (As many as half of the mutants identified by Morgan and Bridges in the pioneering years of the early 20th century are still available in stock centers today!) Although the presence of a sequenced genome now makes chromosomal situs redundant, the principle of drawing inferences by bridging sequence, recombination, and cytogenetic maps remains valid.

For vha55, the single copy gene mapped to 87C on chromosome 3R. This has been an area of intense study, because two copies of the heat shock gene hsp70 are located within 87C, and the 210-kb region had been subjected to saturation mutagenesis (85) in a vain effort to produce flies deficient in hsp70 (unknown to the experimenters at the time, there were two further copies of hsp70 at 87A). It proved possible to show that a P-element insertion that had been mapped cytogenetically to 87C was in fact a disruptant of vha55. This constituted the first documented knock-out of a V-ATPase in an animal (54), and as the P-element insertion was lethal when homozygous, it demonstrated the essential role of V-ATPases in animals.

C. P-element LacZ Reporter Reveals V-ATPase Expression Patterns

All modern P-elements contain a reporter gene (usually lacZ) behind a weak (permissive) promoter, so conferring properties of an enhancer trap element. The elements can thus report on levels of gene expression in a tissue. Importantly, they give information additional to either Northern blots or in situ hybridization. Like nuclear run-on assays, they can indicate the rate of gene transcription rather than the standing levels of mRNA. This is because the level of an mRNA reflects a balance between its transcription and degradation, and although it is traditional to assume that only the former process is dynamically controlled, there is no evidence that this can be
assumed a priori. However, an enhancer trap reporter is a heterologous gene, so its mRNA will not be processed like that of the gene in which it inserts. There is an additional advantage over nuclear run-on assays, because enhancer detectors report on single cells.

Although the LacZ reporter of the lethal vha55 insertion is in opposite sense to vha55, it reports a plausible expression pattern (Fig. 11) that has since been validated with insertions in genes encoding other subunits (61, 65). Vha55 is transcribed at high levels in those tissues in which V-ATPase is thought to play a plasma membrane role, namely, the Malpighian tubules, rectum, and the trichoid sensilla of the cuticle. V-ATPase is also expressed at high levels in other tissues: vha55 is expressed at particularly high levels in the spermatheca and uterus of the female genital tract (54). This may indicate an important role for pH in activation of the fertilized egg as it passes along the uterus (61), a role reminiscent of the activation of sperm as they pass along the epididymis in vertebrates (32).

D. Correlating Functional and Genetic Maps: V-ATPase Expression Levels

At higher resolution, it is possible to map high-level V-ATPase expression within Malpighian tubules, and so test the functional applicability of the genetically derived tubule map described earlier. Within the tubule, only the main segment and lower tubule stain; the initial and transitional segments do not appear to express high levels of V-ATPase (54). This is consistent with our understanding of fluid secretion. As discussed above, only the main and lower tubule segments transport fluid, though in opposite directions (171). The initial and transitional segments are active in calcium transport (72, 73), although the vha55 enhancer detector implies that this transport does not require high levels of V-ATPase activity.

E. V-ATPases Are Often Found in Specialized Cell Types

Within the main segment of the tubule, only the larger principal cells express V-ATPase at high level (54); the intercalated stellate cells do not (Fig. 11F). This is of profound importance for our understanding of the tubule, because all previous work had assumed a uniform transporting cell type. It is now possible to assign electrogenic proton transport to the principal cell and to consider the possibility that the stellate cell does something different. This finding has wide scope, because all Diptera (flies) have stellate cells. Other orders of insect do not have obvious stellate cells, but the possibility that morphologically indistinguishable cells may be functionally diverse (as our enhancer trapping has shown for Drosophila; Fig. 6) has not yet been investigated in detail.

There is a striking parallel between the distribution
of plasma membrane V-ATPase in Drosophila tubules and epithelia of other animals: in each case, the powerfully electrogenic V-ATPase is confined to a particular cell subtype within the tissue (34). In lepidopteran midgut, it is confined to the apical membrane of specialized goblet cells (67, 127). In insect sensilla, it is confined to the tormogen cells of the trichoid (“hairlike”) sensilla (128). In kidney collecting duct, it is found at high levels in intercalated cells (4). In epididymis, it is confined to specialized cells that resemble kidney intercalated cells (32). In fish gill, it is localized on the apical surface of pavement cells, while in elasmobranch gill, it is found in mitochondria-rich cells (89, 218, 248). These latter results provide a wide phylogenetic spread, confirming the generality of the observation within the fish. In C. elegans, expression of the 16-kDa proteolipid is confined to the rectum, the excretory H cells, and a pair of posterior cells (174). There thus seems to be abundant evidence that plasma membrane manifestations of V-ATPase are confined to specialized cells within an epithelium (34). Why might this be the case? The highly electrogenic nature of the V-ATPase might make it an uncomfortable neighbor for other transport proteins. The electromotive force for the V-ATPase may be as high as 240 mV (60), and an electrical field of this magnitude might compromise secondary transport processes. The only exception to this rule is where a secondary transport can be energized directly by the proton-motive force generated by the V-ATPase, as will be outlined below; in this case, it is again desirable to have the V-ATPase in a specialized cell to maximize the efficiency of the coupling. This necessity probably explains the unique morphology of the lepidopteran goblet cells (5, 12, 59, 60).

F. The SzA Locus Provides Multiple Alleles of vha55

In a model organism, the ability to reconcile reverse and forward genetic information provides a potential information resource. It may prove that mutations in a particular gene had been characterized by forward genetics, without foreknowledge of the identity of the gene. For vha55, this proved to be the case. Two P-element insertions at 87C were obtained from stock centers, and one was shown to be a disruptant of the vha55 gene (54). This was then shown by complementation (Fig. 12) to be allelic to SzA, a locus previously documented in the region (85). The earlier work provided an exquisitely detailed description of the vha55 phenotype and constituted the first animal “knock-out” of a V-ATPase subunit; it had the additional advantage that the original experimenters had been “blind” to the identity of the gene whose mutant phenotype was being described (85).

The vha55/SzA locus had been subjected to a saturation mutagenic screen, resulting in the identification of 13 ethyl methyl sulfonate (EMS) alleles (a chemical mutagen that typically induces point mutations or very small deletions), together with two deficiencies (deletions) removing the entire gene. More than half of these mutant alleles had been retained and were available for experimentation 10 years later. Although most alleles were recessive embryonic or larval lethal (implying that zygotic V-ATPase function was essential from late embryogenesis onward), some trans-heterozygotes among the alleles were viable, giving a mild (crumpled wings) or severe (crumpled wings, pigmentation, sterility) phenotype. Additionally, two trans-heterozygotes gave a temperature-sensitive phenotype (85).

Two aspects of the phenotype were particularly instructive. Flies homozygous for the large 210-kb deletion Df(3R)kar3D that spanned the whole of 87C, including vha55, died early in the first larval instar. However, the homozygous EMS alleles showed a range of phenotypes from embryonic lethal to subvital. How could some point mutations be more severe than deletions of the entire gene? The answer came later from our understanding of the V-ATPase structure; there are three copies of the B
subunit in every holoenzyme, and as the V-ATPase is thought to act by a rotational model (161), all must be essential for continued operation of the pump. There is presumably a significant maternal investment of V-ATPase in the embryo, which accounts for survival to the early first instar larva. However, if a correctly folded but inactive V-ATPase B subunit were made, it could act as a dominant negative, so producing a more severe phenotype than absence of the gene (54, 61, 65). We predict that this antimorphic phenotype should be general for V-ATPase subunits present in multiple copies in the holoenzyme (for example, A, B, and C), not just in Drosophila, but in any organism.

A second aspect of the Df(3R)kar3J phenotype is also well explained in hindsight. Dying larvae have transparent Malpighian tubules; this is autonomous in transplants of affected tubules to abdomens of healthy flies (85). The lumens of normal tubules usually contain crystals of uric acid, making them look opaque white; like birds, insects employ a uricotelic excretory system for metabolic nitrogen, to conserve water (this is discussed further in sect. xi). The transported species is probably soluble urate, and the Drosophila genome contains two putative urate transporters. To precipitate uric acid within the tubule lumen, it is necessary to bring down the pH, so the apical V-ATPase could be expected to play a crucial role. Mutations in vha55 could thus be expected to be defective in urinary acidification, and thus to have transparent tubules.

1. An epithelial phenotype in larvae homozygous for lethal mutations in V-ATPase

If knock-outs of vha55 produced a transparent tubule phenotype, it seemed likely that this phenotype could be general to mutations in all V-ATPase subunits that played a role in the plasma membrane V-ATPase. A mutation in vha68–1, one of three tandem genes encoding the V-ATPase A subunit, was also shown to display this phenotype (Fig. 13).

This finding has a more general significance for physiologists, as it identifies a screen, not only for other V-ATPase genes that play plasma membrane roles, but for the associated chaperone, targeting and cytoskeletal proteins essential for the plasma membrane state (63). Although there is an elegant pH-sensitive lethal screen for V-ATPase subunits in yeast (160) (that has indeed given us most prototype subunit genes), yeast are devoid of epithelia. To be able to screen for proteins associated with the plasma membrane role of a V-ATPase requires a multicellular model organism, and at present, the screen outlined here is the only one available.

G. A Human Renal and Auditory Phenotype Is Associated With a Plasma-Membrane V-ATPase

The genetic analysis of V-ATPases in Drosophila has produced results at two levels. In the insect sphere, it has shown the role of V-ATPase in development and renal function, outlined those tissues where V-ATPases are expressed at high levels, and those where they may play a previously unsuspected role. At a more general level, the first knock-out of a V-ATPase subunit has shown that V-ATPases are essential in animals and has predicted a crucial role in certain tissues, notably renal, reproductive, and sensory. These predictions have since been borne out by discovery of human mutations in V-ATPase subunits that have renal and auditory phenotypes (121). Although not yet documented in humans, the Drosophila results predict that some mutations may well exhibit dominant negative phenotypes. Although much work has proven
possible in human [for example, mutations of V-ATPase genes expressed predominantly in kidney show a distal tubular acidosis phenotype but not sensorineural deafness (211), so distinguishing V-ATPase roles], human genetics remains serendipitous. It is thus likely that deeper understanding of the plasma membrane V-ATPase complex can more easily be achieved in a model organism.

IX. HOW DO CHLORIDE AND WATER CROSS THE TUBULES?

The insect Malpighian tubule secretes fluid faster than any other epithelium on a per-cell basis (63, 66). Accordingly, one might expect highly specialized adaptations for shunt conductance and water flux. Classically, it has been considered that chloride moved passively through a paracellular route in Dipteran tubule (239). In this section, we develop the argument that, at least in Drosophila, chloride and water conductance are both transcellular through just the stellate cells of the main segment of the tubule. In this way, the active transport sites of high metabolic activity (the principal cells) can be separated from the passive sites of high flux (the stellate cells). This spatial segregation may represent a specialization for very high transepithelial flux rates, or it may be a consequence of the driving force being provided by V-ATPase, a pump invariably consigned to specialized subsets of cells (see sect. xE).

A. Leucokinins Selectively Increase Chloride Conductance

In most insects studied, it appears that tubule chloride shunt conductance is controlled by the tachykinin-like peptide leucokinin (102), discussed in more detail in section xi. This peptide provides an excellent tool for the selective manipulation of the chloride shunt. In Drosophila there is a single leucokinin (Asn-Ser-Val-Val-Leu-Gly-Lys-Lys-Gln-Arg-Phe-His-Ser-Trp-Gly-amide), encoded by the p gene (226). Leucokinin has three separately measurable effects: 1) it acts rapidly on intracellular calcium in just stellate cells (226), with a maximal response within 100 ms and a EC50 between 10^{-11} and 10^{-10} M. 2) Fluid secretion also rises rapidly (as fast as can be measured) with a similar EC50. 3) As in other insects, the target of leucokinin action is thought to be the chloride shunt conductance, as the tubule’s lumen-positive potential is collapsed toward zero on application of leucokinin. This is a rapid event, complete within 1.5 s (170). 4) Consistent with these results, the Drosophila leucokinin receptor has recently been characterized and has been shown to have an EC50 between 10^{-11} and 10^{-10} M, and to act through intracellular calcium, in cultured cells. Additionally, the receptor is only found in stellate cells within the tubule (181).

B. Patch Clamp Reveals Maxi-Chloride Channels in Tubules

If chloride moves transcellularly, then it would be expected that chloride channels with defined pharmacology could be detected. This is the case: fluid secretion is sensitive to a range of classical chloride channel blockers, at concentrations similar to that effective in vertebrate systems (172). In addition, maxi-Cl channels (Fig. 14), with similar properties to those observed in a range of human tissues (36, 110, 156), are found in apical membrane patches (172). These channels are DIDS sensitive, with a large unit conductance of 280 pS (172). Consistent with the action of leucokinin to raise calcium, vertebrate maxi-Cl channels are known to be Ca^2+ sensitive (36, 110, 156).

Maxi-Cl channels could only be found in a small fraction (2/128) of electrically good patches, although they were abundant in each patch where they were found, suggesting that only a small fraction of the apical membrane contained such patches. Naturally, this suggested the stellate cell apical membrane as a candidate.

C. Self-Referencing Electrode Analysis Shows That Chloride Moves Through Stellate Cells

Self-referencing electrode analysis revealed a relatively small number of hotspots along the tubule (Fig. 15A), and these all mapped to the main segment (see sect. xA4), the site of fluid secretion (see sect. viA8). When hotspots were mapped at high resolution, they were invariably found to be centered on stellate cells (Fig. 15B).
In principle, these currents could be carried either by an influx of chloride or an efflux of a cation. However, ion substitution experiments confirmed that an influx of chloride was responsible (Fig. 15C), and this was confirmed by abolishing hotspot current with chloride channel blockers (Fig. 15D). It thus seems clear that, in Drosophila, chloride flows through channels of classical pharmacology, located in the stellate cells of the main segment.

There is support for this transcellular flux model in other species. Recently, chloride channels were shown in Aedes (mosquito) Malpighian tubule, although these were lower conductance than in Drosophila (169). This may reflect a genuine difference in that chloride was considered to flow mainly paracellularly in Aedes tubules (178, 239). In Drosophila, additional routes for chloride flux have been proposed. A bumetanide-sensitive K⁺/Cl⁻ co-transport was demonstrated in basolateral membrane of principal cells (142); as this is electroneutral, its presence would not be detected by vibrating probe. A sodium-dependent Cl⁻/HCO₃⁻ exchanger, NDAE1, has been cloned and shown to be expressed basolaterally in Malpighian tubule (206). The relative contribution of this exchanger to tubule secretion has not yet been determined. The relative significance of these routes for chloride flux has not been established; however, Cl⁻ channel blockers abolish transport (172), whereas bumetanide only attenuates it (142), implying that, in Drosophila, the stellate route predominates.

D. Tubules Contain CLC Chloride Channels

The molecular identity of the chloride channels in tubules is not yet known. However, an advantage of working in a model organism is that candidate genes can be reliably identified in silico. The Drosophila genome contains three chloride channels (CG6942, CG8594, and CG5284) of the CLC family (Fig. 16). They are evenly spread among the major branches of the human CLC family, suggesting that single genes cover the major roles of the family. There is a clear pattern that the Drosophila genome will frequently have a single gene, perhaps with multiple transcripts, corresponding to typically three similar human genes.

If one of these genes were expressed uniquely in tubules, that would be strong evidence for a candidate locus for an epithelial chloride channel. However (Fig. 16), all three are expressed in tubule. So further analysis will be needed to establish which may play epithelial roles.

E. Bartter Syndrome Type III

Although there are not any extant mutants of the three CLC genes, human studies of CLC mutations may in this case be informative for Drosophila. Bartter syndrome is an autosomal recessive form of (often severe) intravascular volume depletion, due to renal salt-wasting. Patients with Bartter syndrome are often critically ill from birth onward, and their long-term clinical course is also frequently complicated by nephrocalcinosis, leading to renal failure (115, 188). The syndrome encompasses three major classes of mutation in renal transporters or channels; Bartter syndrome type III corresponds to mutations in the CLCNKB gene, encoding a kidney-specific CLC, that impair renal chloride reabsorption in the thick ascending limb of Henle’s loop (209). Given the relative facility with which genes can be mutated in Drosophila, it will be interesting to establish whether similar symptoms are associated with mutations in any of the Drosophila CLCs.

In FIG. 15. Chloride flows through stellate cells. A: vibrating probe survey of an anterior tubule, initial segment to the left and ureter to the right. Current hotspots are confined to the main segment of the tubule. B: higher power view of a current hotspot, showing the currents are centered over a stellate cell. C: chloride substitution experiment, showing that current is carried by chloride. D: niflumic acid, a chloride channel blocker, abolishes chloride current hotspots. Numbers show the current at different times (in min) from the application of niflumic acid. [From O’Donnell et al. (172).]
F. How Does Water Cross the Tubule?

An interesting “back-of-the-envelope” calculation: the volume of cytoplasm in the active region of the tubule is \( \sim 1 \text{ nl} \), and the maximum secretion rate we have observed is 6 nl/min. This means that the fully stimulated tubule pumps its own volume every 10 s! This is very similar to the result obtained for the Rhodnius tubule (153), implying this rate is a characteristic of insect tubules. Such extremely high flux rates pose potential problems. Could cells withstand such high transit rates in the steady state? Could they withstand volume transients as transport was up- or downregulated? There is clearly a benefit in protecting the actively transporting principal cells from rapid fluxes. This could be achieved either by paracellular flux or by aquaporin-mediated flux through a subset of cells in the epithelium. There is no rigorous information yet, but fluid secretion by tubules is known to be sensitive to mercuric ions and organo-mercurials, the best available inhibitors of aquaporins. In silico, it is possible to identify a large gene family of *Drosophila* aquaporins, and most of these are expressed in tubules. Consistent with this, stellate basolateral membrane is stained prominently by antibodies to human aquaporin-3 (Fig. 17) or to buffalo fly aquaporin. If this localization were confirmed, it would provide an elegant solution to the problems of withstanding high flux rates through the tubule.

G. Cation and Anion Transport Are Spatially Separated

It is thus clear that active, electrogenic cation transport is spatially segregated from passive chloride shunt...
conductance, and probably from water flux, in this epithelium. This may confer particular advantages in the spatial optimization of a tissue subjected to uniquely high flux rates, as the cell with intense transport activity, and commensurate intense metabolism, can be shielded from a massive transcellular flux that might compromise its function. Chloride, and we hypothesize also water, could then flow through a far less metabolically active cell, which has the less onerous task of merely staying alive. This is reminiscent of other systems in which the V-ATPase is hypothesized to play a plasma-membrane role and is found in specialized cells (34).

X. PHARMACOLOGY AND CELL SIGNALING MECHANISMS
A. Cell-Specific Calcium Signaling

Calcium signaling and transport processes regulate cellular processes and contribute significantly to renal function (82); furthermore, kidney disease is often associated with channel abnormalities that affect epithelial transport (203). However, there is not a thorough understanding of the complex contribution of calcium homeostasis to vertebrate renal function due to the lack of genetic models for fluid-transferring epithelia. The importance of the Drosophila tubule in such studies is clear: 25–30% of the human proteome constitutes signaling and transport proteins (110a); most of these will have Drosophila tubule homologs. Furthermore, at least 11 Drosophila genes show significant similarity to human renal disease genes (194), suggesting the utility of the tubule in studies of signaling and renal disease.

The importance of calcium signaling and transporting mechanisms in the tubule is demonstrated by the storage of calcium concretions in tubules in many species, including Drosophila (243), Rhodnius (149), and Calliphora (222). Furthermore, Malpighian tubules contain the highest amounts of calmodulin compared with other tissues (249). This suggests that tubules are the main site of calcium storage, and thus may also be the tissue with the highest turnover of calcium (223). More recent work in Drosophila has shown that anterior tubules contain 25–30% of the calcium content of the whole fly (73). Thus, apart from its role in osmoregulation, the tubule is critical to the maintenance of whole body calcium homeostasis.

To define the contribution of calcium signaling processes to renal function, it is necessary to obtain direct measurements of intracellular cytosolic Ca\(^{2+}\) levels ([Ca\(^{2+}\)]\(_i\)). Calcium-binding fluorescent dyes are extruded from insect tubules very rapidly, thus preventing measurements of [Ca\(^{2+}\)]\(_i\) (J. A. T. Dow and T. Cheek, unpublished observations). The extrusion of fluorescent molecules by tubules is thought to be due to the activity of P-glycoprotein, or a related transporter (29). Furthermore, tubule cells are too small to allow reliable measurements by ion-selective microelectrode impalements to be made. Accordingly, a targeted Ca\(^{2+}\) reporter was developed in Drosophila to allow organotypic [Ca\(^{2+}\)]\(_i\) measurements in intact, viable tissue. The luminescent jellyfish...
Aequorea victoria utilizes a photoprotein, aequorin, and produces flashes of blue light that are transduced to green by green fluorescent protein. Critically, the intracellular signal used to elicit this flash in vivo is an increase in \([\text{Ca}^{2+}]_i\). Because the dynamic range of \([\text{Ca}^{2+}]_i\) concentrations in jellyfish is very similar to those in plants and animals (generally 50–500 nM), this means that aequorin is a useful calcium indicator.

Transgenic aequorin was first used in plants (130), but the UAS\(_G\)-GAL4 system for targeted expression (see sect. \(\pi A7\)) allowed a generic indicator to be constructed that could be driven by any GAL4 line of interest. Transgenic *Drosophila* that express (apo)aequorin under UAS\(_G\) control were generated; these lines were crossed into lines expressing appropriate tubule-specific GAL4 drivers (214). This results in expression of the aequorin transgene in specified tubule cell types or tubule regions (190) (Fig. 18), which allows analysis of calcium cycling mechanisms in vivo.

Monitoring of \([\text{Ca}^{2+}]_i\) increases induced by the ER \(\text{Ca}^{2+}\)-ATPase inhibitor thapsigargin, in either principal or stellate cells (Fig. 19), showed that only stellate cells maintained this response in calcium-free medium. This result indicates that in principal cells, the thapsigargin-induced \([\text{Ca}^{2+}]_i\) rise is dependent on extracellular calcium and that calcium stores are emptied too rapidly to be monitored, or that these are too small. In stellate cells, however, significant calcium extrusion from ER stores occurs, which suggests a role for “capacitative calcium entry” in this cell type. The novel and important observation in *Drosophila* that adjacent cells in transporting epithelia may operate distinct calcium cycling mechanisms may well extend to vertebrates; however, this can only occur when technological limitations have been overcome.

### B. Neuropeptide-Stimulated Calcium Signaling

Successful targeted expression of aequorin in *Drosophila* allowed the first organotypic measurements of

![Fig. 18. Cell-specific real-time calcium measurement using UAS-directed apoaequorin in *Drosophila*.](image-url)
tubule $[\text{Ca}^{2+}]$, in any insect. These experiments demonstrated a novel role for the nitridergic neuropeptide CAP$_{2b}$ [pyro-ELYAFPRVamide (53) and below] in renal calcium signaling (190) and showed that CAP$_{2b}$ stimulated an increase in $[\text{Ca}^{2+}]$, in a dose-dependent manner, which only occurred in principal cells in the main, fluid-secreting segment. Elevations in $[\text{Ca}^{2+}]$ were correlated to CAP$_{2b}$-induced increases in fluid transport rates. Furthermore, both CAP$_{2b}$-induced $[\text{Ca}^{2+}]$, and secretion are dependent on extracellular calcium (Fig. 20).

The diuretic neuropeptide leucokinin induces fluid secretion via activation of calcium signaling mechanisms in several insect species (63). However, direct demonstration of the activation of calcium signaling was achieved using targeted aequorin in Drosophila tubules (190). Leucokinin stimulates a rapid millisecond rise in stellate cell...
[Ca\(^{2+}\)]_i from a resting concentration of 80–200 nM (172).

Leucokinin also activates chloride shunt conductance in stellate cells, and chloride channel blockers inhibit leucokinin-stimulated fluid secretion (172). More recently, the gene encoding Drosophila leucokinin, \(pp\), was identified, and the Drosophila peptide (Drosokinin) isolated (226). Expression of \(pp\) has been detected in Drosophila heads (226), suggesting that Drosokinin is synthesized in the central nervous system and released into the hemolymph. Drosokinin elevates [Ca\(^{2+}\)]_i in only stellate cells at an EC\(_{50}\) between 10\(^{-10}\) and 10\(^{-11}\) M to induce high rates of fluid secretion.

C. Calcium Channels

Both anterior and posterior tubules in Drosophila perform transepithelial calcium transport (72). However, the demonstration of a large basolateral calcium flux (entry of calcium into cells via the basolateral membrane) compared with a smaller transepithelial flux (transport of calcium across the epithelium into the lumen) suggests that most of the Ca\(^{2+}\) entering the cell is sequestered within it. Thus plasma membrane calcium entry channels must play an important role in maintaining both calcium and fluid transport by the tubule. Recently, L-type calcium channels, most often associated with neuronal cells (9) in both insects and vertebrates, have been shown to modulate epithelial fluid transport. Similarly, L-type calcium channels are found in vertebrate kidney cells and are associated with calcium reabsorption (256), transepithelial calcium flux (83), and mechanical stress (257); thus L-type channels are not confined to excitable cells.

There are three L-type \(\alpha\)-subunit genes in the Drosophila genome: \(Dmca1A\) (night-blind), \(Dmca1D\), and a novel gene, \(CG1517\). The classical L-type channel blockers, verapamil, and the dihydropyridine nifedipine inhibit CAP\(_{2b}\)-stimulated fluid transport and calcium influx in principal cells (146). The inhibition of response by vertebrate channel blockers suggests conservation of channel function during evolution. Immunolocalization of \(\alpha\)-subunits indicates expression only in main segment, both at the basolateral and apical surface. Use of fluorescent conjugates of verapamil and nifedipine in unfixed, intact tubules reveals both high- and low-affinity sites for verapamil binding in main segment, with low-affinity sites present in basolateral membranes and high-affinity sites located in apical microvilli. However, a major site of dihydropyridine binding occurs in the initial segment, a major site of calcium transport and storage (72, 73). Thus L-type calcium channels not only facilitate neuropeptide-
mediated transport and calcium signaling in principal cells, but it is likely that these also have a role in trans-epithelial calcium transport.

Most recently, a novel epithelial role for the transient receptor potential (TRP) family of calcium channels (94) has been described. TRP represents a highly calcium-selective cation channel, whilst trpl (trp-like) encodes a nonselective cation channel with moderate calcium permeability. The TRP channel family was first identified in Drosophila, and ~20 mammalian TRP proteins have since been identified, which fall into at least three subfamilies. Much of our current understanding of the functional role of TRP in vivo is derived from Drosophila photoreceptors (93), where the light-sensitive current in photoreceptors is completely abolished in trpl:trp double mutants lacking both TRP and TRPL (162, 185). Response to light is dependent on the close interaction of TRP and TRP-related channels with other signaling proteins (rhodopsin, phospholipase C, protein kinase C, calmodulin) mediated through the scaffolding protein INAD (inactivation no-afterpotential D) (93). Recent work has shown that INAD is required for correct localization of TRP-containing supramolecular complexes in the eye (140). Additionally, a newly identified third member of the TRP family, TRP-y, may form heteromultimers with TRPL (251). While much of the work on vertebrate TRP channels has been predominantly based in cultured cell models, the work on Drosophila TRP/TRPL has been performed almost exclusively in an organotypic context.

The role of TRP and TRP-like channels in nonvisual systems is poorly understood. Recently however, a role has begun to be elucidated in transporting epithelia (147). Molecular data show that genes encoding TRP, TRPL, and TRP-Y are expressed in Drosophila tubules. TRP has been immunolocalized to main segment, while TRPL is located throughout the tubule, suggesting roles for TRPL in both calcium transport and signaling. Using mutant alleles of trp and trpl, we have shown that unlike phototransduction, which is dependent on TRP, neuropeptide-stimulated fluid transport is critically dependent on TRPL. Significantly, tubules do not express INAD, suggesting that while transporting epithelia may utilize the same proteins as excitable cells, the complexes formed, if any, are distinct. Also, as INAD has been shown to be critical for TRP function in the eye, the work in tubules suggests that this must be a tissue-specific, rather than a general, requirement for TRP function.

Thus, while the study of calcium signaling events in an organotypic context may be intrinsically difficult, the insights generated are particularly valuable and only obtainable in this way. It is clear that renal epithelia utilize a range of calcium signaling mechanisms that are at least as complex as those in the neuronal context and that these operate in concert with the reabsorption and trans-epithelial transport of bulk calcium.

D. NO/cGMP Signaling

The mobilization of NO/cGMP signaling is a potent mechanism for the control of cell and tissue function. The NO signaling pathway is implicated in neuronal, immune, vascular, and renal function in vertebrates and is so highly conserved throughout evolution that these physiological processes are also NO regulated in insects (52). However, very little is known about NO-mobilizing peptide hormones, especially in relation to renal function. However, the role of cGMP in kidney function, including renin secretion, is beginning to be understood, especially in relation to guanylin action (81). Furthermore, the cellular distribution of NO synthase (NOS) isoforms (13) and soluble guanylate cyclase, cellular targets for NOS-generated NO, have been mapped in kidney (228), although direct demonstrations of NO/cGMP modulation of renal function have not been performed in vertebrates.

In insects, application of exogenous cGMP has been shown to modulate diuresis, in either a stimulatory (Drosophila) (57) or inhibitory (Rhodnius) (180) capacity. The stimulatory role of NO-generated cGMP has also been documented in tubules, via use of NO donors (57).

The only known example of nitridergic peptides in insects to date remains the capa family of peptides. We have identified the gene capability (capa) (123), which encodes three capa peptides, two of which (capa-1, GAN-MGLYAFPRV-amide; and capa-2, ASGLVAFPRV-amide) are structurally and functionally similar to CAP2b. Both capa-1 and capa-2, like CAP2b, stimulate fluid secretion via [Ca2+]i elevation in principal cells. All three peptides activate Drosophila NOS (DNOS), which results in elevation of cGMP levels. Moreover, capa-induced fluid transport is significantly reduced by methylene blue, an inhibitor of soluble guanylate cyclase, supporting the previous observation that NO-generated cGMP plays a significant role in diuresis (53). Four pairs of capa-producing neuroendocrine cells have been identified, one pair in the subesophageal ganglion and the other three in the abdominal neuromeres; this suggests that the capa peptides are released into the hemolymph and act as endogenous neuropeptide modulators of NO signaling (123).

The NO/cGMP signaling pathway has been extensively investigated in tubules. Tubules express DNOS, encoded by a single gene that shares extensive identity with vertebrate NOS1 (52). Intriguingly, NOS is only expressed in principal cells (52). At physiological concentrations, capa-induced increases in intracellular calcium and cGMP signaling are confined to principal cells (123); this, together with the principal cell localization of NOS, suggests that capa-activated NO is an autocrine signal in this cell type.

Investigation of guanylate cyclase (GC) expression in tubule shows that several transcripts, encoding soluble (sGC) and membrane-bound receptor (rGC) forms of the
enzyme are expressed. These are Gycalpha and Gycbeta, encoding α- and β-subunits of sGC, and a novel rGC gene, CG1073 (V. Pollock, J. A. T. Dow, and S. A. Davies, unpublished data). Thus tubules contain bona fide intracellular targets for NO action.

Downstream targets for cGMP include cGMP-dependent protein kinases (cGKs) and cGMP-dependent phosphodiesterases (cG-PDE). Tubules express two genes for cGK, dg1 and dg2 (57). Interestingly, mutations in dg2 result in a transport phenotype which displays hypersensitivity to cGMP (Fig. 21) but not to leucokinin signaling, which is confined to stellate cells. Thus cGK-induced modulation of fluid transport is a result of signaling events in the principal cells in the main segment. In this cell type, the ultimate target of cGMP and CAP2b in tubule may be the V-ATPase (see sect. ix), because an increase in transepithelial potential is associated with cGMP or CAP2b treatment (53).

Inhibitors of PDE activity also affect fluid secretion rates; Zaprinast, which inhibits cG-PDE (PDE5) in vertebrates, accelerates responses in fluid transport when applied to tubules (68). Thus downstream activation of cG-PDE is an important control mechanism for fluid transport rates.

E. Cross-talk Between NO/cGMP and Calcium Signaling

Interactions between cGMP and calcium signaling pathways have been observed in several vertebrate systems, including the visual system (133) and smooth muscle (1). However, although the role of cross-talk in renin secretion has been under investigation (14), the direct effects of cGMP/calcium signaling mechanisms in a renal context have not been fully explored.

In tubules, however, exogenous cGMP has been shown to induce a slow rise in \([\text{Ca}^{2+}]_i\) levels in principal cells, which is dependent on extracellular calcium (148). This calcium signal is verapamil and nifedipine sensitive. Interestingly, cGMP-induced fluid transport is also sensitive to these L-type calcium channel blockers, which suggests that calcium signaling mechanisms contribute to cGMP-induced fluid transport. Expression of the Drosophila gene encoding a cyclic nucleotide gated channel, cng, has been demonstrated in tubules, and it is possible that cGMP-induced transport and calcium responses occur via a CNG channel (Fig. 22). Drosophila CNG is most closely related to vertebrate CNG3, which is highly expressed in non-sensory tissue, including kidney (107). Thus a role for CNG in transporting epithelia is conserved across evolution.

In summary (Fig. 22), the Drosophila tubule contains all the signaling components of the NO/cGMP pathway and remains the only transporting epithelium in which a direct role of NO/cGMP on ion transport has been demonstrated.
F. New Targets for Drug Discovery

Although most pharmaceutical research is at present devoted to analysis of gene products in simple cell-culture models, there is a wide range of diseases where candidate genes may rely for their normal function on other gene products. In this case, analysis of the candidates demands a heterologous expression system that can offer an organotypic context in which the transgene could be expressed. In tubules, there exists a panel of enhancer trap lines that can direct expression of any gene of choice to subsets of cells within the tubules, allowing us to study the physiological phenotype of mutant genes in the context of an otherwise normal fly (see sect. VIII A7), exactly as we proposed to be required for integrative physiology (see sect. III). In particular, the system can be used to express human transport and cell signaling genes ectopically in Drosophila tubules, allowing their mutagenesis and further detailed study. So far, we have successfully expressed two classes of G protein-coupled receptor in tubule cells and have shown that fluid secretion by such transgenic tubules is then sensitive to the appropriate ligands (125). This offers the opportunity both to study the human genes in a heterologous, yet epithelial, context and to manipulate second messenger pathways in specified cell subpopulations of an epithelial model.

G. Insect Tubules as Targets for Selective Insecticides

Insect tubules have long been identified as mission-critical parts of an insect’s physiology that might be good targets for insecticides. Our work has shown that Drosophila tubules share close functional similarity with those of all the 30 or so species of insect so far studied. In particular, their function is effectively identical to those of other Diptera. This means that Drosophila tubules provide a useful model for those of major medical and agricultural pests, such as mosquitoes and tsetse flies, in advance of any transgenic technologies that may be developed for their detailed study later. They can thus act as a proxy for species like Anopheles gambiae with a recently completed genome (27), but technically challenging transgenesis (10). This is discussed in section VIII B.

XI. IN Silico Mapping of Metabolic Pathways

In an organism with a sequenced genome, it is possible to investigate problems at a theoretical level with a precision not heretofore possible. As an example, we will consider the question of purine metabolism. As outlined in section VIII F1, terrestrial insects are considered to have a uricotelic excretory system, in which metabolic nitrogen from the purine pathway is excreted as insoluble uric acid, thereby saving water. However, in practice, terrestrial insects diverge from this ideal: they can excrete the bulk of their nitrogen as a range of compounds, including uric acid, allantoin, urea, and ammonia (43). There is no obvious phylogenetic or life-style trend underlying the preferred compound. In Drosophila, the question is particularly intriguing, as it has a uric oxidase (uricase) gene, that catalyzes the conversion of uric acid to allantoin (237). Urate oxidase (UOX) is known to be expressed in adult eye and in main segment principal cells of Malpighian tubules of third instar larvae and adults only (84). The role in eye is specialized: storage excretion of uric acid allows a white reflective lining for each ommatidium, and UOX presumably plays a role in regulating the deposition of uric acid. The tubules are probably the major sites of nitrogenous excretion and are known to be loaded with uric acid crystals. So why is UOX expressed, and why at only certain times of life? The situation is complicated further by work on related species: in Drosophila pseudoobscura, UOX is expressed only in adult, whereas in Drosophila viridis, it is expressed only in third instar larva (238). To explain this apparently random assortment of facts, it is helpful to take a comparative view of purine metabolism in mammals.

A. Human, Mouse, and Gout

In mammals, there are two parallel pathways for purine metabolism: UOX (usually concentrated in the ribosomes of the liver) and hypoxanthine guanine phosphoribosyl transferase (HPRT). In humans and other primates, UOX is a single copy gene, rendered dysfunctional by two mutations. Accordingly, purine catabolism is through the HPRT pathway, and disruption of this pathway leads to Lesch-Nyhan syndrome, with mental retardation, cerebral palsy, choreoathetosis, uric acid urinary stones, and self-destructive biting of fingers and lips (136). Partial deficiency can lead to Kelley-Seegmiller syndrome, featuring renal stones, uric acid nephropathy, renal obstruction, and gout (124). In contrast, in rodents, UOX is functional and the HPRT pathway is insignificant. Accordingly, mutation of rodent UOX leads to neurological symptoms highly reminiscent of Lesch-Nyhan syndrome (250). These findings show that there are alternative, potentially redundant, pathways of purine catabolism; it is only the overall function that is necessary for life.

B. Drosophila, Mice, Humans, and Gout

In the three Drosophila species, it is highly unlikely that UOX is essential, since closely related species express it at different life stages and have a visibly func-
tional uric acid excretory pathway. The most likely explanation is that the UOX pathway to allantoin provides extra capacity at life stages appropriate to the organism’s lifestyle. Insects generally feed most intensively in their last larval instar, and scaling arguments imply that problems in excretion are exacerbated by size. It is thus not unreasonable to assume that this semi-redundant pathway gives a slight selective advantage that is decisive in wild populations.

An alternative explanation, offered for both human and Drosophila, is that urate is protective under conditions of oxidative stress. There is experimental evidence in favor of the model in Drosophila: mutants deficient in synthesis of urate showed reduced tolerance to oxidative stress from any of three sources (paraquat, ionizing radiation, and hyperoxia) (104). Furthermore, synthetic lethality was obtained by crossing urate-deficient flies with superoxide dismutase (SOD) mutants, implying that both pathways provide partial protection against oxidative stress (104). This is only a partial explanation, because it still does not explain why different, closely related species of Drosophila have very different expression patterns, or why just the tubules and eye should need to be afforded such extra protection. However, these results show that urate provides real protection against oxidative stress in Drosophila.

C. Disorders of Purine Metabolism

Obviously, it would be of interest to extend the mutational data on UOX to Drosophila, but there are no known Drosophila UOX mutants. However, purine metabolism is a multistep pathway, and it is reasonable to assume that mutations at other key steps in the pathway might elicit similar symptoms to a UOX blockade. In vertebrates, urate is generated by xanthine oxidase, and defects in this enzyme lead to xanthinuria type I (55), characterized by low serum urate and very high levels of urinary xanthine, frequently forming yellowish stones. Thus, in the absence of urate oxidase to the kidney, such as urethral lithiasis (154) and hydronephrosis (155). In Drosophila, severe rosy mutants have shortened and malformed Malpighian tubules that contain yellow to orange-colored globular inclusions in the lumen (92). Like their human counterparts, ry flies are sensitive to dietary purines (198). Untreated, severe ry alleles are semilethal and temperature sensitive. It is thus clear that, despite apparently gross differences between their anatomy, the cause and symptoms of xanthinuria type I are conserved between human and fly.

However, there is an added value to fly work compared with human observational studies. In humans, the penetrance of mutant phenotypes can vary widely, confounding systematic study. This is presumed to be because of variation at other predisposing loci. In Drosophila, it is possible to test for interactions between loci by controlled crossing. Accordingly, rosy mutations have been crossed to similar eye color mutants, and synthetic lethality has been observed in crosses with cinnamon (cinn), low xanthine dehydrogenase (lxd), and maroon-like (mal) (87). The molecular identities of these loci are not all known, but they presumably encode cofactors of rosy that together are essential for normal function. Such results may thus provide novel insights into the distressing group of human xanthine metabolism disorders (7).

D. Does Drosophila Have a Xanthine Oxidase Mutant?

Interestingly, Drosophila does have a xanthine oxidase mutant; it is the eye-color mutation rosy (ry), first characterized by Bridges, and the subject of 612 papers. The phenotypes of human and fly mutations are strikingly similar. In human patients, there are low serum and urinary levels of urate and very high levels of xanthine, causing formation of yellowish xanthine calculi (55). These frequently cause distortion and obstructive damage to the kidney, such as urethral lithiasis (154) and hydronephrosis (155). In Drosophila, severe rosy mutants have shortened and malformed Malpighian tubules that contain yellow to orange-colored globular inclusions in the lumen (92). Like their human counterparts, ry flies are sensitive to dietary purines (198). Untreated, severe ry alleles are semilethal and temperature sensitive. It is thus clear that, despite apparently gross differences between their anatomy, the cause and symptoms of xanthinuria type I are conserved between human and fly.

E. Virtual Metabolomics: an Index of Possibilities

The principle illustrated here with xanthine oxidase and urate oxidase in Drosophila can readily be extended. It is possible to sketch out in silico a potential metabolic pathway for purines, or for any pathway of interest, and interrogate the genome to see whether any candidate gene exists for a given step in the pathway. This principle suggests fast and easy experiments, for example, primer design for RT-PCR, to test which elements of a pathway are present in a tissue of interest. Although this procedure falls far short of a proof that a particular pathway operates as outlined in that tissue, the absence of a particular gene in the genome, or the absence of expression of a gene at levels detectable by PCR, would be powerful negative evidence. This technique, which we name “virtual metabolomics,” gives rapid insights into an index of metabolic possibilities in a tissue of interest. It is, of course, only as authoritative as the genome being analyzed, and so is presently most useful in human and other genetic model organisms for which complete sequence is compiled.

The application of this technique to the purine metabolism pathway in Drosophila is shown in Figure 23.

This analysis can be reconciled with physiological data. At different stages in its life cycle, different genes are expressed. Thus, in the absence of urate oxidase (expressed only in third instar larvae and adults), it is not
possible to form allantoin, so uric acid is likely to be the only by-product. In contrast, in third instar and adult, allantoin can be produced, and the presence of an allantoinase implies that urea could be formed.

The analysis also highlights the impact of mutations in diverting metabolites with pathological consequences. In rosy flies, xanthine oxidase is absent, so xanthine accumulates to levels that produce xanthine stones. When V-ATPase is defective, then although urate is formed, it does not precipitate as luminal uric acid.

F. Eye Color Mutants Have Interesting Causes

The first Drosophila mutation described (white) was for eye color (158); appropriately enough, the gene encodes an ABC transporter with both eye and Malpighian tubule transport phenotypes, and now over a hundred distinct loci are known that produce an eye color phenotype. Interestingly, many of the eye color mutants have tubule color phenotypes (242); indeed, most classical Drosophila geneticists would consider the tubule as a
larval indicator that predicts adult eye color. The reason for the congruence between eye and tubule is not entirely clear, but it may reflect an original excretory function of the pathways that generate and transport the major classes of metabolite (omnochromes and pteridines) that were subsequently pressed to a specialized role in pigment granule formation. [An alternative explanation, that purine metabolism may allow urate to accumulate and perhaps provide protection against oxidative stress, has been outlined above (104).]

With the benefit of the genome project, it has recently proven possible to reexamine the massive literature on eye color mutations (there are 2,374 references for the white gene alone) and assign them to one of three broad classes. These are as follows: metabolic/biosynthetic enzymes that form the pigments or their precursors; transporters, frequently of the ABC class, that move pigments or precursors to their targets; and vesicle trafficking proteins, which place the pigment granules in their correct destinations (see Table 3).

One of these categories is particularly topical, as vesicle trafficking is an area of intense biomedical interest. For example, garnet encodes a δ-subunit (143), carmine a ω3-subunit (159), and ruby a β-subunit (134) of the recently discovered AP-3 adaptin complex. It is thus pleasing to think that a valuable experimental resource may be provided in the examples above, by direct descendants of flies first isolated in 1916 (33), 1927 (157), and 1910 (C. B. Bridges, unpublished data).

However, there is a further twist: the rekindled interest in vesicle trafficking has focused on the eye phenotype, rather than the tubule. This reflects both the overt nature of the eye phenotype and the widely held Drosophila perception that only the brain is a respectable alternative topic to development in flies. However, this perception was not always held; the early papers on eye color mutants invariably sought tubule as well as eye phenotypes. This open-mindedness is entirely laudable, as the insect eye is a highly specialized and unique tissue; perhaps the more general and physiologically relevant information will be gained from studying vesicle trafficking in a differentiated, transporting epithelium? Our view of epithelial function increasingly emphasizes the importance of shuttling differentiated classes of vesicles, carrying precisely determined cargoes, to specific targets (44, 88, 144, 216). For example, antidiuretic hormone triggers the translocation of aquaporin-2-containing vesicles to the plasma membrane of renal cortical collecting duct, a signal that is compromised in diabetes insipidus (232). At present, much work on membrane trafficking is performed in cell lines (144, 216) that have manifest differences from the differentiated epithelial state. Perhaps the tubule will be a good place to study some of these translocation and targeting processes. After all, some of the vesicles are even color-coded.

**XII. DROSOPHILA AS A MODEL FOR HUMAN DISEASE**

It is not news that the simple fruitfly, Drosophila melanogaster, is a useful model for humans, both in health and disease. However, nearly all Drosophila groups in the world study developmental biology, and most of the remainder, brain and behavior. So it is not surprising to find that the similarities between these very different organisms (which diverged 400 million years ago) have been drawn most compellingly in the context of developmental biology. However, the purpose of this review is to develop an alternative position, that useful parallels can be drawn in renal function and epithelial biology. These are areas of great medical significance, but the similarity of these two organisms has only been hinted at previously (132).

Despite huge publicity for each success, surprisingly few genes have been identified as causes of human genetic disease to date. However, a very large fraction of these genes have homologs in genetic model organisms. These similarities have been curated (46, 184) and are available on the internet (http://homophila.sdsc.edu/). [A similar curation for zebrafish has been published (208).]

Of 929 known human genes with at least 1 mutant allele, 714 (74%) had closely similar matches (P < 10^{-10} by BLAST search) in Drosophila. Of these, a useful fraction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Eye Color</th>
<th>Tubule Color</th>
<th>Function</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>brown</td>
<td>Brown</td>
<td>Slightly paler than wild type</td>
<td>ABC transporter for guanine and xanthine</td>
<td>217</td>
</tr>
<tr>
<td>claret</td>
<td>Deep reddish yellow</td>
<td>Colorless</td>
<td>Required for uptake of radiolabeled kynurenin</td>
<td>217</td>
</tr>
<tr>
<td>carnation</td>
<td>Deep ruby</td>
<td>Pale yellow</td>
<td>Vacular sorting protein, vps3</td>
<td>21</td>
</tr>
<tr>
<td>carmine</td>
<td>Carmine</td>
<td>Very pale yellow</td>
<td>Chaperone: clathrin adaptor protein AP3 subunit μ3</td>
<td>157</td>
</tr>
<tr>
<td>cinnabar</td>
<td>Bright scarlet</td>
<td>Pale yellow</td>
<td>Kynurenine 3-monoxygenase</td>
<td>31</td>
</tr>
<tr>
<td>deep orange</td>
<td>Orange</td>
<td>Nearly colorless</td>
<td>Vacular membrane protein PEP3</td>
<td>26</td>
</tr>
<tr>
<td>garnet</td>
<td>Brownish</td>
<td>Colorless</td>
<td>Adapin</td>
<td>33</td>
</tr>
<tr>
<td>Henna</td>
<td>Brown to black</td>
<td>Wild type to deeper</td>
<td>Phenylalanine/tryptophan monoxygenase</td>
<td>20</td>
</tr>
<tr>
<td>light</td>
<td>Yellowish pink</td>
<td>Colorless</td>
<td>Vacular assembly protein vps41</td>
<td>56</td>
</tr>
<tr>
<td>lightoid</td>
<td>Clear, light, pink</td>
<td>Colorless</td>
<td>Likely kynurenine transporter</td>
<td>217</td>
</tr>
</tbody>
</table>

TABLE 3. Some examples of Drosophila eye color mutants for which the loci have been characterized
had extant mutants: 153 had known alleles, and a further 56 had nearby P-elements that might be useful. Interestingly, although most work in Drosophila has focused on development, most of the cross-matches in the Homopila database are for metabolic, signaling, or transport enzymes. There is thus ample scope for new work in Drosophila.

A. Can Drosophila Cure Human Disease?

A critic of the simple model approach for integrative physiology might argue that no human disease has been cured by such studies. However, this is a little disingenuous, because the vast majority of funded biomedical research neither cures nor sets out to cure disease. It is acknowledged that most research is fundamental, aimed at understanding the causes of disease, so it would be unreasonable to apply dual standards. Obviously, it would not be appropriate (for example) to model the interaction of vasopressin and the baroreceptor reflex system in the regulation of arterial blood pressure in a fly, as this is too narrowly phrased a question. However, there are plenty of examples of human disease models where insights from simple organisms are likely to accelerate understanding or reduce animal use (Table 4).

B. How Different Can You Be and Still Be the Same?

A common criticism of the extension of a comparative physiological approach to invertebrates is the lack of conservation of body plan. Insects, for example, lack backbones; they are not mammals; and they lack a developed vascular system. However, all eukaryotic organisms must accomplish similar functional tasks to stay alive. The human and Drosophila renal epithelia perform fundamentally analogous tasks, and this review should have provided sufficient evidence for useful commonality in function. However, the similarities between disparate phyla have more spectacular exemplars. For example, the visual systems of fly and humans are about as dissimilar as it is possible to be. The former is a faceted eye in which hundreds of rhabdomeres have distinct but overlapping visual fields, each lined with multiple photoreceptor cells in a precisely deterministic pattern (Fig. 24, B and C). The latter has a single transparent lens, two fluid-filled spaces, and a complex, multilayered retina. However, there is remarkable commonality in the genes that specify eye development. In both fruitfly and human, there are several single-gene mutations, each capable of blocking eye formation. In humans, these rare syndromes are known as microphthalmias. Accordingly, these genes are considered

### Table 4. Some recent examples of Drosophila models of utility in the study of human disease

<table>
<thead>
<tr>
<th>Drosophila Model</th>
<th>Human Disease</th>
<th>Utility of Model</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hedgehog</strong></td>
<td>Holoprosencephaly (and others)</td>
<td>A signaling pathway of major interest to the fly community. Accordingly, mutants available in all points of the pathway predict value for identification of human genes, interactions</td>
<td>15</td>
</tr>
<tr>
<td><strong>ninaD</strong></td>
<td>Blindness through vitamin A deficiency</td>
<td>NinaD encodes a class B scavenger receptor for carotenoid uptake. NinaD mutants are blind. Predicts similar phenotypes for human SR-B1 or CD36</td>
<td>126</td>
</tr>
<tr>
<td><strong>Mechanoreception</strong></td>
<td>Deafness</td>
<td>Aspects of mechanoreception are conserved from worm to human</td>
<td>114</td>
</tr>
<tr>
<td><strong>Flies transgenic for triple repeat a-Synuclein transgenic flies</strong></td>
<td>Huntington’s disease (and triplet expansion diseases)</td>
<td>Basic understanding, but also allow identification of modifier loci through suppressor screens</td>
<td>42, 103, 122, 195, 210</td>
</tr>
<tr>
<td><strong>Flies transgenic for human tau protein Technical knockout</strong></td>
<td>Parkinson's disease</td>
<td>Flies transgenic for a-synuclein show neuronal loss and inclusion bodies, recapitulating human disease. Transgenic flies also show humanlike pharmacology. Symptoms are rescued by overexpression of human or fly hsp70, implying that chaperones may protect against neurodegeneration</td>
<td>11, 91</td>
</tr>
<tr>
<td><strong>Bestrophin</strong></td>
<td>Alzhiemer’s disease</td>
<td>Flies recapitulate human disease (formation of neurofibrillary tangles)</td>
<td>112</td>
</tr>
<tr>
<td><strong>Linkage disequilibrium (LD)</strong></td>
<td>Deafness</td>
<td>Defective in hearing: model for mitochondrial deafness</td>
<td>229</td>
</tr>
</tbody>
</table>

**Physiol Rev • VOL 83 • JULY 2003 • www.prv.org**
to be high-level developmental switches that trigger eye development. Remarkably, the genes underlying these mutations are similar across 400 million years of divergent evolution.

Vertebrate homologs of Drosophila eyes absent genes are capable of functionally complementing the corresponding Drosophila mutations (Fig. 24), restoring both morphology and normal function (28). So even in areas of physiology where there would seem a priori to be little ground for expecting similarity in function, there is a level at which the comparison with simple genetic models can be informative.

C. Functional Genomics Offers a Future for Comparative Physiology

The utility of genetic models extends phylogenetically in two dimensions. On one hand, the comparison with humans provides reverse genetics for human functional genomics. On the other hand, simple genetic model organisms can provide a valuable resource for classical physiology in closely related species. This can be important where the species is of medical, veterinary, or agricultural significance but lacks the thousands of researcher-years of classical genetic or genomic work that is the hallmark of a genetic model.

What about physiologists who find that they simply must work on a target organism that is not a genetic model? It is still possible to obtain many of the benefits of the genome project, even in related species. Genome projects are now spread widely though the Phyla, and it would be hard to find a species that is not phylogenetically related to a genetic model organism and an associated genome project (Fig. 25). Studies in related species can then be seen to “cluster” around the models. It is possible to analyze a particular process in the target organism, to obtain candidate cDNAs from the phylogenetically closest genome project to use as probes in libraries from the target organism. Expression patterns can usefully be mapped by Northern or in situ in both species, with the comparative approach providing useful information. Models of function can then be tested by reverse genetics in the nearest model, and similarities inferred with the target organism.

Increasing numbers of species are now being sequenced, beyond the elite list of genetic models in Table 1. This is often for sound economic or medical reasons, as in the case of the malarial vector Anopheles gambiae or the Ascidian Ciona intestinalis. Does this weaken the need for the prototypic, “reference” genetic models as in Figure 25? On the contrary, it strengthens the case. Reverse genetics is still central to the discovery of function of novel genes, and although suppression of expression [e.g., by morpholinos, injection of antisense, or RNA in-
terference (79, 80)] can provide some utility in generating hypomorphs, this is only a partial solution. As has been illustrated earlier, the depth of genetic knowledge, the availability of mutants from stock centers, and the powerful reverse genetic technologies add value to the neighboring organisms with recently sequenced genomes. We illustrate this concept of “model hopping” below, using an important set of Drosophila’s phylogenetic neighbors: Dipteran vectors of parasitic disease.

1. Vector biology

Insects make up the large majority of all living species on earth, compared with animals, plants, fungi, or microbes. The “constituency” of Drosophila thus spans perhaps 30 million species. In particular, Drosophila is a thoroughly typical fly, or Dipteran. Many insect pest species are Dipteran, and so are very closely related to Drosophila. Simply by inferring similarity between gene sequences and functions in a given target (pest) species and Drosophila, it is possible now to draw on many of the benefits of the Drosophila genome project in advance of the completion of the Aedes and Anopheles genome projects.

2. The lower tubule domain is conserved among Diptera

In the context of the tubule, our understanding of the Drosophila tubule now outstrips that of any other insect species. Is it possible to roll out the understanding of Drosophila to major vectors of parasitic disease, and so offer new possibilities for selective insecticides? One of the key insights is the enhancer-trap-derived genetic map of tubule domains. It would be exciting to establish whether other Diptera, or other insects, were similarly organized. However, enhancer trapping depends on transposon mutagenesis and remobilization, and although these technologies are being pioneered in other Diptera, they are far from routine (168). A purely morphological approach is not possible, because there are several regions and cell types in tubules that are morphologically identical, but which can be distinguished on the basis of gene expression (Fig. 6). However, enhancer trapping can lead to the underlying genes, and this in turn can suggest histochemical, immunocytochemical, or functional assays that can be applied to other species. As an example, the two enhancer trap lines that mark the lower tubule domain are insertions in alkaline phosphatase (see sect. IX A8); and in Drosophila, alkaline phosphatase expression (determined histochemically) maps precisely to the domain marked out by the relevant enhancer traps (Fig. 26). This provides a simple histochemical assay to test whether the lower tubule domain is present in nondrosophilid insects. Although the tsetse and mosquito tubules are 10 times longer, the absolute size of the lower tubule domain is similar.

In Drosophila, the lower tubule domain marked by alkaline phosphatase is reabsorptive (see sect. IX A8). Although these pilot data fall short of proof of conserved function, they suggest interesting and informative experiments.

Another valuable feature of Drosophila has been the ability to use the GAL4/UAS reporter system to direct genes of choice to particular genetically defined domains. In particular, the UAS-aequorin system has allowed cell-specific aspects of neuropeptide signaling to be delineated. In the absence of routine GALA enhancer trapping in other insects, how could this be emulated?

Most insect neuropeptides show some cross-reactivity in other species, although (for example) not all insect leucokinin stimulate secretion by Drosophila tubules (63). Once this principle is established for Drosophila tubules (Fig. 27A), then it is possible to use transgenic Drosophila tubules, containing stellate cell-directed ae-
D. Functional Genomics and Integrative Physiology Are Linked

This line of argument highlights a close linkage between two major areas of contemporary life science, but one that may not have been perceived. Both functional genomics and integrative physiology are united in their dependence on genetic model organisms. (Of course, even this term is a double-edged sword, as it might imply wrongly that the organism was of interest only because of its genetics. We hope that this review will have convinced the reader otherwise.) Although the emphasis in functional genomics is presently on high-throughput genomics, and then on large-scale target gene selection (for example with “chip” technologies), there is no doubt that ultimately a functional analysis of each gene of interest at an individual level will need to be undertaken. In most cases, this will demand a reverse genetic approach, and thus a genetic model. Conversely, the requirement of integrative physiology to move away from the single molecule, and back to the organism, will often require a set of transgenic and gene-targeting technologies that overlap heavily with those of functional genomics. Paradoxically then, the move away from analytical, molecular, physiology toward a more holistic analysis of organismal function may require a greater level of molecular biological sophistication than is presently required to perform analytical science.

Although this argument may sound persuasive, it is not one that has previously been aired extensively. In the United Kingdom, the Biotechnology and Biological Sciences Research Council mounted two workshops in the summer of 1998: one each on Functional Genomics and Integrative Physiology. Significantly, there was almost no overlap in the delegates!

E. A Plan for Physiologists

From this argument, it is clear that those physiologists who can handle the concept of a genome project have exciting new opportunities at their disposal.

1. How can genome projects help physiologists?

At its most simple, any physiologist with basic molecular training (or a qualified collaborator) can scrutinize the sequences output from the genome projects as they are published, request the DNA clones (they are available for only a modest charge), sequence, and characterize the genes.

At the next level, the use of transgenic technologies afforded by model organisms allows the posing of new questions in physiology and allows the limitations of cell culture to be shrugged off with true organotypic transgene expression.

At a further level, by selecting and developing physiological analyses and assays of a particular tissue within a genetic model, they will be able to harvest the pick of the novel genes that are already tumbling from the genome projects. In doing so, they not only future-proof their careers, but provide the bridge between molecular biology and organismal biology that so many of us feel needs to be built and strengthened.

2. How can physiologists help genome projects?

For physiologists who can translate their research into an appropriate model, and so develop a useful phenotype, the genome projects can provide rapid access.

---

**FIG. 27.** Cross-specificity of neuropeptide action allows for *Drosophila* bioassays of tsetse peptides. *A:* secretion of *Drosophila* tubules is stimulated by extracts of *Glossina* central nervous system (CNS). *B:* *Glossina* CNS extract elicits calcium signals in *Drosophila* tubules and are measured with transgenic aequorin. (From J. A. T. Dow and S. A. Davies, unpublished data.)
For example, a collaboration between SmithKline and the European Mouse Genome project is presently producing thousands of mutant mouse lines, with random mutations. At present, each line is being screened for obvious neurological defects using a 15-min battery of tests designed to detect inadequacy in a range of indicator behavioral phenotypes (37, 167). However, these mice are equally likely to have mutations in almost any gene of interest. The collaboration is thus anxious to attract researchers to “hotel” in the Genome center and screen the mice for any informative phenotype, while the mice still exist. Simple economics dictate that only the most interesting lines can be kept alive for any length of time.

In *Drosophila*, with its smaller genome size and lower maintenance costs, the genome project operates slightly differently. It is possible to contemplate a stock center holding lethal mutant lines that represent all (or at least a large fraction) of all essential *Drosophila* genes. As a result of some heroic large-scale screening and characterization work, there are now at least 5,000 such lines available from stock centers. Rather than invoking a “hotel” approach, these lines are simply made available, free of charge, to the *Drosophila* community. So the onus is on individual experimenters to realize that they need a particular line by virtue of its documented phenotype.

Other genome projects can be expected to operate somewhere on this continuum, based on the trade-off between genome size, maintenance cost, and ease of mutagenesis of the genetic model organism.

**XIII. FUTURE PROSPECTS**

Our analysis suggests a bright future for functional (physiological) research, with benefits both to the physiological and to the genomics communities. However, there is a deeper message: that physiologists, rather than agonizing over whether molecular biology is a friend or foe, must set their horizons very much wider. The linkage we have demonstrated between functional genomics and integrative physiology is just an example of the integration of the life sciences themselves. Physiology, genetics, and molecular biology are combinations of philosophies and techniques that are convergent and will become unified in the postgenomic “new biology.”

**XIV. CONCLUSIONS**

Integrative physiology and functional genomics converge on their need for genetic models, transgenics, and physiology. The *Drosophila* tubule demonstrates the usefulness of genetic technology to physiology. *Drosophila* is an ideal model for study of human diseases, and not just of development or brain.

We thank the referees for their helpful comments. This work was supported by the Biotechnology and Biological Sciences Research Council and the Wellcome Trust.

Address for reprint requests and other correspondence: J. A. T. Dow, Div. of Molecular Genetics, Institute of Biomedical and Life Sciences, Univ. of Glasgow, Glasgow G11 6NU, UK (E-mail: j.a.t.dow@bio.gla.ac.uk).

**REFERENCES**


724 JULIAN A. T. DOW AND SHIREEN A. DAVIES


64. DOW JAT AND O’DONNELL MJ. Calcium homeostasis...


157. NGCHORN A, HEALY MJ, AND DAVIS RL. The cyclic AMP phosphodies-
INTEGRATIVE PHYSIOLOGY AND FUNCTIONAL GENOMICS


202. Seibal A, Price JL, Man B, and Young MW. Loss of circadian...


