Genetic approaches to the study of epithelial function in *Drosophila melanogaster*

by

David Christopher Kelly

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The research reported in this thesis is my own original work except where otherwise stated, and has not been submitted for any other degree

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Abstract

The goal of a complete and integrated genetical, biochemical and physiological understanding of a model epithelium is still some way from completion. In this study, the Malpighian tubule of *Drosophila melanogaster* is proposed and tested as a candidate for whole organ *in vivo* studies.

Firstly, current genetic technologies are harnessed in an attempt to allow expression of any transgene specifically in Malpighian tubules using an endogenous promoter sequence. This sequence does induce expression of a reporter gene in *Drosophila*, though the expression pattern within tubules is different to that expected.

Secondly, the GAL4/UAS binary expression system of *Drosophila* is used to express a human serotonin receptor gene in Malpighian tubules to investigate the possibility of manipulation of the pharmacology of Malpighian tubule cells. Supply of the agonist to tubules expressing the receptor results in a measureable change in levels of an intracellular second messenger molecule.

Lastly, the mechanism of fluid secretion in Malpighian tubules is investigated genetically. A cDNA with sequence homology to water channel gene sequences is cloned from an adult *Drosophila* cDNA library, and this cDNA is mapped to 47F9-16 of chromosome 2R.

Analysis of P-element containing fly lines and lines bearing deletions in this region of chromosome two reveal a line with enhancer detection in Malpighian tubules, and two lines hemizygous for gene sequences.

The study demonstrates that the Malpighian tubule from *Drosophila melanogaster* is an accessible and versatile model system which is amenable to genetic, biochemical and physiological analysis.

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Chapter 1

Introduction

Genome analysis has for nearly two decades involved the identification of novel genes and expression patterns, and the prediction of protein sequences. This nucleic acid based approach is limited by the number of genes available to be identified, and as the various genome projects achieve near saturation coverage of their subjects' genetic material, the scientific community is beginning to consider the possibility of using their genetic data and technology to answer questions more traditionally studied in physiology, anatomy and biochemistry laboratories. For example human geneticists have identified genes which are mutated in individuals suffering inheritable disease. Having cloned and sequenced these genes they are now looking at the effect of mutation on physiology , and in the case of diseases such as cystic fibrosis, are interested in using genetic therapy to offer relief to patients (Caplen *et al.*, 1995).

In *Drosophila*, for which the techniques available for analysis through genome manipulation are possibly the most advanced for any complex eukaryotic organism, decades of classical genetics and observation, mutagenesis and genome analysis have generated a sophisticated understanding of many aspects of the animal's life. Unfortunately the attributes which make *Drosophila* an attractive model system to geneticists are also the principal objections used by physiologists and biochemists against its use. The small size of the animal allows the geneticist to keep large numbers in relatively small spaces reasonably inexpensively; however, this small size makes collection of sufficient tissue for biochemical or physiological analysis arduous, and in most cases is prohibitive.

The goal, however, of a complete and integrated genetic, physiological and biochemical understanding of complex processes such as epithelial fluid transport would demand the sophistication that *Drosophila* genetics can offer, though the difficulties of speedy access to sufficient tissue would need to be solved before this organism could be easily used as a model system.

1.1 Malpighian tubules of *Drosophila*

Renal tubules have been extensively used as a model system for the study of fluid secretion and neurohormonal control in insects (Maddrell and O'Donnell, 1992).

The four renal tubules in *Drosophila* are located in the abdomen , and are arranged into two pairs. Each tubule is a blind-ended epithelium, one cell thick comprising in total about 120 cells. Each pair of tubules is attatched to the gut at the junction between the midgut and hindgut by a shared ureter (Figure 1.1). Malpighian tubules are the primary site of osmoregulation and excretion in insects.

Circulating haemolymph is cleansed of nitrogenous waste and other toxins by active uptake across the basal membrane of cells of the tubule and transport across the apical

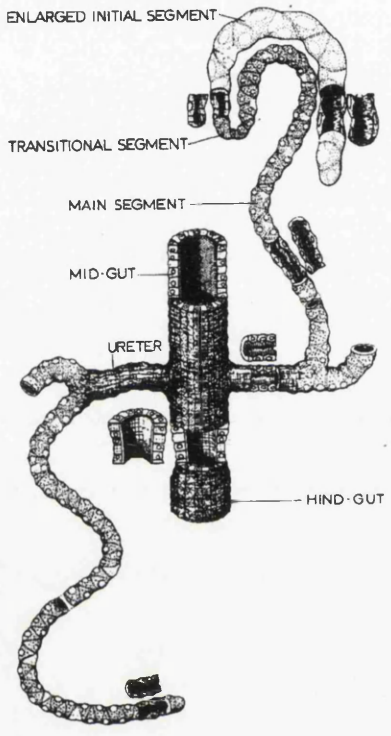


Figure 1.1
Diagram of Malpighian tubules in *Drosophila*. The four tubules are blind ended epithelia, one cell thick, and arranged into two pairs, an anterior facing and posterior facing pair.
The anterior pair have an enlarged initial segment , which appears white. The main segment is separated from the initial segment by a short transitional segment.
Both pairs share main segment morphology. Each pair of tubules shares a ureter which is attached to the gut at the junction between the midgut and hindgut.

(Diagram from Wessing and Eichelberg, 1978)

membrane of the tubule cells into the lumen. From here the precursor 'urine' passes along the tubule, through the ureter into the gut, where it joins ingested dietary material, with which it is egested (Figure 1.2).

The two pairs of tubules are morphologically distinct (Wessing and Eichelberg, 1978); one pair face anteriorally and each tubule of this pair displays an enlarged initial segment of approximately 22 cells (Sozen *et al.*, manuscript in preparation) towards the blind end, furthest from the confluence with the gut. This initial segment is identified by conventional microscopy to be separated from the main length of the tubule by a number of cells; this domain is known as the transitional segment. The transitional segment is not a visually apparent feature of the other pair of tubules - which are oriented posteriorally along the abdomen. Despite these differences, the main length of the tubules share common morphology. The main segment is known to be made up of two types of cells: principal cells; and secondary, or stellate cells. The principal cells appear to form the main stuctural basis of the

tubule, whereas the secondary cells are dispersed along the tubules at different intervals along their length, but the periodicity is approximately once in every 2-4 principal cells. Along the main segment, these cells appear star like, with projections which appear to extend along the junctions between primary cells.

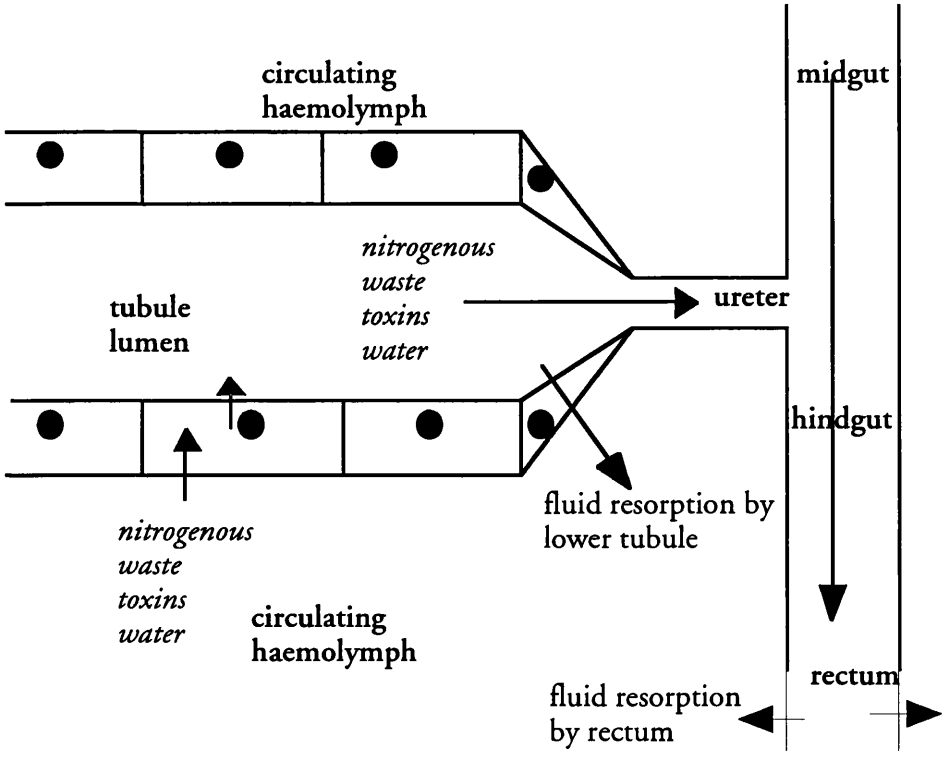


Figure 1.2

Schematic cross-section of a Malpighian tubule in *Drosophila melanogaster*. The organ is a single cell thick epithelium, attached to the gut at the junction between the mid and hindguts. Water, toxins and nitrogenous waste products are transported across the cells of the main segment of the epithelial wall into the tubule lumen, and this fluid passes through the ureter into the gut: evidence suggests that the cells of the lower tubule reabsorb some of the water transported into the lumen by the cells of the main segment; the rectum is also a site of water resorption. The waste is egested along with digestive waste material.

Ultrastructural studies demonstrate subcellular differences between principal and secondary cells. Electronmicrographs of tubule cross sections show electron dense cytoplasm of principal cells, packed with mitochondria. The surface area of both basal and lumenal membranes of principal cells is greatly increased by complex infolding. In contrast secondary cells have less electron dense appearances, have fewer

mitochondria than primary cells and do not have a significantly increased surface area on either surface.

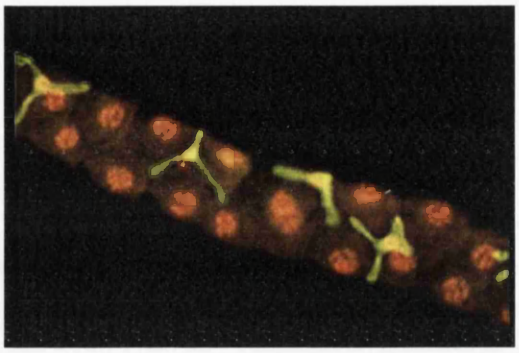


Figure 1.3

Main segment stellate cells imaged by fluorescence microscopy with fluorescence coupled anti-β-Gal antibody. Principal cell nuclei are visible by ethidium bromide staining. Stellate cells occur at intervals along the tubule, have smaller nuclei than principal cells, and have finger-like projections which appear to extend along the junctions between cells. (Picture M.A. Sozen)

1.2 Malpighian tubules are amenable to physiological study.

Extension of the study of Malpighian tubules demands a phenotype which can be scored or quantified to allow meaningful analysis at a cellular and molecular genetic level. The tubule secretion assay, allowing quantitation of secretion rates from Malpighian tubules, was developed from the techniques developed by Ramsay (Ramsay, 1954) to collect fluid produced by the Malpighian tubules of *Dixippus morosus* (stick insect); the assay was adapted to allow study of more than one tubule at a time, and used extensively for study of the Malpighian tubules of *Drosophila hydei* (Wessing, 1987), a larger though genetically less well understood relative of *Drosophila melanogaster*. The assay has been adapted for use with the tubules of *Drosophila melanogaster* (Dow *et al.*,1994). In this assay, pairs of tubules are dissected in a rich saline solution, and placed in a drop of aqueous medium under paraffin, one tubule of each pair is drawn into the paraffin and secured with a dissecting pin to leave the main length of the other tubule remaining in the saline with the ureter under paraffin (Figure 1.4).

Fluid is secreted at the ureter as a direct result of the physiological action of the Malpighian tubule segment within the saline. This secretion can be inhibited by supplying metabolic inhibitors to the bathing medium, demonstrating that the fluid

secretion is the result of active processes in the tubule (Bertram *et al*, 1991). Aqueous solutions are perfectly spherical under paraffin, therefore volumes of small drops of aqueous solution in paraffin can be easily established by measuring the diameter of the drop using an eyepiece graticule in a microscope, and using this figure, together with the optical settings of the microscope to give "real" diameter. The volume of a sphere is calculated by the formula $4/3(\pi r^3)$, where r is the radius of the sphere. Thus, if droplets of secreted fluid are removed at known intervals from the ureter, their volumes can be calculated, and secretion rates computed.

This assay has been the vehicle for the generation of most of the physiological and pharmacological data available for *Drosophila melanogaster* Malpighian tubules.

Unstimulated tubules secrete steadily for up to 15 hours, and a typical secretion rate observed in this kind of experiment is 0.74±0.03 nl/min (mean±SEM, n=217)(Dow *et al.*, 1994). No difference is observed between the secretion rates of anterior and posterior tubules, and both types can be used in the assay.

Using this assay, the maximal secretion rate has been determined (Dow *et al.*, 1994), and this figure has been used to estimate the maximal rate of water transport across the Malpighian tubule plasma membranes on a per-cell basis. This estimate suggests that each cell is capable of transporting its own volume of fluid in less that 15 seconds. This is probably the fastest rate recorded for a fluid secreting epithelium; and so suggests that this tissue is an excellent model for analysis.

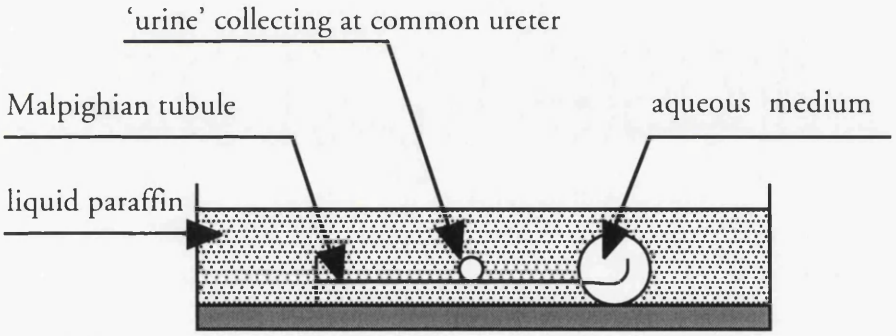


Figure 1.4

Schematic diagram of a typical tubule secretion assay. A pair of tubules is dissected and placed in a drop of aqueous medium under paraffin. One of the tubules is drawn into the paraffin and secured with a dissection pin. The tubule remaining in the aqueous medium secretes fluid which collects at the common ureter. Drops of fluid can be removed from the ureter at intervals, and secretion rates computed. The effect of agents on tubule secretion can be determined following their direct addition to the bathing saline, and analysis of subsequent secretion rates.

Active transport across plasma membranes is ATP dependent, therefore implicating the presence of one or more types of ATPase in *Drosophila* Malpighian tubules.The

effect of certain metabolic inhibitors on secretion rate of Malpighian tubules has been determined using the secretion assay, and this data allows the presence or absence of certain membrane transporters, identified as integral in the transport processes in animal cells, to be established.

1.3 V-ATPases in *Drosophila* Malpighian tubules

Specific inhibitors are identified for most of the characterised ATP-ases. F- ATPases use the electrochemical gradient of H^+ (Greville *et al.*, 1969) or Na^+ (Hilpert *et al.*, 1984) to generate ATP, and their reversible action can be inhibited by azide and N,N'-dicyclohexylcarbo-diimide (Linnett *et al.*, 1979). P type ATPases, which include the Na^+/K^+ ATPase, cycle through conformational states which include the formation of phosphorylated intermediates; this enzyme is not inhibited by azide, but is specifically inhibited by vanadate (which is a transition state analog of phosphate), and in most cases by N -ethylmaleimide (Goffeau *et al.*, 1981; Serrano, 1984; Jorgensen, 1982; Ikemoto, 1982; Epstein, 1985; Hugentobler *et al.*, 1983). The Na^+/K^+ ATPase is inhibited by cardiac glycosides such as ouabain (Sweadner and Goldin, 1980).
A third class of ATPases, the vacuolar ATPases (V-ATPases) were originally shown to hydrolyse ATP to generate a proton gradient used for acidification of subcellular compartments (Bowman *et al.*, 1986; Mellman *et al.*, 1986; Sze, 1985).

Later work (Wieczorek *et al*, 1991) has shown that this class of ATPase is involved in the generation of a K^+ gradient across an animal (*Manduca sexta*) membrane, however the potassium ion transport is not a part of the ATPase activity, and occurs through the action of a K^+/nH^+ antiporter which relies on the electrochemical proton gradient generated by the vacuolar ATPase (Figure 1.5).

This type of ATPase, the V-ATPase , is not inhibited by azide , vanadate or ouabain (Anstee *et al.*, 1979); nonspecific inhibitors include N,N' -dicyclohexylcarbodiimide and N-ethylmaleimide (Bowman *et al.*, 1988). Bafilomycin A_1, a macrolide antibiotic with a 16 membered lactone ring, specifically inhibits members of this class of ATPase (Werner *et al.*, 1984; Bowman *et al.*, 1988).
Therefore, a series of specific inhibitors exist which allow the identification of an ATPase subtype important in the energetics of fluid transport across Malpighian tubules.
Fluid transport by *Drosophila melanogaster* Malpighian tubules, like those of all other insects analysed, is inhibited by Bafilomycin A_1 at $5x10^{-5}$ M (Dow *et al*, 1994). Oubain does not inhibit fluid secretion, even at concentrations as high as 10^{-3}M.

These data therefore suggest the presence of a V-ATPase in the Malpighian tubule cells of *D.melanogaster*.

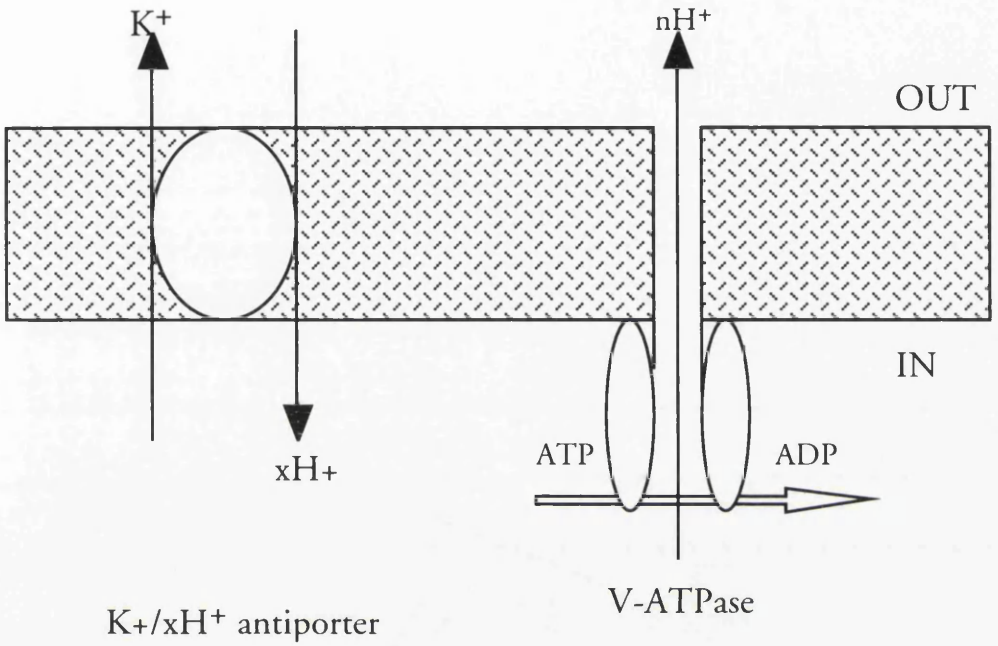


Figure 1.5

The Vacuolar-ATPase uses the energy of ATP hydrolysis to transport hydrogen ions from the cytoplasm, across the plasma membrane to the outside of the cell. The hydrogen ions exchange through a K^+/xH^+ antiporter, which is not directly coupled to the V-ATPase, generating a K^+ gradient across the membrane (after Wieczorek *et al.*, 1991).

Degenerate pairs of oligonucleotides were designed from conserved sequences of V-ATPase genes (Gluck, 1992) and used to screen Malpighian tubule cDNA by PCR for the presence of V-ATPase transcripts. This work confirms the presence of V-ATPase subunit gene expression in *Drosophila melanogaster* Malpighian tubules (J. Dow, Y. Guo, personal communication).

1.4 How do tubule cells secrete fluid?

In *Manduca*, V-ATPase action does not acidify, as proton transport is coupled to ion translocation *via* a K^+/nH^+ antiporter which builds up a net potassium cation ion

gradient across the membrane. In *Drosophila* Malpighian tubules, the secretion of fluid is insensitive to bumetanide, an inhibitor of $Na^+/K^+/2Cl^-$ cotransport, but sensitive to amiloride, a non specific inhibitor of Na^+ -selective transport processes. Secreted fluid is known to be K^+ rich with minimal Na^+ content (Maddrell and O' Donnell, 1992). Taken together, these data suggest that in Malpighian tubules "ion translocation is energised by an apical, bafilomycin-sensitive V-ATPase, which drives net K^+ secretion *via* an alkali metal cation/proton exchanger" (Dow *et al.*, 1994; Wieczorek *et al.*, 1991). The maintenance of a K^+ gradient across the membrane establishes a favourable electrical gradient for the movement of Cl^- from the cytoplasm of Malpighian tubule cells into the lumen, and water follows passively as a consequence of the osmotic gradient set up across the membrane.

1.5 Metabolic control of secretion rates of Malpighian tubules

The Malpighian tubule, viewed as a whole, acts to transport potentially large volumes of fluid across its membrane surfaces, such potent and inducible transport would be expected to be regulated by sophisticated mechanisms; indeed, it has been shown that each of three second messenger signalling pathways can influence secretion rates of Malpighian tubules of *Drosophila melanogaster.*

Cyclic AMP (cAMP) has been identified in many species of insect as a stimulatory second messenger in tubules (Maddrell *et al.*, 1971; Coast, 1991). cAMP added to the bathing saline/Schneiders of the tubule secretion assay increases secretion rates of *Drosophila melanogaster* Malpighian tubules. Forskolin, an activator of adenylate cyclase, when added to the bathing medium in the secretion assay gives a similar response (Dow *et al.*, 1994).

An increase in secretion rate is also observed when cyclic Guanosine MonoPhosphate (cGMP) is used in the same way, furthermore, coincubation with Zaprinast, an inhibitor of cGMP phosphodiesterase accelerates this response; however the maximal rates observed in the presence and absence of Zaprinast are the same, implying that cGMP is acting as a dedicated second messenger in this tissue (Dow *et al.*, 1994a).
This result is confirmed by exposing Malpighian tubules to sodium nitroprusside, a nitric oxide (NO) donor. Nitrosylation of the haem group of soluble guanylate cyclases by nitric oxide causes the enzyme to accelerate catalysis of Guanosine TriPhosphate (GTP) to cGMP (Moncada *et al.*, 1991). Its interaction with guanylate cyclases is specific to the soluble (cytoplasmic) form of the enzyme, and no stimulation in secretion rate is observed when agonists of membrane bound forms

are used in the tubule secretion assay. The effect seen with sodium nitroprusside can be inhibited by co-incubation with methylene blue, which inhibits the stimulatory effect of NO on soluble guanylate cyclase, demonstrating that the action of sodium nitroprusside is *via* the release of NO and its interaction with a soluble guanylate cyclase (Dow *et al* 1994a). RT-PCR studies with degenerate primers designed to recognise guanylate cyclases identifies a novel sequence when Malpighian tubule mRNA is used as a template (M.Smith, J.Dow, unpublished observations). Therefore, four separate lines of evidence suggest the involvement of cGMP in regulation of secretion rate in Malpighian tubules.

A cardio acceleratory peptide, (CAP_{2b}), has been identified in *Manduca sexta* (Tublitz *et al.*, 1992; Tublitz *et al.*, 1992a). This peptide increases the secretion rate of Malpighian tubules from *Drosophila*, and this increase in secretion rate is accompanied by a rise in intracellular cGMP concentration (Davies *et al.*, 1995). The observed effect of CAP_{2b} on Malpighian tubules is inhibited by co-incubation with methylene blue (S. Davies, personal communication). These data provide evidence for the existence of a ligand/receptor activated, and nitric oxide mediated, cGMP elevation leading to increased secretion rates.

A third independent signalling pathway in Malpighian tubules has been shown. Thapsigargin, which inhibits reuptake of Ca^{2+} by the endoplasmic Ca^{2+}-ATPase therefore elevating cytosolic calcium concentration (Thastrup *et al.*, 1990), elevates secretion rates of Malpighian tubules (Davies *et al.*, 1995). Leukokinins are a family of insect neuropeptides (Hayes at al., 1989) which are thought to act through the elevation of intracellular Ca^{2+} (O'Donnell *et al.*, 1996; S.-A. Davies and P.Rosay, personal communication). An increase in secretion rate of Malpighian tubules is seen when Leukokinin IV is used in the tubule secretion assay (Davies *et al.*, 1995). There are therefore two lines of evidence suggesting the presence of a $[Ca^{2+}]_i$ signalling pathway in Malpighian tubules.

1.6 Evidence for the independence of the three signalling pathways.

At least three elements of signalling pathways in the stimulation of fluid secretion by Malpighian tubules have been shown. It is possible that two or more of these elements may be working in the same pathway, or that each is acting separately, in parallel and different signalling pathways.

The tubule secretion assay has been used to distinguish between these possibilities. Maximal stimulation of an element of a signalling pathway may result in an increase in tubule secretion rate. Further action to stimulate the same pathway will not result in an increase in secretion rate, as maximal stimulation has been achieved. Thus

additivity in stimulation of secretion rate of two or more agents is indicative that these agents are acting on different signalling pathways.

1. The stimulation with cAMP is not additive to that of forskolin (Dow *et al.*, 1994), nor is the enhanced secretion seen when cGMP is used in the assay additive to the effect of sodium nitroprusside (Dow *et al.*, 1994a). These data suggest that cAMP and forskolin, and cGMP and SNP are acting to stimulate the same pathways respectively.

2. Stimulation with cAMP is additive to the effect seen with leukokinin, though it is partially additive to the effect of cGMP. This suggests that the cAMP signalling pathway can be separated from the Ca^{2+} signalling pathway, and that elements of the cAMP and cGMP signalling pathways overlap (Davies *et al.* 1995).

3. An additive response is also observed when tubules are stimulated with leukokinin and cGMP (Dow *et al.*, 1994a).

4. The effect of Leukokinin IV is not additive to that of thapsigargin in the tubule secretion assay, though its effect is additive to that of the cyclic nucleotides cAMP and cGMP.

Therefore, it has been demonstrated that in Malpighian tubules of *Drosophila melanogaster*, there are three separable secretion rate elevating signalling pathways, involving cAMP, cGMP and calcium.

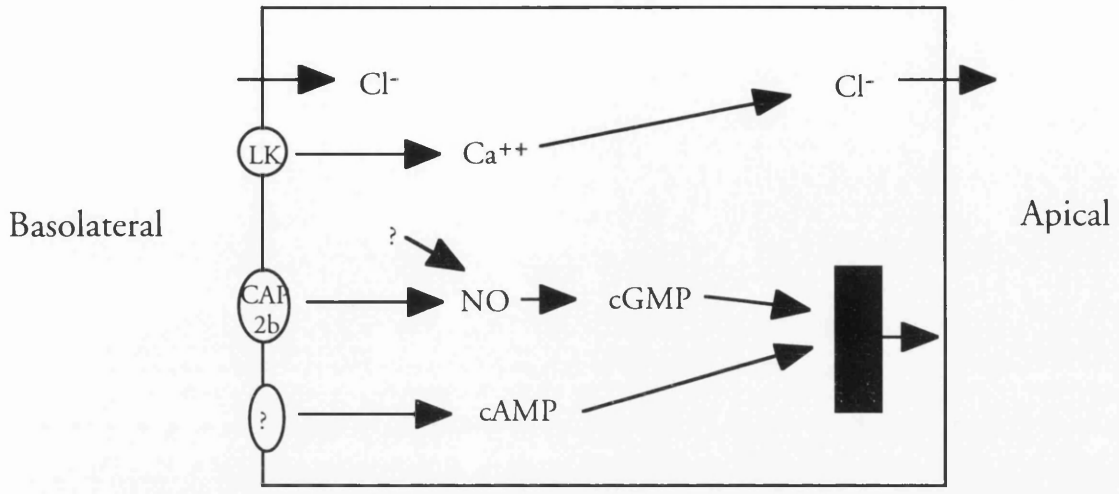


Figure 1.5.1

Schematic representation of a stylized Malpighian tubule cell of *Drosophila melanogaster* showing the interactions of, and between, the three signalling pathways thought to govern the activity of the tubule cells. Leukokinin IV (LK) elevates intracellular calcium; this leads to a reduction in the Cl^- component of the transepithelial potential difference (O'Donnell *et al.*, 1996). CAP_{2b} acts through NO to elevate intracellular cGMP levels leading to an increase in fluid secretion rate; an elevation of intracellular cAMP also causes an elevation in tubule secretion, however the effects of cAMP and cGMP are only partially additive, suggesting that they share an element/elements of secretion effecting pathway.

1.7 Further access to Malpighian tubule cells

The approaches described above give clear information about the nature of Malpighian tubule cells. These approaches however, suffer from the limitations of the tubule secretion assay, i.e. that the tubule is seen as a homogenous unit, so that no difference in cell/domain function can be easily identified or exploited. Maddrell and O'Donnell (O'Donnell and Maddrell, 1995) sucessfully adapted the assay for a relatively crude series of experiments which demonstrated that upstream main segments secrete potassium cation rich fluid, that cells in the lower tubule reabsorb significant amounts of potassium and water, and that cells of the initial segment do not participate in urine production in the tubule. This adaption was also used to show that the lower tubule acidifies the secreted fluid + secretes Ca^{2+} into it. The adaption was based on localising the drop of aqueous medium in the assay to a

particular domain of the tubule, in this case the lower tubule (close to the ureter), or main segment. This level of experimentation is not practical for large scale analysis of tubule physiology, and it must be acknowledged that the local placement of the bathing medium cannot be accurately repeated in each sample.

Thus, spatial resolution of both pharmacological intervention and physiological analysis are thus severely limited and therefore, further modifications of the tubule secretion assay are unlikely to provide significant new benefits to the advancement of understanding of tubule physiology.

Genetic approaches are being sucessfully used in the study of *Drosophila* development, brain anatomy and physiology, and these approaches have been adapted for the study of Malpighian tubules in *Drosophila*.

Modern *Drosophila* genetics has benefitted from the identification and characterisation of the P transposable element, responsible for the phenomenon of P-M hybrid dysgenesis.

1.8 P-M hybrid dysgenesis

In the P-M sysytem of hybrid dysgenesis, dysgenesis occurs when males of a P strain are mated to females of an M strain. The germ line cells of the progeny of such crosses develop aberrantly resulting in elevated rates of mutation as well as reduced fertility and chromosome rearrangement (Kidwell, *et al.*, 1977). P strains bear multiple copies of the P- transposable element dispersed along chromosome arms which are absent from M-strains (Engels, 1979; Engels and Preston, 1980). Transposition of P-elements in P strains is repressed; however, when chromosomes bearing P transposable elements are introduced into M strains the elements become derepressed and transpose at high rates. The transposed elements induce mutations by insertion into gene sequences and thus disruption of normal gene function. These transposable elements can excise from their point of insertion both precisely and imprecisely, resulting in either reversion to wild type phenotype or inheritable stable mutation (O'Hare and Rubin, 1983).

1.9 Isolation of P elements

P elements were identified (O'Hare and Rubin, 1983) as a family of elements, heterogenous in size but with strictly conserved DNA sequence. A complete P transposable element is 2907 nucleotides long, and smaller elements are thought to have arisen by internal deletion events. The complete P-element contains 4 separate open reading frames punctuated by introns which together code an 87kD transposase polypeptide (Karess and Rubin, 1984; Rio, Laski and Rubin 1986). The transposase polypeptide is vital to the mobility of the element; therefore, internally deleted P elements with no ability to code a transposase are stable in the genome, unless a transposase is supplied *in trans* by another element. The observation that hybrid dysgenesis is restricted to cells of the germ line is accounted for by the differential splicing occurring in somatic and germ line cells . RNA splicing to remove the third intron is restricted to cells of the germ line (Laski, Rio and Rubin, 1986). Failure to splice this intron results in an ineffective, 66kD polypeptide, therefore there is normally no transposition in somatic cells (Rio, Laski and Rubin, 1986).

Transposition competent P-elements are flanked by perfectly conserved 31bp inverted repeat sequences (Mullins *et al.*, 1989). These sequences are required absolutely for P-element mobility, and their removal or mutation renders an element stable, even in the presence of a functional transposase protein .

The observation that the transposase need not be supplied *in cis* is an important one, as it opens up the possibility of using P elements as vectors for transfer of exogenous DNA to the *Drosophila* genome.

1.10 Extrachromosomally supplied P-elements can cause transposition events

In 1982 Spradling and Rubin (Spradling and Rubin, 1982) demonstrated that a stably inserted P element could be mobilised by microinjection of a P element bearing plasmid. The stable insertion caused the *singed weak* (sn^w) phenotype, a mutation in bristle morphology; the stability of this insertion was a consequence of a P-element internal deletion. Mobilising a P element can potentially bring three separate classes of progeny; parental, due to non mobilisation; a more extreme phenotype due to imprecise excision of the element; and wild type, due to precise excision of the element. When sn^w embryos were microinjected with a plasmid bearing an intact 2907bp element, the progeny of these individuals showed all three possible phenotypes, demonstrating that the microinjected plasmid had mobilised the P-element *in trans*. In this experiment the stable element became unstable in future generations, and this was later shown to be due to the insertion of the precise P-element sequence alone from the microinjected plasmid into the genome.

1.11 Use of P elements in transfer of exogenous sequences

Given that internally deleted P-elements are capable of transposition when supplied with a source of transposase *in trans*, it was thought likely that the P-element would provide a vehicle for germ line transformation of *Drosophila* with exogenous sequences. This was proved to be correct (Rubin and Spradling, 1982) when a mutant P-element made by inserting a chromosomal sequence bearing the gene for xanthine dehydrogenase was sucessfully germ line transformed when co-injected with an intact P-element plasmid. Mutation in the gene coding xanthine dehydrogenase causes an eye colour deficiency known as *rosy* (*ry*). Germ line transformation was scored by reversion of a mutant eye colour phenotype in *rosy* individuals to the wild type due to non-cell autonomous expression of the *rosy* sequence. This finding led to the development of P-element vectors bearing selectable markers for easy identification of insertion of DNA sequences which may themselves not confer an easily scorable phenotype.

1.12 Sources of transposase for germ line transformation experiments

A synthetic P element was constructed *in vitro* which lacked the intron between the 3rd and 4th exons, and was named Δ2-3 (Laski, Rio and Rubin, 1986). This element could code functional transposase in all tissues, and caused somatic mosaicism when it was introduced into a genetic background containing non autonomous P-elements. Thus, the Δ2-3 element is capable of producing functional transposase which is active in all tissues and not in the germ line alone. Removal of the intron did not effect the transposability of the Δ2-3 element, however one stable Δ2-3 insertion was generated which was capable of providing competent transposase, but itself did not transpose (Robertson *et al.*, 1988). This element gave much higher transposase activity than an intact P-element insert.

This Δ2-3 element produces a high activity, somatically active transposase. It can also be supplied on a plasmid by co-injection during germ line transformation experiments. The intact Δ2-3 element however is capable of self transposition, therefore leading to inheritable instability. This is not desirable for stable germ line transformation. Using a stable chromosomal source of Δ2-3 is beneficial, as the source of transposase can be crossed away from the insert, which can then be maintained stably. This system is not wholly ideal, as insertion of DNA into the same chromosome as the source of transposase causes complexity; also when Δ2-3 insertion causes homozygous lethality, the chromosome is maintained over a suitable

balancer. The inherent homozygous lethality of balancer chromosomes reduces the efficiency of the transformation process.
Thus, an ideal source of transposase would be a self transposition incompetent plasmid which coded a high activity source of transposase. Such a plasmid has been constructed (Rubin, unpublished observations; Karess and Rubin, 1984), and is widley used by members of the *Drosophila* community.

1.13 P-element vectors

The availability of P-elements as vectors to supply DNA sequence of choice to the *Drosophila* germ line, the generation of a stable high activity source of transposase *in trans*, and the transposibility of sequences flanked by P-element repeats allowed the development of P-element vectors which allow DNA sequences of choice to be transferred to the *Drosophila* genome. The ability to transfer exogenous DNA to the germ line of *Drosophila* can be used in many ways. Mutations can be "rescued" by supply of non mutated DNA. Reversion to wild type after germ line transformation can prove that the cloned DNA, when mutated *in vivo* is involved in the generation of the mutant phenotype. Also, the consequences of expression of sequences from other species can be examined in a whole animal *in vivo* context by controlled expression - either by fusion of the sequence to *Drosophila* tissue/developmental stage specific promoter, or under heat shock promoter control.

1.14 An early use of P-element vector technology

The plasmid pLacA92 was a P-element derived plasmid bearing an in frame translational fusion of the *lacZ* gene to the second exon of the P-element, both ends of the P-element which are necessary for P-element transposition, the *rosy* gene as an eye colour marker, and the trailer sequences and polyadenylation site of the *Drosophila hsp70* gene (O'Kane and Gehring, 1987). When this plasmid was germ line transformed, expression of the *lacZ* gene in independent single insertion lines was established.
The P-element promoter is relatively weak; however, under the influence of genomic regulatory elements its strength as a promoter is enhanced. In this experiment distinct tissue-specific *lacZ* staining patterns resulting from genomic positional effects were observed.
Thus, regulatory sequences present in the *Drosophila* genome can be uncovered by random P-element mutagenesis using this or a similar reporter construct containing

P- element. This technique is known as enhancer trapping, and has been applied in plant and vertebrate model systems (Sundaresan *et al.*, 1995; Joyner, 1991).

1.15 Greater sophistication in P-element mediated mutagenesis

The enhancer trap technique generates pseudo-random mutation, and can uncover the expression pattern of a gene or genes either nearby or at a distance. An enhancer trap element will often generate a reporter gene product distribution similar to that of the gene within which it has inserted. This property of the element has made them valuable tools in the isolation of developmental stage specific and cell type specific genes, and in the mapping of genetic boundaries *in vivo* (O'Kane and Gehring, 1987).

This technology relies on a fortuitous insertion which drives expression of the reporter. Genetic mapping of a tissue in this way has relatively limited benefits for subsequent genetic manipulation, although flanking sequences can be removed by imprecise excision of the element. It is desirable to ectopically express exogenous sequences specifically in marked cells, for example; expression of an introduced sequence in a cell type specific manner which reverts a mutant phenotype not only demonstrates that the original genomic copy of the sequence is mutated and responsible for the phenotype, but also assigns a functionality to the cell type; ablation of specific cell types by local expression of toxins allows viability in the absence of this cell type to be assessed; and ectopic cell specific expression of sequences can allow biochemical intervention leading to a greater understanding of cell/tissue function.

The use of P-element enhancer traps to drive expression of many different gene sequences can be achieved if the induction of enhancer trap by genomic regulatory elements is separated from the induction of expression of the selected sequence. Such a system has been developed in *Drosophila.*

1.16 The GAL4/UAS$_G$ system

UAS elements in yeast can be DNA binding sites for *trans* acting regulatory proteins, such as the yeast transcriptional activator GAL4. The GAL4 protein binds four related 17bp sites within the UAS element, which is required for the activation of the divergently transcribed galactose metabolising genes GAL10 and GAL1 (Giniger *et al.*, 1985; Bram *et al.*, 1986). Transfection studies in HeLa cells (Webster *et al.*, 1988) have shown that GAL4 protein provided *in trans* is capable of activating transcription from otherwise inactive promoters of a reporter gene placed downstream of the UAS$_G$ sequence. This binary approach to activation of a reporter gene was first applied in *Drosophila* by Fischer *et al.* (Fischer *et al.*, 1988). The GAL4

coding sequence was placed under the control of the promoter of the endogenous *Drosophila* gene alcohol dehydrogenase (adh), and this construct was germ line transformed. The promoter, when fused to *lacZ* and germ-line transformed, activates *lacZ* gene expression in the fat body, Malpighian tubules, anterior and middle midgut of larvae.

A second transformed line was generated. This line had the *lacZ* gene downstream of an inactive *hsp70* promoter. Immediately upstream of the inactive promoter were placed 4 of the 17bp GAL4 binding sites of UAS_G.

These two transformed lines could be stably maintained without detriment to either line. No expression of the *lacZ* reporter was seen in the UAS-*lacZ* construct alone.

The protein and its binding site were brought together by crossing the two lines. In lines bearing both the UAS-*lacZ* and the adh-GAL4 elements, high levels of reporter activity were observed in the fat body and anterior midgut. Low levels of *lacZ* activity were reported in Malpighian tubules and middle midgut, however no reporter activity was seen in any tissue other than those expected.

Therefore, the GAL4/UAS_G system provides in *Drosophila* a practical binary system for the activation of many different gene sequences in a predictable and precise tissue/cell type specific manner.

1.17 Second generation enhancer trap elements

Following the demonstration that the GAL4/UAS_G binary system could be applied in *Drosophila*, a second generation of enhancer trap elements were constructed (Brand and Perrimon, 1993). The plasmid pGawB was derived by modification of the *lacZ* enhancer detection vector plwB (Wilson *et al.*, 1989), the *lacZ* detector element was removed and replaced in frame by the GAL4 coding sequence followed by the *hsp70* terminator. Neither the P transposase promoter nor the P-element terminal repeats were affected in the cloning process.

This p{GAL4} vector once germ-line transformed can be mobilised by a source of P transposase provided *in trans*, therefore allowing enhancer detection by the GAL4 protein.

1.18 Applications of the p{GAL4} system

The GAL4/UAS_G enhancer detection system has been successfully used in the study of mushroom bodies of *Drosophila* brain (Yang *et al.*, 1995).

The mushroom bodies are implicated in insect associative learning and in other aspects of behaviour (Erber *et al.*, 1987; Mizunami *et al.*, 1993). Classical physiological analysis had suggested that the bodies were made up of homogenous

arrays of specific neurons. In this study, the p{GAL4} element was mobilised to generate 1400 new lines. GAL4 distribution patterns in these lines were analysed by GAL4 mediated *lacZ* activation, by crossing the p{GAL4} element into a UAS-*lacZ* background. Several lines gave *lacZ* expression patterns more or less restricted to specific anatomical domains of the brain, and in a few, the mushroom bodies were clearly revealed. Study of mushroom body staining patterns has revealed that tissue is far from homogenous, as parallel subcomponents exhibit discrete patterns of gene expression.

These findings have generated enough data for the proposal of a model for mushroom body function. As the lines studied activate *lacZ* expression under the influence of GAL4 protein, any transgene can be activated in the domains identified, allowing the model to be tested and developed.

Thus the second generation enhancer trap is a powerful tool in the dissection and analysis of the complex anatomies and physiology of *Drosophila.*

1.19 Enhancer detection in Malpighian tubules

In a parallel study (Sozen *et al.*, manuscript in preparation), 700 of the lines generated in the mushroom body study were stained for GAL4 mediated *lacZ* induction in the Malpighian tubules. About 150 individual lines showed some staining in tubules, with a third this number demonstrating region or cell type specificity of expression within the tubule. Only 5 lines showed *lacZ* expression which was restricted to the tubules alone in all developmental stages analysed. This analysis has confirmed the physiological differences along the tubule described by conventional physiology, and has generated lines in which staining patterns suggest genetic compartmentalisation of cells within the tubule which had not been realised before.

There follows a description of the results obtained in this study, the results of conventional physiological studies of the organ have already been sumarised in 1.1.

In most of the lines studied, the initial segement was identified by its failure to stain, although in several lines this segment was identified by *lacZ* staining. The transitional segment is also apparent both from its exclusion in a line where main and initial segment cells are stained (C825) (Figure 1.6) , and also as a separate staining entity in line C507. Analysis of lines demonstrating concerted gene expression in initial and transitional segments of anterior tubules, showed analagous boundaries in the posterior tubules (Figure 1.6). The main segment of both sets of tubules are marked by many lines. The lower tubule is seen as distinct from the main segment and ureter in lines C507 (Figure 1.7A) and C232. The ureter is distinct by staining, and is seen to be subdivided in lines C649 and C601 (Figures 1.7 B and C).

This technique has marked the secondary (stellate) cells (lines C710 and C724) (Figure 1.3). Secondary cells are not present in the lower tubule, and the position of the first stellate cell on the tubule respects the boundary seen between the main segment and lower tubule.

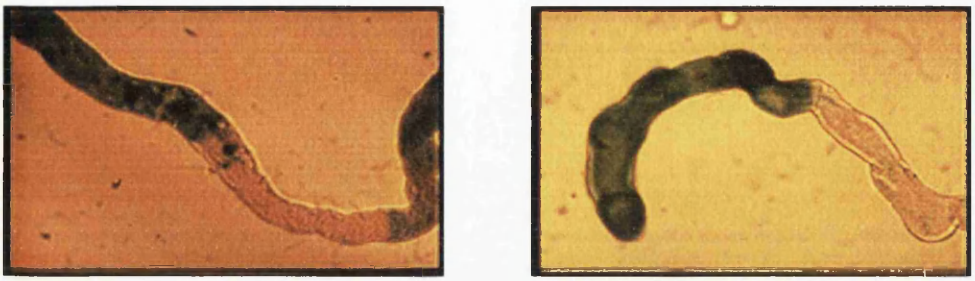


Figure 1.6
Malpighian tubules stained for β-Galactosidase (β-GAL) activity with the chromogenic substrate X-Gal. The lines from left to right are C825 and C155Y. Line C825 defines the transitional segment of an anterior tubule by its exclusion in staining for β-Gal activity. Line C155Y shows compartmentalised staining of a posterior tubule. This staining pattern demonstrates the existence of initial and transitional segments in posterior tubules at a genetic level, even though this distinction cannot be made by conventional physiological methods. (all pictures on this page, M.A. Sozen)

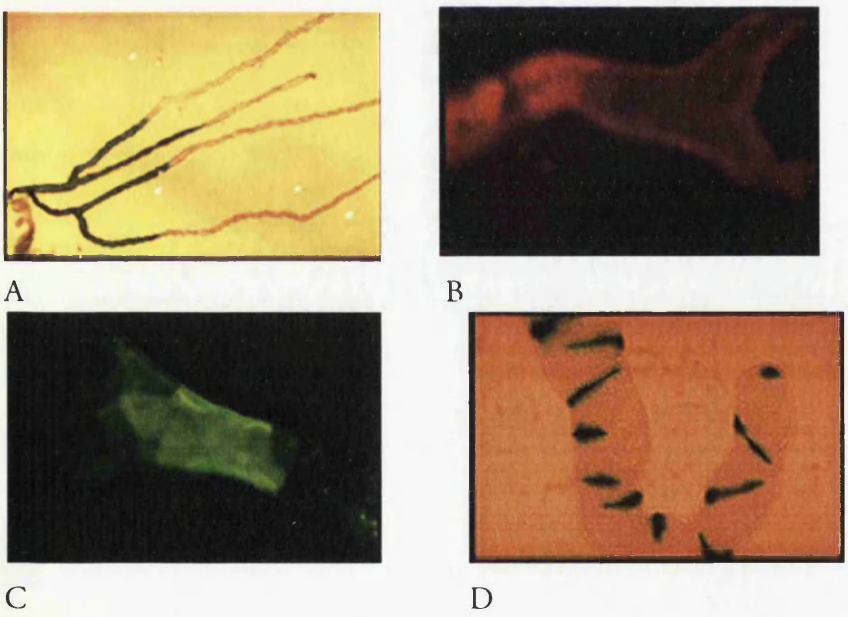


Figure 1.7
A shows GAL4 distribution in the line C507. This staining pattern demonstrates that the lower tubule is a distinct domain of the organ in both pairs of tubules. B and C are fluorescence antibody visualised to uncover GAL4 expression pattern of the ureter in lines C601 and C649 respectively. These figures show that the ureter can be subdivided into at least two distinct domains . GAL4 expression in line C601 is restricted to the cells closest to the junction with the gut. Line C649 shows a GAL4 distribution extending from the bifurcation to the boundary defined in line C601. C649 GAL4 expression does not appear to overlap with that of C601. D shows bar shaped stellate cells of initial segment disclosed by staining tubules of C710 flies (adult) with X-Gal

There is a difference in stellate cell morphology observed in the anterior tubules, where the stellate cells of the initial and transitional segments are bar shaped (Figure 1.7D), though their periodicity remains the same as along the main length of the tubule. In posterior tubules, these segments (initial and transitional) are much smaller than in the other pair, there are fewer stellate cells, and there is no difference in their morphology.

Another class of cells, termed tiny cells, which had not been observed before were disclosed in this analysis. These cells were found in lower tubules and posterior midgut, had reduced cytoplasm with a compact nucleus, and high resolution studies showed a thin neurite-like process extending from the cell (Figure 1.8).

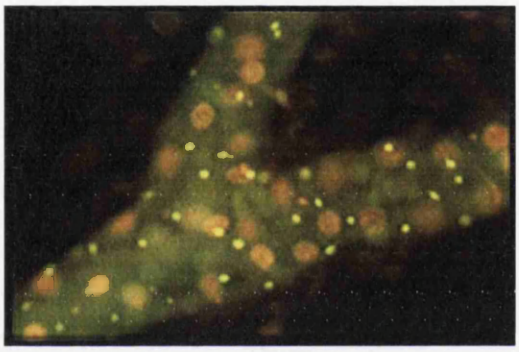


Figure 1.8

Fluoresence microscopy of line C710 showing GAL4 distribution in cells at the bifurcation of a pair of Malpighian tubules. Cell nuclei are counterstained with ethidium bromide. This line marks the "tiny cells" which had not been described before.

The Malpighian tubule is therefore a relatively simple model epithelium with well characterised physiology and genetics. Thanks to the painstaking work of Sozen *et al.* a resource of well characterised and tubule domain specific p{GAL4} lines have been generated and characterised. The availability of an assay of whole organ function in combination with these p{GAL4} lines makes the possibilities for further organ analysis relatively easy and potentially rewarding.

1.2 Summary

This work explores the possibility of using and adapting the recently developed transgenic technology to intervene in the activity of Malpighian tubule cells of the adult fly.

At the outset of this study, p{GAL4} lines with tubule domain-specific expression patterns had not been characterized, and so an attempt was made to design tubule specific GAL4 expression by transforming flies with a tubule-specific promoter-GAL4 construct (Chapter 3). Tubule-specific control sequences of the urate oxidase gene have been identified and characterized, and these control sequences were used in the construct described above.

The idea of this approach is to make available a p{GAL4} line with GAL4 distribution identical (or at least similar) to that of the urate oxidase gene. The gene is expressed in the main segment of all 4 tubules in the third larval instar and adult stages; tubules of animals at these developmental stages are sufficiently large to be amenable to physiological analysis, and expression of transgenes in this temporal and spatial pattern may minimise the potential for developmental abnormalities.

The results of the study undertaken by Sozen *et al.* (manuscript in preparation) has provided many lines with p{GAL4} element insertions, with unique tubule and domain-specific expression patterns. Therefore that study has provided the resource required of this work; however, the initial choices of UAS/GAL4 technology allow fly lines from both studies to be applied interchangeably.

In Chapter 4, GAL4/UAS technology is exploited to express a human receptor gene in cells of the Malpighian tubule. Physiological analysis of the *Drosophila* Malpighian tubule has generated an understanding of the biochemistry of cellular processes of fluid secretion; therefore, a genetics-based approach is taken in an attempt to intervene in the biochemistry of the tissue. The receptor gene chosen is the 5-HT1Dβ receptor gene, which in mammalian cells causes a decrease in intracellular cAMP levels when stimulated with the agonist 5-hydroxytryptamine. In this study the efficacy of the transgenic receptor is analysed by quantitation of intracellular cAMP concentrations after stimulation of tubules expressing the receptor with the agonist. This approach to intervening in the biochemistry of Malpighian tubule cells has much potential. The p{GAL4} lines identified by the study of Sozen *et al.* allow expression of the same transgene in many different cell populations within the tissue; indeed, this study was the first to provide evidence that adjacent principal cells of the tubule may not be identical at the molecular genetic level. It is hoped that sucessful intervention in cellular biochemistry using a transgenic approach could be adapted to help assign physiological function to different domains of the tissue.

The transgenic approaches described above complement well the existing gene cloning technologies; an understanding of tissue function and physiology predicts that the membranes of Malpighian tubule cells are not sufficiently permeable to account for the high rates of fluid flow across them, and in Chapter 5 the

experiments leading to the cloning and characterization of an integral membrane protein aquaporin gene expressed in Malpighian tubules are described.
Isolation of genes integral to the function of Malpighian tubules is likely to lead to a greater understanding of tissue function; also, a characterization of DNA sequences directing expression of tubules is of interest as;

1), it may lead to the generation of a greater variety of promoter-GAL4 fusions available for future work,

2), it may allow consensus DNA sequences for tubule expression to be uncovered, and so lead to an understanding of the transcriptional mechanisms employed in directing expression of DNA sequences in Malpighian tubules.

Thus, the work described in this thesis uses varied and modern approachs to understanding the mechanisms of epithelial function in this model system.

Chapter 2

Materials and Methods

2.1 Purification of plasmid DNA

Plasmids were prepared by alkaline lysis (Ish-Howowicz and Burke, 1981; Sambrook *et al*, 1989).
In all cases plasmids were prepared from host *E.coli* strains (XL1-Blue, DH5α, or INFαF') from LB-broth liquid culture shaken overnight at 37°C. Plasmids containing the β-lactamase gene were grown in the presence of 50 μg/ml ampicillin.

2.1.2 Small scale preparation

The cells of 1.5 ml of overnight culture were pelleted by centrifugation and resuspended in 100 μl of Solution I (50 mM glucose, 25 mM Tris.Cl (pH 8), 10 mM EDTA (pH 8)) and incubated at room temperature for 5 mins. The suspension was lysed in 200 μl of Solution II (0.2M NaOH, 1% SDS) and placed on ice for 10 mins. 150 μl of Solution III(3 M K^+, 5 M Acetate) was then added, mixed thoroughly and incubated on ice for a further 10 mins. After centrifugation at 13,000 rpm for 10 mins the supernatant (450 μl) was precipitated in 1ml absolute ethanol. Nucleic acid was recovered by centrifugation at 13,000 rpm at room temperature, the supernatant discarded, and the pellet was resuspended in 50 μl sterile distilled water. RNase A was added to a final concentration of 20 μg/ml.

2.1.2a Large scale preparation

The initial stages of large scale preparation use the same solutions I, II and III described above.
The cells from 500 ml of liquid overnight culture were harvested by centrifugation and resuspended carefully in 10 ml of Solution I. After incubation at room temperature for 5 mins the suspension was lysed with 20 ml of Solution II and placed on ice for 10 mins. The lysate was precipitated with 15 ml of ice cold Solution III and the preparation incubated on ice for a further 10 mins. Precipitate was removed by centrifugation and the nucleic acid of the supernatant precipitated in 0.6 volumes of propan-2-ol. Precipitated nucleic acids were pelleted by centrifugation, the supernatant discarded, the pellets washed in 70% ethanol and air dried before resuspension in 3 ml of sterile distilled water.
High molecular weight RNA was removed by adding 3 ml of ice cold 5 M lithium chloride. The precipitated ribonucleotides were pelleted by centrifugation and the nucleic acid content of the supernatant was again precipitated, with an equal volume of propan-2-ol. The DNA solution was again centrifuged at 10,000 rpm for 10 mins, the supernatant discarded and the pellet resuspended in 0.5 ml of sterile distilled

water containing DNAse free RNAse at 20 mg/ml. After 30 mins at room temperature sodium chloride and polyethyleneglycol 8000 (PEG) were added to 0.8 M and 7.5% respectively in a total volume of 1 ml. Plasmid DNA was recovered by centrifugation at 13,000 rpm for 5 mins at room temperature. The pellet was suspended in 0.5 ml sterile distilled water and extracted once each with phenol and chloroform. The plasmid was precipitated by addition of the aqueous phase after chloroform extraction to 1 ml of absolute ethanol with 150 ml of 3 M sodium acetate. At this stage the plasmid could be seen as a stringy white precipitate and was removed from the ethanol/sodium acetate mixture using the end of a pasteur pipette which had been formed into the shape of a hook in a bunsen flame. The plasmid DNA was resuspended in 0.5 ml sterile distilled water and stored at -20°C until required.

2.1.2b Caesium Chloride density gradient purification of plasmid DNA

When necessary plasmids which had already been purified by PEG precipitation (2.1.2a) were further purified by equilibrium centrifugation in a caesium chloride/ethidium bromide gradient.

DNA prepared by alkaline lysis and PEG precipitation (approximately 0.5-1 mg in 0.5 ml) as above was added to a further 4.5 ml sterile distilled water. 5 g of caesium chloride was slowly added to the 5 ml of plasmid solution. Ethidium bromide was added to a final concentration of 740 μg/ml and any precipitate removed by centrifugation at 8,000 rpm for 20 mins at room temperature. The supernatant was centrifuged for 14-16 hours at 45-49,000 rpm at 15°C, in the Ti70 rotor of a Beckman L7-55 ultra centrifuge. The plasmid band was removed using syringe and needle, and ethidium bromide was extracted repeatedly in water-saturated butan-1-ol. DNA was precipitated by adding 3 volumes of sterile distilled water and 2.5 times the resultant volume of absolute ethanol, and incubation at 4°C for 30 mins. The plasmid was recovered by centrifugation at 10,000 rpm for 10 mins, the supernatant discarded, and the washed and dried pellet resuspended in sterile distilled water. The plasmid was kept at -20°C until required.

2.1.3 Isolation of total fly genomic DNA

Adult flies were homogenised in DNA homogenisation buffer (10 mM Tris.Cl pH 7.5, 60 mM NaCl, 10 mM EDTA) at room temperature. Debris was removed by centrifugation at 1,000 rpm for 1 minute. The supernatant was removed to another

tube and spun for 5 mins at 8,000 rpm. The cell pellet was resuspended in 0.5 ml of DNA homogenisation buffer and proteinase K added to a final concentration of 100 μg/ml. The solution was made up to 1% SDS (from a 10% stock) and incubated for 1 hour at 37°C. The digest was extracted twice with phenol and once with chloroform; the aqueous phase was precipitated in 2 volumes of absolute ethanol/ 0.1 volumes of 3M sodium acetate.

The thread-like precipitate was removed to a clean tube and resuspended in sterile distilled water. Genomic DNA was left overnight at 4°C before use to allow complete resuspension , and was stored at 4°C.

2.1.4 Isolation of P1 clone DNA

P1 were clones were grown overnight in small scale LB-broth liquid culture in 25 μg/ml kanamycin. The following day LB-broth/25 μg/ml kanamycin was innoculated with 1/1000 volume of the overnight culture and shaken at 37°C for 3 hours. After this time IPTG (isopropyl β-D-thiogalactopyranoside) was added to a concentration of 1 mM and the culture shaken for a further 3 hours at 37°C. Small quantities of DNA were prepared using the small scale plasmid purification protocol given above, and larger quantities of P1 DNA were prepared using the PEG isolation method of 2.1.2a.

2.2 DNA digestion protocols

2.2.1 Plasmid/P1 DNA

Up to 1 μg of plasmid DNA was digested with 10 units of restriction endonuclease in the manufacturers recommended buffer at single strength, by incubation for 1 hour at 37°C.
Plasmid DNA which was digested by two enzymes with incompatible buffers was digested separately in each enzyme. Buffer salts and enzyme from the first digestion were removed by phenol/chloroform extraction and the plasmid precipitated before resuspension in the appropriate buffer for the second nuclease, and digestion by that enzyme.

2.2.2 Fly Genomic Digests

Up to 10 μg of genomic DNA was digested per reaction using 20 units of enzyme in the appropriate buffer at single strength, by incubation for at least five hours at 37°C. A sample to be digested in two enzymes with mutually incompatible buffers was first digested in one of the enzymes before the DNA was extracted in phenol and chloroform, precipitated, resuspended, and digested with the second enzyme in the appropriate buffer.

2.2.3 Agarose Gel Electrophoresis

DNA was electrophoresed horizontally through 1% agarose/1xTBE (1xTBE, 0.09 M Tris-borate, 0.002 M EDTA) gel containing 0.1 mg/ml ethidium bromide.

2.2.4 Purification of DNA from agarose gels

DNA bands were excised from the 1% agarose gel using a scalpel blade. The agarose plug was placed in a 0.5 ml eppendorff tube and either snap frozen on dry ice or liquid nitrogen or slowly frozen in a -20°C freezer overnight. The bands were thawed and a hole punctured in the bottom of the tube with a number 2 needle. The 0.5 ml tube was then placed inside a 1.5 ml tube from which the lid had been removed, and the assembly centrifuged for 10 mins at 6,500 rpm. Liquid collecting in the 1.5 ml eppendorff tubes was precipitated in 1/10 volume 3 M sodium acetate and 3 volumes of absolute ethanol at -20°C for 30 mins. DNA was recovered by centrifugation at 13,000 rpm and the pellet resuspended in sterile distilled water.

If particularly clean DNA was required from this procedure the liquid collected in the 1.5 ml tubes after the first centrifugation was phenol/cholroform extracted.

2.2.5 Southern Blotting of DNA gels

Agarose gels were visualised and photographed under ultraviolet irradiation. The gels were denatured for an appropriate time in denaturing solution (1.5 M NaCl, 0.5 M NaOH). Denaturing solution was discarded and the gels washed in distilled water before incubation in neutralising solution (1 M Tris.Cl (pH 8.0), 1.5 M NaCl). Gels were then capillary blotted onto a nylon membrane (Hybond N(Amersham)) in 20xSSC (20xSSC; 3 M NaCl, 0.3 M tri-sodium citrate) buffer using standard procedures. After transfer, DNA was fixed to the filter in a UV crosslinker (1200 μJcm^{-2}). Blots were stored at room temperature until required.

2.2.6 Hybridization of Southern blots

Fixed Southern blots were placed inside hybridization tubes, with the DNA side of the filter facing inward. Blots were prehybridized, hybridized and washed at 65°C in a Techne hybridization oven. Prehybridization and hybridization were in Church buffer (7% SDS, 1% BSA, 1 mM EDTA, 0.25 M Na_2HPO_4 (pH7.2)) , and washing was in 0.1% SDS and appropriate strength SSC. Washed blots were air dried, wrapped in Saran wrap and exposed to film for the required time.
After hybridization and signal analysis, bound probe was removed from the filter by incubation of the filter in boiling 0.1% SDS for 15 minutes with shaking.

2.2.6b Detection of Digoxigenin (DIG) labelled DNA

DNA probes were labelled with digoxigenin-dUTP (Boehringer Mannheim) using the protocols supplied by the supplier. Hybridization, washing and developing were exactly as recommended by the supplier of the DIG-dUTP, except 1% milk power was substituted for the supplier's recommended blocking compound. (Detailed protocols for use of the DIG system can be found in The DIG System User's Guide for Filter Hybridization - which can be obtained directly from Boehringer Mannheim)

2.2.7 Radioactive labelling of DNA for hybridization

30-50 ng of purified DNA was labelled with ^{32}P-α-dCTP by random priming in the method of Feinberg and Vogelstein (Feinberg and Vogelstein, 1984; Feinberg and Vogelstein, 1983). Labelled DNA was denatured by boiling, and snap cooled on ice before addition to the hybridization tube.

2.3 Ligation of DNA into plasmid vectors

2.3.1 Preparation of plasmid DNA for ligation

DNA was cleaved with the appropriate restriction endonuclease, and the linear DNA band excised from an agarose gel to minimise contamination. Plasmid DNA which was to be used to clone an insert with differing "ends" was cut a second time using double the normal enzyme concentration, and for double the normal time. Double and singly cut DNAs were treated with calf intestinal phosphatase in single strength buffer before extraction with phenol and chloroform. Plasmid DNA was concentrated by precipitation in 0.1 volume 3 M sodium acetate and 3 volumes of absolute ethanol, resusupended in sterile distilled water, and its approximate concentration determined by DNA electrophoresis.

2.3.2 Preparation of insert DNA for ligation

PCR-generated DNA fragments were first cloned into the vector pCRII with the TA cloning kit of INVITROGEN and then treated as DNA fragments in subcloned plasmid.

Subcloned fragments were excised from their parent plasmid with the appropriate restriction endonucleases to maintain desired orientation of the DNA fragment in the new vector. The insert band was purified from an agarose plug after electrophoresis using the method of 2.2.4. DNA was precipitated, resuspended in sterile distilled water, and its approximate concentration determined by agarose gel electrophoresis.

2.3.3 Ligation of plasmid and insert

Vector and insert DNAs were added to a ligation mixture at relative concentrations which gave a 1:1 molar ratio of DNA ends. A maximum of 100 ng DNA was added per ligation.
Plasmid and insert prepared in the above method were ligated in single strength supplied buffer (containing 5 mM ATP) with the T4 DNA ligase of GIBCO-BRL at 14°C overnight.

2.3.4 Preparation of competent *E.coli*

50 ml of LB-Broth was innoculated with 50 μl of an overnight culture of XL1-Blue or DH5α *E.coli* and shaken until reaching an ODλ600nm of between 0.3 and 0.4. The cells were pelleted by centrifugation at 1,600 rpm at 4°C and resuspended in ice cold 100 mM $CaCl_2$. After incubation for 40 mins on ice the cells were pelleted in the same way as before and again resuspended in 100 mM $CaCl_2$. The cells were either used immediately for transformation or dimethyl-sulphoxide (DMSO) added to 35 μl/ml cell suspension, incubated on ice 15 mins and a further 35 μl DMSO/ml cell suspension added. Cells were aliquoted to ice cold tubes and snap frozen on liquid nitrogen before being stored at -70°C until required. In this case cells were thawed on ice before use.

2.3.5 Bacterial transformation of plasmid DNA

5-10 μl of ligation mix was added to 100 μl of competent *E.coli,* and incubated on ice for 40 mins. The bacteria were heat shocked at 37°C for 60 seconds and plunged immediately onto ice. After 5 mins on ice, 800 μl of LB broth was added to the bacteria and the bacterial/broth mixture shaken at 37°C for 1 hour. After this expression step the bacterial culture was spread onto LB-agar plates containing the appropriate antibiotic. When blue/white colour selection was used 120 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in dimethyl-formamide was added to the bacteria after the expression step.
Agar plates were inverted and incubated overnight at 37°C, and stored at 4°C until required.

2.4.1 Synthesis of Oligonucleotides

Custom oligonucleotides were synthesised in house on an ABI 391 or 392 DNA sythesiser on 0.2 μM columns (Cruachem).

2.4.2 Deprotection of Oligonucleotides

Glass beads were removed from the synthesis column and incubated at room temperature for 2 hours-overnight in 1 ml of aqueous ammonia. After this incubation the ammonia solution was removed to another tube and a further 1 ml of ammonia added. The 2 ml of ammonia solution was incubated at 55°C overnight.
Before use the ammonia was removed from the oligonucleotides in a rotary evaporator and the DNA resuspended in sterile distilled water.

2.5 DNA sequencing

Manual sequencing

DNA was sequenced in the di-deoxy method of Sanger using the protocols and components of the Amersham/USB Sequenase 2.0 kit and ^{35}S-α–dATP. 7.5 μg of double stranded DNA was denatured in 0.2 M NaOH for 30 minutes at 37°C and precipitated according to kit protocols. Labelling mix was diluted 1/15 rather than 1/5.
Products of the sequencing reaction were separated by electrophoresis through a vertical 6% acrylamide (19:1 acrylamide:bis-acrylamide), 7 M urea, 1xTBE gel cast in a Bio-Rad sequencing apparatus at 50°C.
Gels were dried onto Whatman 3MM paper in a vacuum drier and exposed to film overnight.

Automated sequencing

Single stranded PCR was performed using template for sequencing and PCR/termination mix (ABI) containing fluorescently labelled dideoxy nucleotides. Sequence from this reaction was resolved by polyacrylamide gel electrophoresis as above, and detection in an ABI automated DNA sequencer.

2.6 Polymerase Chain Reaction

Standard protocols for polymerase chain reaction (PCR) were used throughout. Reactions were performed in single strength buffer provided by the supplier of Taq DNA polymerase. Each dNTP was supplied at a concentration of 125 μM and approximately 6 pmoles of each primer were used per 20 μl reaction.

2.7 DYNAL oligo dT magnetic bead system

mRNA was isolated from Malpighian tubules using the protocols and materials supplied with the DYNAL mRNA direct kit. mRNA captured on magnetic particles was added directly to the reverse transcription reaction so that the dT moiety of the magnetic bead primed first strand cDNA synthesis. Approximately 20 tubules were used per preparation, and 30 μl of resuspended beads used in the capture and synthesis stages.

2.8 Reverse transcription of *Drosophila* mRNA

Reverse transcription of *Drosophila* mRNA was carried out using the dT moiety of the magnetic bead to prime cDNA synthesis. Extracted mRNA on beads was added to a solution containing 20 units RNAsin (Promega), 1 mM each dNTP, 5 mM DTT, 1x reverse transcription buffer (supplied with enzyme). The enzyme used was GIBCO-BRL Superscript II reverse transcriptase, 20 units of which was added to the reaction mix at 42°C, and the reaction left for 30 minutes. cDNAs covalently attached to the magnetic particles were concentrated with the magnetic particle concentrator and washed and resuspended in sterile distilled water. PCR from cDNA used the PCR protocol above with 1 μl bead suspension per reaction as template.

2.9 Library Screening

20 ml of LB-broth/10 mM $MgSO_4$ was innoculated with 0.5 ml of an overnight NM621 liquid bacterial culture. The cells were grown at 37°C with shaking for three hours and then pelleted by centrifugation.
The pelleted cells were resusupended in 5 ml of ice cold 10 mM $MgSO_4$ before use.

0.5 ml of prepared cells were incubated at room temperature with the required volume of phage stock (previously titred). After 15 minutes the cell/phage suspension was added to 7.5 ml top agar (1:1 vol/vol LB-agar/LB-broth in 10 mM $MgSO_4$) at 55°C. This mixture was quickly poured over prepared LB-agar plates and the top agar left to set completely before the plates were inverted and incubated overnight (maximum 12 hours) at 37°C.

Plates to be screened by hybridization of plate lifts were left at 4°C for several hours before use. Precut nylon filters were prewetted before being placed carefully onto each phage plate. The filters were marked in a unique manner allowing precise realignment with the parent plate after the hybridization. After careful removal from the plate the filter was placed, DNA side up, on a piece of filter paper saturated in DNA denaturing solution (1.5 M NaCl, 0.5 M NaOH). After incubation for five minutes the filter was removed to a piece of filter paper saturated in DNA neutralising solution (1 M Tris, 1.5 M NaCl) for a further five mintues. The filter was gently washed in 3xSSC, air dried and the DNA fixed to the filter by UV irradiation.

The filters were then hybridized according to protocols 2.2.6

Positive colonies were picked with a pasteur pipette and placed in Phage buffer (20 mM Tris.Cl (pH7.4), 100 mM NaCl, 10 mM $MgSO_4$) and left overnight at 4°C before further use.

2.10 Excision of phagemid pBS from λZapII

XL1-blue *E.coli* were grown in small scale overnight culture in 12 μg/ml tetracyclin. 200 μl of the cells were added to 100 μl of a single phage plug in phage buffer solution and 1 μl (approx $7.5x10^7$ pfu/ml) of helper phage (Stratagene, (R408 Interference Resistant Helper Phage (Russel *et al.*, 1986))) and incubated at 37°C for 15 minutes. The culture was added to 5 ml of 2xYT medium and shaken at 37°C for 3 hours. 1.5 ml of this culture was incubated at 70°C for 20 minutes. The cells were separated from the culture by centrifugation, and 200 μl of the supernatant added to a further 200 μl of overnight XL1-Blue culture and incubated at 37°C for 15 minutes. After this time the cells were plated onto LB-agar/ampicillin plates and grown overnight at 42°C. Single colonies were grown in small scale overnight culture in LB-broth with shaking at 42°C, and phagemid DNA prepared using protocol 2.1.1

2.11 Medium scale preparation of DNA from bacteriophage Lambda.

Host Cell Preparation

100 ml of LB-broth was innoculated with 1 ml of a fresh NM621 overnight and grown for approximately 3 hours. The cells were centrifuged at 3K rpm and resuspended in ice cold 10 mM $MgSO_4$ to a final optical density ($A\lambda 600nm$) of 1.0.

Growing Lambda lysates

$2x10^6$ eluted phage were added to 0.5 ml of plating cells prepared as described above. The mixture was incubated at 37°C for 30 minutes before being used to innoculate 37 ml of LB-Broth/10 mM $MgSO_4$, this innoculate was shaken at 37°C overnight.

Isolation of Phage DNA

0.1 ml of $CHCl_3$ was added to the lysate and mixed well. Nucleic acid in the lysate was digested by incubation with DNase1 and RNase A for 30 mins at 37°C by addition of 0.37 ml of a nuclease stock solution (stock solution; 50 mg DNase1, 50 mg RNase A in 10 ml 50% glycerol, 30 mM NaOAc, pH6.8). 2.1 g of NaCl was added and mixed until dissolved. The mixture was centrifuged at 4K rpm at 4°C for 20 mins and the pellet discarded. The supernatant was transferred to clean tubes bearing 3.7 g PEG 8,000, which was dissolved by gentle agitation. Once the PEG had dissolved the mixture was incubated on ice for an hour. PEG precipitate was collected by centrifugation at 10,000 rpm for 20 mins at 4°C, and resuspended in 0.5 ml phage buffer. 0.5 ml of $CHCl_3$ was added to the phage buffer in a microfuge tube and the suspension spun for 5 mins at top speed in a microfuge.
The aqueous layer was transferred to a new tube, to it were added 20 µl 0.5 M EDTA, 5 µl of 20% SDS, and 2.5 µl of 10 mg/ml proteinase K. This mix was incubated at 65°C for 30 mins before extraction with phenol and chloroform. DNA was precipitated in ethanol/0.3 M NaOAc and collected by centrifugation. The DNA pellet was washed in 70% ethanol and resuspended in 0.3 ml water.

2.12 Fly husbandry

Fly stocks were kept at 18°C or 25°C in dedicated incubators on normal yeast/agar flyfood (recipie, appendix 4).

2.12.1 Virgin selection

Vials containing late pupae were emptied of adult flies and all eclosing flies were examined at 30 minute intervals. Females were selected by absence of sex combs, and were kept isolated for several hours to mature before crosses were set up.

Males were selected on the basis of presence of sex combs.

2.12.2 Procedures for microinjection

The strain of flies bearing the w^{1118} (Hazelrigg *et al.*, 1984) mutation were used throughout.

2.12.2a Selection of embryos

Adult flies were kept on grape juice/agar plates seeded with yeast paste at room temperature for several days before embryos were required. On days when embryos were required, food plates were changed every 30 minutes and embryos removed with fine forceps. All subsequent embryo manipulation for microinjection took place at 18°C.

Undechorionated embryos were lined up along a straight edge of a piece of grape juice/agar food with the spiracles overhanging the edge. Before microinjection the embryos were removed to a coverslip; this was achieved by smearing the coverslip with glue (sellotape glue dissolved in heptane) and touching lightly to the embryos. The coverslip was then secured to a glass slide with a drop of oil.

2.12.2b Preparation of DNA

All DNA for microinjection was purified twice, once by PEG purification (protocol 2.1.2a) and then by ethidium bromide density gradient centrifugation (protocol 2.1.2b). DNA was resuspended in sterile distilled water. Helper plasmid and P-element plasmid were co-precipitated with ethanol/sodium acetate, pelleted by

centrifugation and washed several times with 70% ethanol. The pellet was air dried and resuspended in injection buffer (5 mM KCl, 0.1 mM PO_4 (pH7.8)) to give final DNA concentrations of

1, P-element plasmid 200 ng/μl

2, helper plasmid 50 ng/μl.

2.12.2c Preparation of needles

Needles were pulled on a Campden Instruments computer controlled electrode puller, model 763, from borosilicate glass capillary tubes(G100T-10, Clark Electromedical Instruments) (1.0mm (O.D.) x 0.78mm(I.D.)) with the following programme.

pre-pull heat	30
time	5
force	50
displacement	30.

The needles were back filled with DNA solution prepared as above and the remaining space filled with oil.

2.12.2d Microinjection

The embryos were covered in Voltalef 10S oil and the slide placed on the stage of an inverted stage Nikon microscope. The needle was mounted on the apparatus and the tip placed in the same plane of focus as the edge of the slide, the tip was touched gently to the slide and the end broken. The broken needle was placed under the oil and the flow rate adjusted by increasing/decreasing the hydraulic pressure of the oil behind the DNA in the needle, to give approximately 1 drop per second. Each embryo was pierced at the posterior pole with the needle exuding DNA.

2.12.2e Post injection care

Each coverslide of microinjected embryos was removed from the slide and placed on a grape juice/agar plate seeded with yeast paste. The embryos were kept at 18°C on this plate until hatching and developing to the third larval instar, whereupon each larva was removed individually to a yeast/agar food containing vial.

Animals which survived to adulthood were mated to males or virgin females of the w^{1118} strain, and the eye colour of the progeny scored; any progeny with revertant eye colour were propagated.

2.14 Dissection of *Drosophila* tissues

Drosophila tissues were dissected in normal phosphate buffered saline (PBS), except Malpighian tubules for use in the tubule secretion assay - which were dissected in standard *Drosophila* saline (Dow *et al.*, 1994).

2.15 β-Gal visualisation with X-Gal

Tissues were dissected in PBS and fixed in 4% paraformaldehyde (4% paraformaldehyde in PBS, pH 7.5) for 15 minutes. Fixed tissues were washed 3 times for 5 mins per wash in PBS before incubation in Fe/NaP buffer/ 0.2% X-Gal (Fe/NaP buffer: 10 mM sodium phosphate buffer pH7.2, 3.1 mM $K_4(Fe[II](CN)_6)$, 3.1 mM $K_3(Fe[III](CN)_6)$, 150 mM NaCl, 1 mM $MgCl_2$; X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside)) at 37°C for 2 hours to overnight. Tissues were washed in PBS before examination of staining pattern.

2.16 Sectioning of *Drosophila* tissues

Dissected tissue was embedded and frozen in Tissue Tek O.C.T. compound. Sections were taken on an Anglia Scientific Cryotome 620, and the sections placed on gelatinised slides. The sections were fixed in 1% glutaraldehyde in PBS at 4°C for 15 minutes, and washed in PBS three times for 5 minutes per wash at 4°C before use.

Detail of fly lines used

Details of the fly lines used are given in the thesis where these lines are introduced. More information on the genotype of the fly lines is available from the online database FLYBASE (http://www.ebi.ac.uk:7081/) with the exception of the line P1386, for which more specific details are given below.

P1386 cn[1]P{ry[+t7.2]=PZ}l(2)04738[04738]/CyO;ry[506]

Chapter 3

Results 1

3 Combining tissue specific and binary expression

As explained in Chapter 1, tissue specific expression patterns uncovered by enhancer trap elements can be used to drive ectopic expression of genes of interest in *Drosophila.* The identification of GAL4 lines generating tissue specific expression in the target organ or cells is a labour intensive task, and relies on pseudo-random transposition; however, ectopic gene expression using the GAL4/UAS system can also be targetted by the inclusion of tissue specific promoter sequences upstream of the GAL4 sequence in non generic P{GAL4} elements (Brand and Perrimon, 1993).

3.1.1 Vectors for the construction of non generic promoter-GAL4 fusion elements

Vectors have been constructed which make fusion of a promoter sequence to the upstream of the GAL4 cDNA relatively easy; pGaTB is an example of this type of vector (Figure 3.5).

Any DNA sequence of choice can be placed upstream of the GAL4 coding sequence in pGaTB (see Figure 3.5) and a promoter-GAL4-hsp-terminator construct can be removed using unique *Kpn1*, *BamH1* and *Not1* restriction endonuclease sites. This construct can be inserted into any one of the pCaSpeR (Appendix 1) vectors freely available and germ-line transformed. In the transgenic animal, the GAL4 expression pattern is determined by the sequences cloned upstream of it. Brand and Perrimon (1993) themselves demonstrated the usefulness of this strategy by germ line transformation of a promoter-GAL4 fusion construct. The promoter was from the gene *Rh2,* which is expressed in the photoreceptor cells of the ocelli. GAL4 distribution was visualised by crossing transgenic lines to a UAS-*lacZ* bearing line, the ocelli of animals with both the GAL4 and UAS sequences stained dark blue after only 15 minutes incubation with the chromogenic substrate, implying strong selective activation of the *lacZ* gene.

This approach has been extensively used by the *Drosophila* community, and among others, GAL4 expressing lines in the pattern of developmentally important genes such as *decapentaplegic* (*dpp*) (Staehling-Hampton *et al.*, 1994).

3.1.2 Targetted gene expression in Malpighian tubules

This approach can be used to target expression of the GAL4 transcription factor, and therefore ectopic expression, to Malpighian tubules once a suitable tubule specific promoter sequence has been identified. The urate oxidase gene is a well characterised gene with Mapighian tubule specific expression (Wallrath *et al.*, 1990).

3.1.3 Urate Oxidase, an enzyme with strictly regulated expression patterns

In *Drosophila melanogaster*, particular purines serve as precursors of nucleotides and pterins (Johnson and Friedman, 1983). Excess purine is converted to uric acid which is stored, excreted or catabolised depending on the developmental stage. The enzyme urate oxidase (UO) catabolises uric acid to allantoin. In *Drosophila melanogaster* urate oxidase(E.C. 1.7.3.3) activity is detected only in the Malpighian tubules.
UO activity is detected only in the Malpighian tubules of third instar larvae and the adult. As the late third instar larva undergoes metamorphosis, UO activity declines abruptly and is no longer detectable 24 hours after pupariation , though the Malpighian tubules remain intact and are carried over from the larva to the adult. The loss of UO activity is accompanied by accumulation of uric acid during pupariation (Friedman, 1973).

3.1.4 Cloning of sequences of the gene coding urate oxidase

To isolate a cDNA clone coding for urate oxidase, mRNA from mid-third instar larvae was extracted and used to generate cDNA. A cDNA library was constructed in the *E.coli* plasmid pBR322. Analysis of the library for urate oxidase gene sequences gave a single plasmid bearing a 520 bp insert. This fragment was used to rescreen the library and this analysis gave two 1100 bp fragments. Ckral et al., 1986)
Northern analysis with mRNA from total *Drosophila* at various developmental stages using the 520 bp fragment as probe demonstrated that there is a single band of UO mRNA of approximately 1400 bp in larva and adult. mRNA for UO was not detected in first instar larvae or prepupae and was barely detectable in second instar larvae, however there are high levels detectable in third instar larvae and adults.
A genomic clone isolated by screening a *Drosophila* genomic DNA library with the 520 bp fragment, hybridised at position 28C on the left arm of chromosome 2 in an *in-situ* hybridisation to salivary gland chrmomosmes (Kral *et al.*, 1986).

3.1.5 Structure of the gene coding urate oxidase

The urate oxidase gene of *Drosophila melanogaster* is structurally compact consisting of two exons separated by a 69 bp intron between nucleotides 608 and 677 of the coding sequence. The 5' flanking region of the UO coding sequence contains a 13 base pair direct repeat (AAGTGAGAGTGAT) at -138 and +11. Each direct repeat element includes a perfect direct repeat of the sequence AGTGA with an axis of symmetry centred at the G nucleotide at positions -132 and +17 (Wallrath *et al.*, 1990). The sequence of the direct repeat motif is similar to proposed 20-hydroxyecdysone consensus sequence found upstream of six 20-hydroxyecdysone inducible genes of *Drosophila melanogaster* (Pongs, 1988). The similarity of these motifs to proposed steroid hormone receptor binding sites in vertebrates (Beato *et al.*, 1989), and the position of the two direct repeat motifs relative to the transcriptional start site of the UO gene suggest that these elements may be important for the transcriptional repression of the UO gene by 20-hydroxyecdysone.
Downstream of the direct repeat element is a single long open reading frame, beginning 34 nucleotides 3' of the UO transcription start site. The gene is transcribed to yield UO mRNA of 1,224, 1,227 and 1,244 nucleotides long and codes for a 352 amino acid sequence.

3.1.6 Domains of expression of the UO gene in *Drosophila melanogaster* Malpighian tubules

Hybridisation of antisense RNA from a restriction fragment covering the coding region of the UO gene to Malpighian tubules demonstrated the following expression patterns for the gene: hybridisation was observed along the entire length of the posterior pair of tubules; there was hybridisation only to the mid segment of the anterior tubules with no observed signal in the transitional or initial segments; there was a small degree of hybridization to cells of the ureter (Wallrath *et al.*, 1990).

3.1.7 Regulatory sequences essential for correct expression of a urate oxidase transgene

A 3.2 kb *Pst1 Drosophila* genomic DNA restriction fragment was germ-line transformed into white deficient flies. The *Pst1* fragment covered the UO gene with the 5' site at position -826 and the 3' site some 1.2 kb downstream of the UO transcribed region. Northern analysis with mRNA from transgenic flies and an

oligonucleotide probe which exclusively recognised the transgene demonstrated that the regulatory sequences included in the transgenic fragment are sufficient to direct expression patterns faithful to those of the wild type gene(Wallrath et al., 1990)

3.1.8 Strategy for targetted gene expression in Malpighian tubules

The urate oxidase gene is therefore a gene with a well defined, tubule-specific, expression pattern. Previous analysis of the DNA sequences of the gene have defined a short DNA segment capable of driving high levels of tissue and stage specific expression of an introduced construct.

The control element of the urate oxidase gene is an ideal candidate for use to drive ectopic expression of the yeast transcription factor GAL4; not only is the gene tubule specific, but it also is expressed in the segments of the tubule thought to be actively involved in urine production - these segments are of most interest in studies of Malpighian tubule function; expression of the gene is restricted to the final larval instar and adult flies, stages tractable to physiological study; ectopic expression in this temporal pattern is desirable as it reduces the potential for morphogenic abnormalities and should have a lesser effect upon viability.

Therefore the urate oxidase DNA control element is an ideal candidate for use in promoter fusion studies to generate a tubule wide expression of GAL4 protein.

In this study, the sequences upstream of the urate oxidase coding region which have been shown to direct Malpighian tubule specific expression of a transgene have been cloned upstream of the GAL4 cDNA sequence in the vector pGaTB, and germ lined transformed in the vector pCaSpeR4 (Figure 3.4.2).

3.2.1 Cloning of Urate Oxidase upstream sequences

The regulatory sequences upstream of the urate oxidase gene were cloned by PCR using specific oligonucleotides and genomic DNA as template. Oligonucleotide selection from the published DNA sequence is shown below (Figure 3.1). Sequences used to design oligonucleotides are in bold and underlined, successful PCR using these oligonucleotides and genomic template DNA yields a product of 860 bp.

C**TGCAGTTGCTATGCCAA**CCTTTTATTCCCTTTACTAAAAGGGTATACTAGGCTTA
CTGAACAGTATGTAACTGGTAAAGTAAAGCGTTTCCGATTCTATAAATTATATATC
TAAACTTTTGATCAGTCGAATCCATCTGAACACATTCTGTCACATTAGATTATTCC
AGAAACTCAACTTAAACATGTGTATTTTTTAAGACCATTATCAAGGATATTAAAAA
TGGTCTCCTAAAATTTAATAAACAAAGTGTCACATCAAATTTAAGACGTAAATTAT
ATTTTTTTTCTATGGTGAAATAATGTTATTTTCCAATGTTGTGAAATAATAAATGT
ATCTTTTCAACGCACACATTTTCAAGGTTTTAATAATAATAGTGACTCGTGCGTGA
ATAAGAGAGAAATTAAGATTTTAAAAAAGAATAAAATTCAGAGATGTGATCTGTAA
AAATTATTTACCAATTTTCATTTACCCCCGAAAGTGATGCTAATGGTTAAAACGGC
ATTTGCGACTTATCTCCTACGTAATATTGCAAAAATAAGGATTTGGTTAGATGAGT
GTGAAGTAAACAAGATGCAAAGTTTTGGAGATAGAAAACATAGCCTTGGAGTTTGG
TCATGTTTACTTGGCACCAGGCCGCAATTATCAGCGCTACTAGTCGTAATTTGAGT
TAGACCTTTAATACTCTAAGTGAGAGTGATGATATACGATTTCCCAGCCACTTGCT
TTCTACGAAATGCGCTAAAAAAAATCCCTAACTACACAAAGATTTGTGTT***GTTATC***
CAGGTGTTCTGATATAAAAGGCGGCAAGGAAATTGATGGCATCATCAGTATCAAAG
TG**AGAGTGATTGCAGTCACA**^{ATG}*TTTGCCACGCCCCTCAGACAGCCAGCTGCGGCTAACCACCAGACCCCA*
AAGAATTCCGCCGGCATGGATGAGCATGGTAAGCCGTATCAGTACGAGATTACCGATCACGGATACGGCAAGGAT....
.....GCAAAACCATATTTCGAGATCACCCTTGCCTTGGCTGAATAAATCCAAATTATATCAGAACTATTT
TCTCGAGTCAGTGTGCCAGGACGTTACCTGTTGCCCGACACCAGAAAGACCTACGACAATATGGTGGCCAATATTCACT
CGACCATGAAGTGTAAGCTGTTGTGCTAAAAACTTAACCAGAAACTTTCAAAACCAATCAATCATTTA

Figure 3.1

DNA sequence of the urate oxidase gene (from Wallrath *et al*, 1990). Upstream element sequence in plaintext, coding sequence in reduced size and italics. ATG start codon is emphasised as a superscript. DNA sequences used to design forward and reverse PCR primers in bold and underlined. The DNA sequence used to design an outward facing sequencing primer (UOOR) is shown in bold and italics.

3.2.2 Cloning strategy for PCR product

The strategy for isolation and primary cloning of the urate oxidase regulatory sequences is shown schematically below (Figure 3.2)

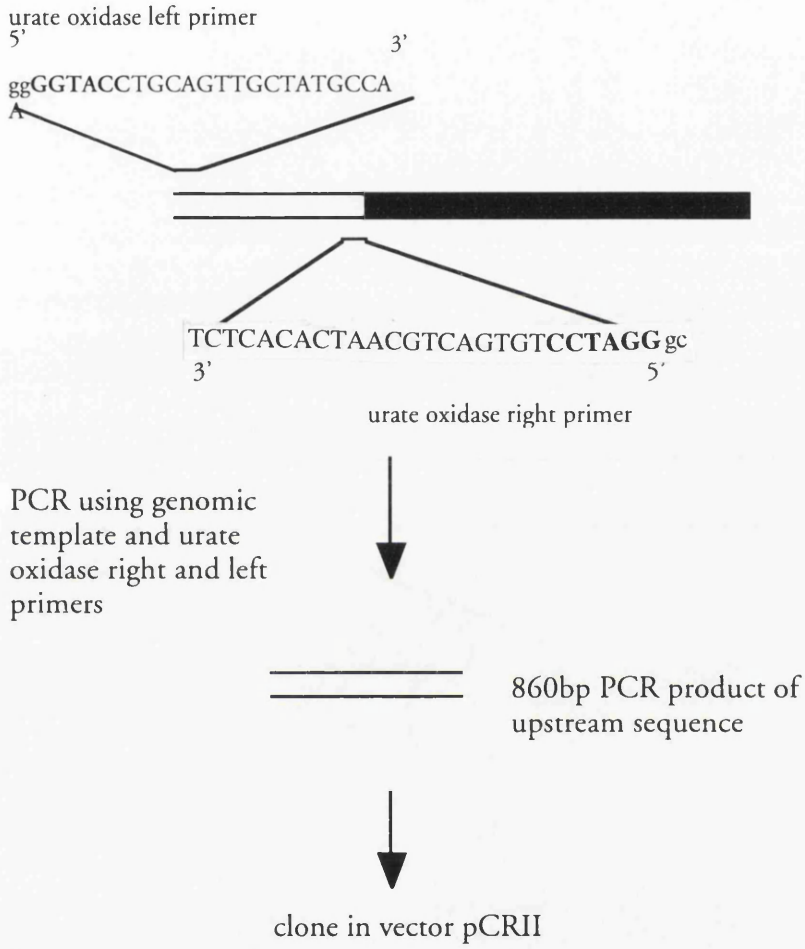
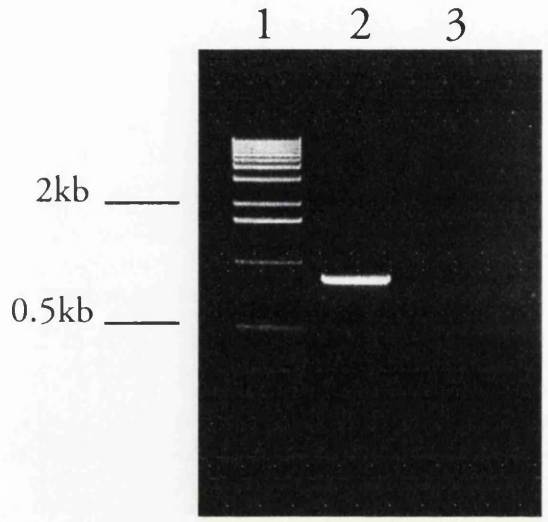


Figure 3.2

Mutagenic oligonucleotides direct PCR from *Drosophila* genomic DNA to isolate an approximately 860 bp sequence immediately upstream of the urate oxidase coding sequence. Oligonucleotide sequences shown; sequences in bold are recognition sites for restriction endonucleases (GGTACC recognition site of *Kpn1*, GGATCC recognition site of *BamH1*), and bases in lower case added to improve efficiency of endonuclease action on PCR product. The PCR product was cloned into the vector pCRII(Appendix 2)

3.2.3 PCR using Urate Oxidase Left and Right primers and genomic template



Lane 1, DNA 1kb ladder (GIBCO BRL)

Lane 2, product of PCR reaction with urate oxidase left and right primers and genomic DNA template

Lane 3, negative control PCR, no template

Figure 3.3

PCR using urate oxidase right and left primers and genomic template DNA amplifies a single product of approximately 860 bp.

3.2.4 Cloning of PCR product

The 860 bp PCR product was cloned into the plasmid vector pCRII (INVITROGEN, see appendix 2). This vector allows direct cloning of PCR products for sequencing. The product can be excised with the restriction enonucleases *Kpn1* and *BamH1* as sites for these enzymes have been designed in the oligonucleotides. This approach to the generation of PCR product with sticky ends for subcloning works with much greater efficiency than direct clevage of PCR product, as the restriction endonucleases cleave sites at the end of a linear molecule with lower efficiency than those within a circular molecule.

3.2.5 Sequence analysis of cloned 860bp fragment

The 860 bp fragment in pCRII was sequenced using the pCRII forward and reverse primers (forward, AGCTCGGATCCACTAGTA; reverse, CTCGAGCGGCCGCCAGTG, see appendix 2).
The sequence determined from the forward primer is shown below.

Figure 3.4a

Sequence from pCRII forward primer
CCACCTTTTATTCCCTTTACTAAAAGGGTATACTAGGCTTACTGAACAGTATG
TAACTGGTAAAGTAAAGCGTTTCCGATTCTATAAATTATATATCTAAACTTTTG
ATCAGTCGAATCCATCTGAACACATTCTGTCACATTAGATTATTCCAGAAACT
CAACTTAAACATGTGTATTTT

Figure 3.4b
BLASTN alignment of above sequence with database sequences

```
emb|X51940|DMUROX Drosophila melanogaster DNA for urate oxidase (EC 1.7.3.3)
           Length = 2602

  Plus Strand HSPs:

 Score = 896 (247.6 bits), Expect = 5.6e-67, P = 5.6e-67
 Identities = 180/181 (99%), Positives = 180/181 (99%), Strand = Plus / Plus

Query:     9 CCACCTTTTATTCCCTTTACTAAAAGGGTATACTAGGCTTACTGAACAGTATGTAACTGG 68
             | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    16 CAACCTTTTATTCCCTTTACTAAAAGGGTATACTAGGCTTACTGAACAGTATGTAACTGG 75

Query:    69 TAAAGTAAAGCGTTTCCGATTCTATAAATTATATATCTAAACTTTTGATCAGTCGAATCC 128
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    76 TAAAGTAAAGCGTTTCCGATTCTATAAATTATATATCTAAACTTTTGATCAGTCGAATCC 135

Query:   129 ATCTGAACACATTCTGTCACATTAGATTATTCCAGAAACTCAACTTAAACATGTGTATTT 188
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   136 ATCTGAACACATTCTGTCACATTAGATTATTCCAGAAACTCAACTTAAACATGTGTATTT 195

Query:   189 T 189
             |
Sbjct:   196 T 196
```

The DNA sequence from the forward primer aligns almost perfectly to the published sequence of the urate oxidase gene. The sequence mismatch may be the result of a PCR error inducing a base change, or from a sequencing error; therefore the 860 bp PCR product is genuinely derived from the regulatory sequences of the urate oxidase gene. Sequence data using the reverse primer (data not shown) confirms that the fragment sequence is exactly as expected.

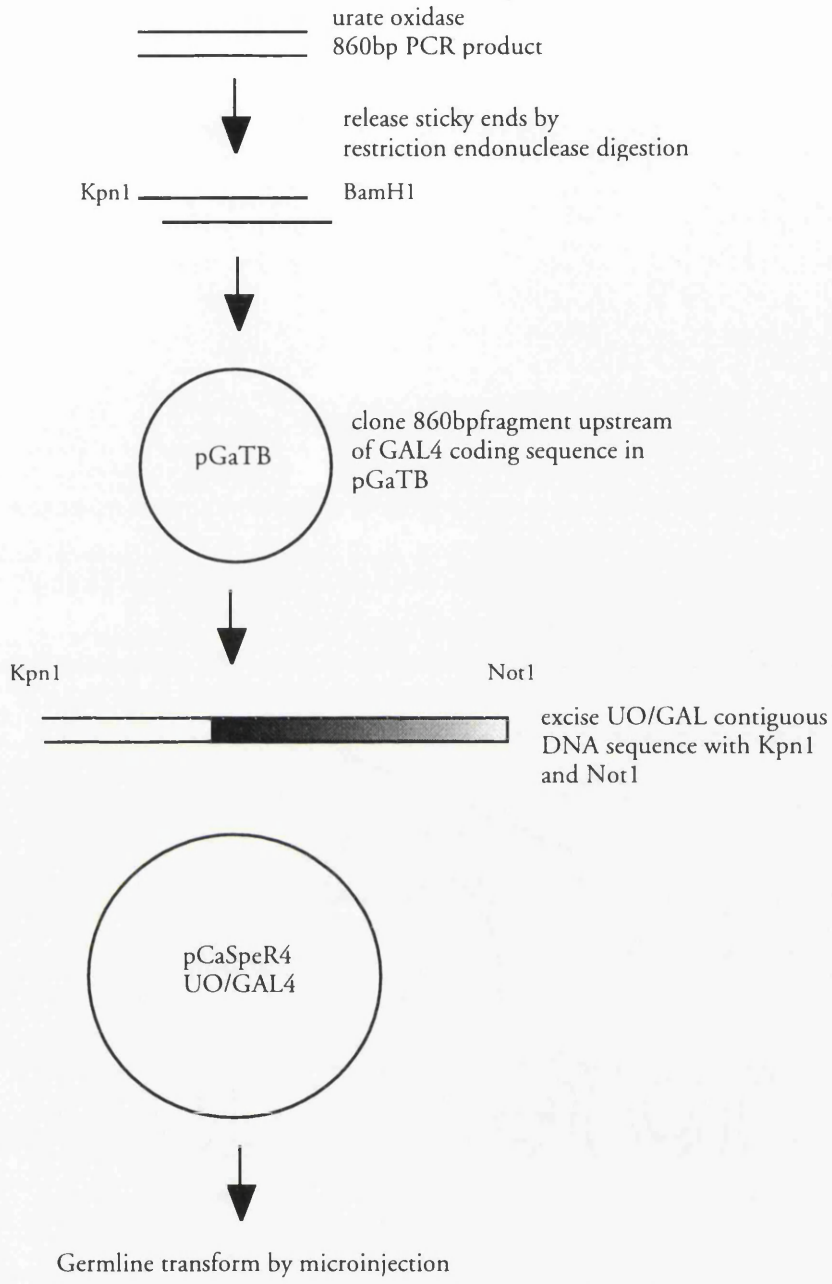


Figure 3.4.2

Flow diagram of construction strategy of pCaSpeR4-UO/GAL4. The 860 bp UO PCR fragment is excised from the vector pCRII using the restriction endonucleases *BamH1* and *Kpn1*. This fragment is cloned into the vector pGaTB, and a *Kpn1/Not1* fragment removed which bears the GAL4 coding sequence downstream of the urate oxidase control element. This fragment is cloned into the P-element transformation vector, pCaSpeR4 , and germ line transformed.

3.2.6 Constrution of UO promoter-GAL4 sequence

The vector pGaTB was obtained as a gift from Andrea Brand at the University of Cambridge. This vector was constructed by cloning of the GAL4 coding sequence (supplied on a *HindIII* fragment) into the vector pHSREM (Knipple and Marsella-Herrick, 1988). Upstream heat shock sequences were removed from the resulting molecule by restriction endonuclease digestion, and replaced with a unique *BamH1* site (Brand and Perrimon, 1993). The vector therefore contains unique *BamH1* and *Kpn1* restriction endonuclease recognition sites upstream of the GAL4 coding sequence; the GAL4 sequence is followed by a heat shock promoter polyadenylation signal, and a unique *Not1* recognition site.

The UO promoter *Kpn1/BamH1* fragment was cloned directionally upstream of the GAL 4 coding sequence.

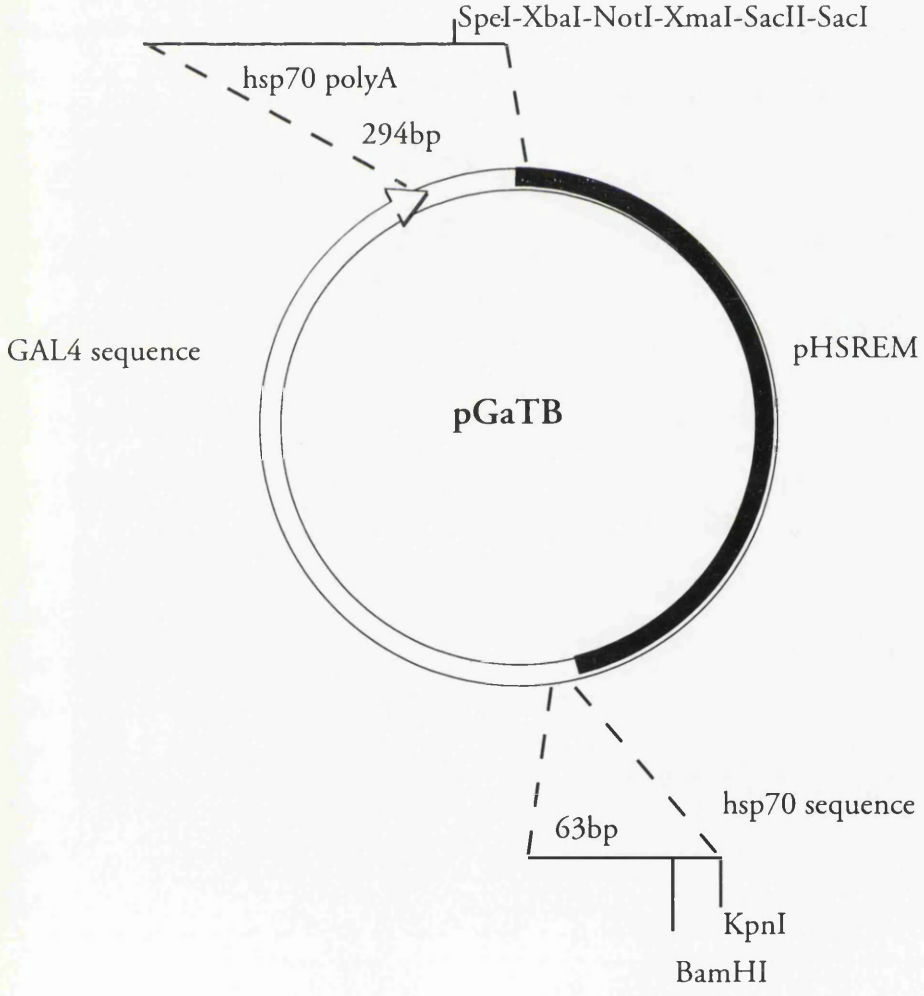


Figure 3.5

The vector pGATB. Urate oxidase regulatory sequences were cloned into the *Kpn1* and *BamH1* sites upstream of the GAL4 coding sequence. The UO-GAL4 construct can be removed as one fragment by the enzymes *Kpn1* and *Not1*.

3.2.7 Characterization of plasmid UO/pGaTB

The plasmid UO/pGaTB was prepared as described 2.1, and sequence analysis with the primer UOOR (GTTATCCAGGTGTTCTGATA, Figure 3.6.1) was performed. This oligonucleotide was designed to prime sequence outward from the urate oxidase derived sequence, across the *BamH1* site of the urate oxidase right oligonucleotide (urate oxidase right, see Figure 3.2), and into the GAL4 coding sequence. Sequencing with this oligonucleotide demonstrates that the urate oxidase regulatory sequences have been cloned in the desired orientation, and are immediately upstream of the GAL4 coding sequence.

Figure 3.6

Detailed map of the UO/GAL4 region of plasmid UO/pGaTB

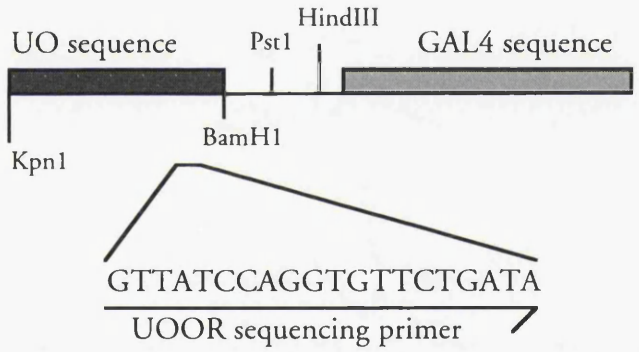


Figure 3.6.1

GTGATTGCAGTCACA***GGATCC***CGGTAAGCAAATAAACAAGCGCACGTGAACAAGCT

AAACAATT***CTGCA***GCCC***AAGCTT***GAAGCAAGCCTCCTGAAAG^{ATG}

DNA sequence of the plasmid UO/pGaTB using UOOR as sequencing primer. The sequence underlined is derived from the urate oxidase right primer (Figure 3.2). The sequences in bold and italics are recognition sites of restriction endonucleases; GGATCC-*BamH1*, CTGCA-*Pst1*, AAGCTT-*HindIII*. The italicised and superscripted ATG is the GAL4 sequence start codon.

From this sequencing data it is apparent that the construction of urate oxidase regulatory sequences immediately upstream of the GAL4 coding sequence has been effectively completed.

The UO-GAL4 sequence can be transferred to the germ line of *Drosophila* in a suitable transformation vector. The sequence is therefore completely excised from UO/pGaTB using the enzymes *Kpn1* and *Not1*, and cloned into the vector pCaSpeR4.

3.2.8 UO/GAL4 in pCaSpeR4

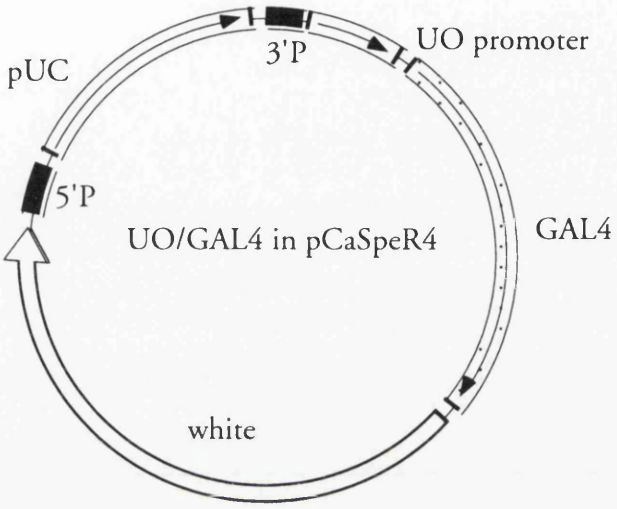


Figure 3.7a

Schematic representation of urate oxidase regulatory sequences upstream of GAL4 in the *Drosophila* germ line transformation vector pCaSpeR4.

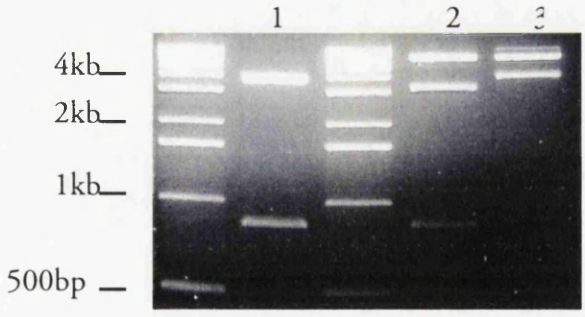


Figure 3.7b

Agarose gel showing restriction fragments of the plasmid shown in 3.7a. Lane 1, The urate oxidase (uo) 860 bp promoter fragment is excised from the plasmid pCRII as a size marker for the uo fragment excised from the pCaSpeR vector; lane 2, UO/GAL4/pCaSpeR cleaved with endonucleases *BamH1*, *Kpn1*. *BamH1* and *Kpn1* release the 860 bp uo fragment, and the GAL4 gene fragment is released with *BamH1* ends because of a *BamH1* site in the multiple cloning site in pCaSpeR 3' of the introduced GAL4 gene sequence (approx 3kb); Lane 3, UO/GAL4/pCaSpeR plasmid cut with restriction endonucleases *Kpn1* and *Not1*. These enzymes release the contiguous UO/GAL4 sequences and the vector pCaSpeR.

3.2.9 Transformation ♂ flies with plasmid UO/GAL4/pCaSpeR

Drosophila of the w^{1118} strain were transformed with the UO/GAL4/pCaSpeR plasmid as described in 2.12. Eight flies with eye colour reversion phenotype following microinjection procedures were observed. Separate lines from these flies were generated and maintained.

3.2.10 Demonstration that insertion of UO/GAL4/ pCaSpeR is cause of eye colour revertant phenotype

Southern hybridization to genomic DNA is a useful method for disclosing the presence or absence of differently sized genomic restriction fragments. Transgenic flies bearing an insert derived from the UO/GAL4/pCaSpeR plasmid will have a different restriction fragment length profile because of the mutagenesis performed. It is predicted that genomic DNA fragment of 860 bp will be present in genomic DNA cleaved with *Kpn1* and *BamH1*, which should not be present in wild type flies. This novel fragment length for a *Drosophila* sequence is due to the introduction of novel sites for these enzymes in the PCR reaction used to clone the urate oxidase fragment. A genomic Southern blot was made with wild type DNA (Oregon R) and DNAs from each of the transgenic lines maintained; the DNA in all cases was cut with *BamH1* and *Kpn1*, and the blot was hybridized with labelled urate oxidase 860 bp fragment DNA (Figure 3.8).

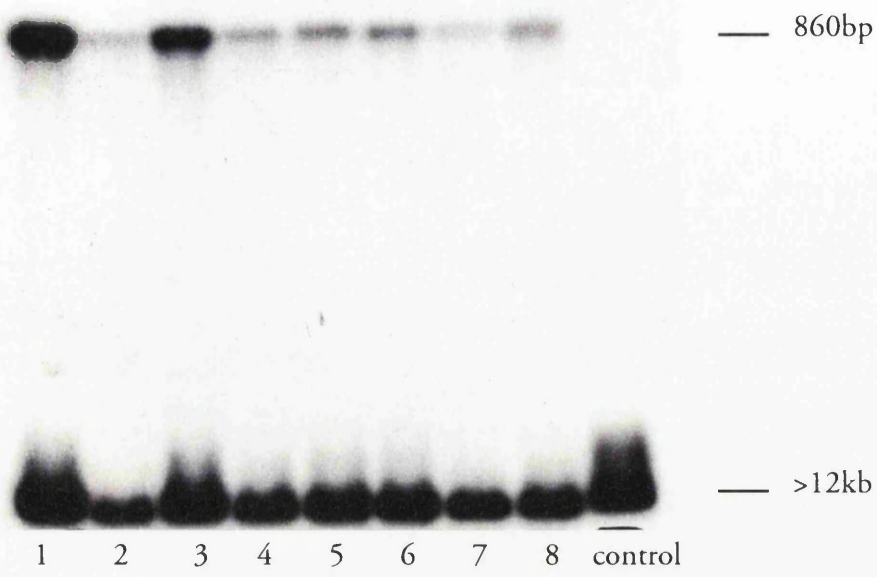


Figure 3.8

Genomic Southern hybridization of *Drosophila* genomic DNAs. Lanes 1-8, DNAs from flies derived from a parent showing eye colour phenotype reversion following microinjection with UO/GAL4/pCaSpeR; control, DNA from Oregon R flies. All DNAs are doubly digested with *BamH1* and *Kpn1*, the probe used in the hybridization is the 860 bp urate oxidase fragment. The blot was hybridized and washed at 65^{o}C, washed to 0.1 X SSC and exposed to film for 6 nights.

The Southern hybridization shows that in all lanes a single *BamH1/Kpn1* fragment is present. Only in the DNAs of the transgenic flies is there a supernumary band which is diagnostic for the insertion of the UO/GAL4/pUAST plasmid.

3.2.11 Activity of the UO-GAL4 construct *in vivo*

To assay the effect of the UO-GAL4 construct *in vivo,* flies bearing the insertion are crossed with homozygous flies bearing a UAS-*lacZ* insertion. The presence of the GAL4 protein can be assayed by detection of β-Gal activity in the GAL4/UAS-*lacZ* flies as described 2.15.

Flies transformed with the UO/GAL4 construct were mated to UAS-*lacZ* flies and the Malpighian tubules of the progeny scored for β-Gal activity (Figure 3.9)

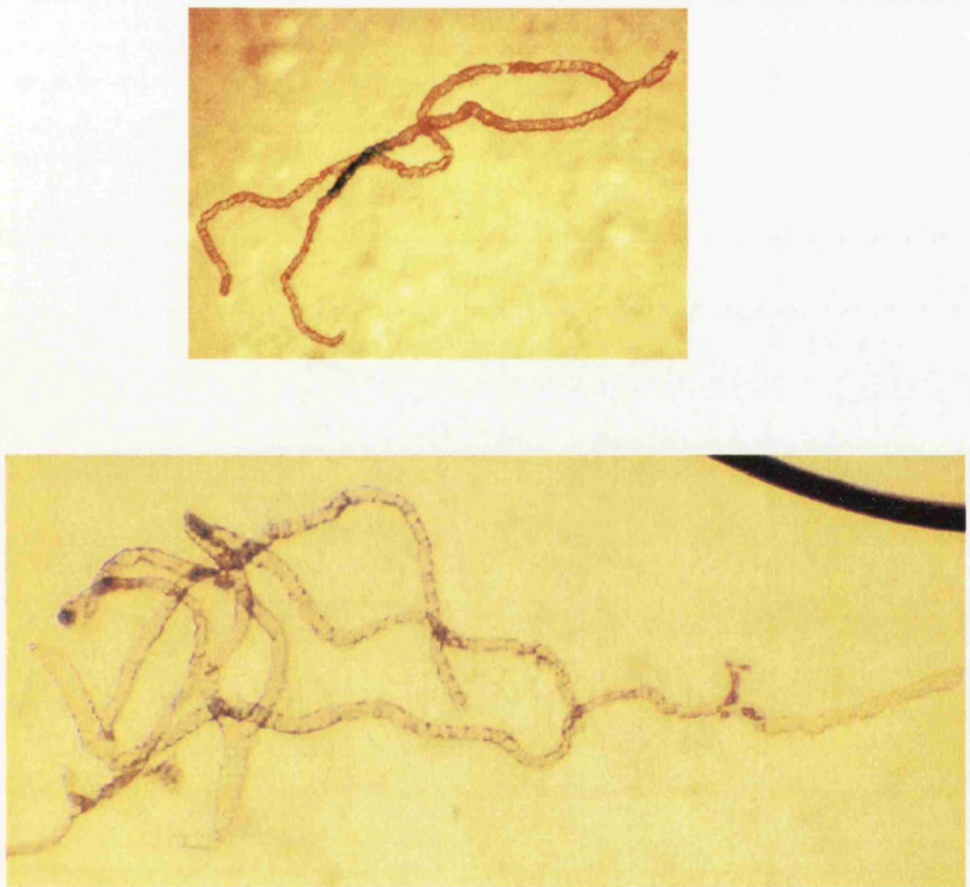


Figure 3.9

Tubules of *UO/GAL4:UAS-lacZ* flies with detectable β-Gal activity. The tubules were dissected, fixed and incubated overnight with X-Gal.

Only lines with a degree of tubule expression are shown. Of all the lines tested, two show a degree of β-Gal activity. In the pair of posterior tubules shown from one of the staining lines, a small segment of the main segment of one tubule can be seen to have stained (Figure 3.9, upper picture). The staining intensity is not as great as that

seen with some of the tubule specific P{GAL4} lines previously characterized (Sozen *et al.*, in preparation) In the other line (lower picture), there is very weak particulate expression throughout both tubules.

The other tissues of these flies observed and all tissues observed of the non tubule staining UO/GAL4 fly lines did not show unusual β-Gal activity.

3.3 Discussion

These results confirm those of Brand and Perrimon (Brand and Perrimon, 1993), that the GAL4 gene-promoter fusions can be constructed in the vector pGaTB, and that this construct can be transferred to the *Drosophila* genome in the germ line transformation vector pCaSpeR4.
In two of the transgenic fly lines obtained, a degree of tubule specific GAL4 distribution is observed in adult flies. The intensity of expression dictated by the urate oxidase promoter in these lines is relatively low when compared to the intensity of β-Gal activity disclosed in similar studies with enhancer trapped p{GAL4} elements in *Drosophila* Malpighian tubules (Sozen *et al.*, in preparation). In tubules of adult flies, expression of the urate oxidase gene is seen in the main segment of anterior tubules and along the whole length of posterior tubules. Thus, the urate oxidase 860 bp promoter fragment when fused to the GAL4 gene sequence in the vector pGaTB and germ line transformed does not direct a high level of expression in the normal pattern of the urate oxidase gene. The element does however drive two distinct patterns of expression in the tubule.

The difference in tubule β-Gal activity distribution between the two lines described above suggests that the 860 bp of the urate oxidase gene do not alone act to upregulate expression of the GAL4 coding sequence in transgenic flies bearing the insert. This is confirmed by the lack of expression in the other lines derived from insertion of the UO/GAL4 element. Therefore, it appears that the UO/GAL4 element relies on genomic positional effect to enhance expression of the GAL4 gene. It appears that this positional effect acts to direct expression within different domains of the tubule, rather than enhance the transcript number in the expression pattern of the urate oxidase gene. In an enhancer trap mobilisation experiment, approximately 10% of 700 different lines tested showed tubule specific expression from novel P{GAL4} enhancer traps (Sozen *et al.*, in preparation). In this study, two of eight lines show a degree of tubule specific expression; the proportion of lines generating tubule specific GAL4 distribution pattens may in fact be higher than 25%, as the number of unique insertions of the UO/GAL4 element in the lines derived has not been established. Therefore, this element may be useful in enhancer mobilisation studies to generate new discrete domains of GAL4 expression in the Malpighian tubule.

In the study in which the upstream sequence necessary to direct the expression pattern of the urate oxidase gene was determined (Wallrath *et al.*, 1990), 826 bp of 5' flanking DNA and 1200 bp of 3' flanking DNA were transformed along with the urate oxidase gene in a single contiguous fragment. Expression of the transgene was identified using oligonucleotides which hybridize specifically to 3' expressed sequences of the transgene. This study localized expression of the transgene in the expected tubule domains as wild type urate oxidase.

A further study (Wallrath *et al.*, 1991) in which the urate oxidase genes of *D. pseudoobscura* and *D. virilis* were transformed into *D. melanogaster* along with their own 5' and 3' flanking DNA sequences showed expression detected by northern analysis and urate oxidase enzyme activity assays in Malpighian tubules. Domains of expression from the transgene within the tubules were not established in the study. Deletion of the upstream flanking sequences of the *D. virilis* clone reduced detectable levels of *D. virilis* transcript in tubules, and showed expression in tissues other than Malpighian tubules.

In the studies described above, 5' and 3' flanking sequences of the urate oxidase genes are used, and the upstream sequences are replaced in the vector pCaSpeR. In this study only 5' sequence is used, and the inserted promoter and gene sequences are separated by 63 bp of *hsp70* TATA box sequence as a consequence of the use of pGaTB.

From the observations with a *D. virilis* clone in *D. melanogaster,* it is unlikely that the inclusion of 3' sequences of the UO gene in the UO/GAL4 construct would have a significant effect on the level and tissue distribution of GAL4. It is possible, however, that synthesis of this construct in pGaTB acts to reduce the promoter effect of the 860 bp sequence used, either as a result of the PCR based mutagenesis which created terminal *BamH1* and *Kpn1* restriction endonuclease recognition sites on the molecule, or as a consequence of the structure of the vector pGaTB. Alternatively, all promoter-reporter constructs may be subject to genomic positional effects upon insertion, and analysis of a larger number of independent UO/GAL4 insertions may result in characterization of a UO/GAL4 bearing fly with a GAL4 distribution identical to that of the urate oxidase gene

3.4 Further Work

Many p{GAL4} enhancer trap lines with discrete domains of tubule specific GAL4 expression have been characterized, although none of these lines give tubule specific expression in all of the main segment of both tubules. Generating a GAL4 line with an expression pattern similar to that of urate oxidase could therefore still be of interest.

It is possible that inclusion of a longer 5' flanking sequence of the urate oxidase gene in a UO/GAL4 construct may have a stronger effect in enhancing tissue specific expression of GAL4 in the pattern of the urate oxidase gene. Therefore, if the experiment is to be repeated, the cloning of genomic sequences around the urate oxidase coding sequence, and construction of a new UO/GAL4 element using - for example - 5 kb of 5' genomic sequence could be performed.

Alternatively, the existing line appears to have a greater tubule specificity than the generic P{GAL4} lines used in the studies of Sozen *et al.*, and simple mobilization of this new element may generate a family of novel insertions throughout the genome. This increases the diversity of positional genomic effect, and may result in many new GAL4 expression patterns in Malpighian tubules.

Chapter 4

Results 2

4.1 Introduction

There are already many model systems for study of cell surface receptors, mostly involving the use of cultured cells. Cells from both mammalian and insect species are routinely used for expression and characterization of receptor pharmacology and to identify intracellular signalling events following receptor activation by an agonist. Experimentation in cell culture is a well tested and trusted system for the generation of receptor specific data. Cell culture systems provide a homogenous genetic and cytologic environment for these studies, but do not reflect subtle differences in receptor biology which may be a consequence of different sites of wild type expression (Levy *et al.*, 1992).

This study assesses the potential of *Drosophila* Malpighian tubules for use as a reconstitution system for the study of heterologous expression of cell surface receptors. The Malpighian tubule has a relatively simple biology which is becoming well understood, and is unique among genetically manipulable model systems in that a functional assay (the tubule secretion assay) has been developed which allows quantitation of live whole organ function. Expression and analysis of a cell surface receptor in *Drosophila* Malpighian tubules may give important information about its behaviour in a multicellular environment. A long term goal of this approach is to use transgenic receptor biology to characterize the contribution to tubule function made by genetically identified compartments of the tubule (Sozen *et al.*, in preparation).

Typical experiments performed in cell culture based heterologous expression studies use radiolabelled ligand species to calculate binding affinity of a ligand to intact cells expressing a receptor, or to membrane preparations of cells expressing the receptor. Competitive binding experiments in the presence of a radiolabelled ligand and unlabelled ligands are used to generate specific pharmacological information, and assays for levels of second messenger molecules following receptor activation can be performed in whole cells, cell homogentates, or in membrane preparations from cells (Levy *et al.*, 1992; Peroutka *et al.*, 1988).

Transfection of receptor gene constructs to cell culture cells is easy, and cells bearing these constructs can be selected for due to the presence of resistance marker genes for specific cytotoxic agents in the plasmid constructs used in transfection. This type of transfection experiment can be followed by experimentation which generates data specific to expression of the receptor in a single cell type.

By contrast, the *Drosophila* whole organ approach may give functional information of the effect of the transgenic receptor on whole organ physiology. Also, it has been shown (Davies *et al.*, 1995) that levels of cyclic nucleotide second messengers can be measured in Malpighian tubules at the order of femtomoles per tubule with sensitive antibody-based assays. Membrane preparations of Malpighian tubule are not practical. Transformation of *Drosophila* Malpighian tubules relies on whole-organ germ-line transformation by microinjection of embryos with suitable constructs. This procedure is laborious and time consuming; however, it is usually successful, and sophisticated techniques have been developed for specific spatial and temporal activation of transgenic constructs.

The genetic technology to be used in this study is the GAL4/UAS system which has already been discussed. In particular, a receptor gene sequence is to be cloned downstream of the upstream activation sequence (UAS) in a *Drosophila* germ line transformation vector, before introduction to the *Drosophila* genome. The vector used is pUAST (Brand and Perrimon, 1993).

A serotonin receptor was chosen as the receptor species for expression in Malpighian tubules. Serotonin (5-hydroxytryptamine, 5-HT) is a neuromodulator present in most animal phyla which is involved in a variety of functions including sleep, appetite, learning, pain perception and vascular contraction (Kravitz, 1988; Saudou *et al.*, 1992). In tubule secretion assays using tubules of the Hemipteran *Rhodnius prolixus,* secretion rates are elevated by the specific interaction between 5-HT and cell surface expressed receptors (Maddrell *et al.*, 1991). Serotonin elicits an insignificant response when applied to *Drosophila* Malpighian tubules in a secretion assay (Simon Maddrell, personal communication). Therefore, the lack of physiological reactivity to serotonin makes the transfection of a serotonin receptor to the tissue a viable proposition, as the effect of the receptor can be examined in the absence of significant background.

In addition to being widely distributed, serotonin activates multiple receptor subtypes that are differentially expressed and couple to different intracellular signalling systems (Julius, 1991). The resultant changes in levels of second messengers such as inositol triphosphate or cAMP influence the activity of a battery of kinases that will in turn phosphorylate variable targets depending on the cell type in which the receptor is expressed (Saudou *et al.*, 1992).

Of numerous 5-HT receptors identified, four which belong to a large group of neurotransmitter and peptide hormone receptors whose biological effects are mediated by guanine nucleotide binding proteins (G-proteins) have been characterized; these subtypes are 5-HT1A, 5-HT1B, 5-HT1D and 5-HT2 receptors (Witz *et al.*, 1990; Julius, 1991).

Agonist binding to a specific G-protein coupled receptor results in the activation of heterotrimeric G-proteins (composed of subunits α, β, and γ) acting as transducers and signal amplifiers; activated G-proteins then modulate the activity of cellular effectors (Linder and Gilman, 1992). Serotonin receptors 5-HT1A, 5-HT1B, 5-HT1D and 5-HT2 belong to the extended family of G-protein coupled receptors that consist of a single polypeptide chain containing seven putative membrane spanning α helices. Other members of this class include the visual opsins, muscarinic cholinergic receptors, adrenergic receptors, tackykinin receptor and the dopamine D2 receptor (Julius, 1991).

In a preliminary experiment (data not shown), a *Drosophila* serotonin receptor gene sequence was cloned into the vector pUAST, and the construct germ-line transformed. Tubules of progeny of a cross between the p{GAL4} line C724 (stellate cells only) and the UAS-5-HT receptor bearing line showed elevated secretion rates when compared to the secretion rates of tubules from the w^{1118} strain, when challenged with millimolar serotonin. The UAS-5-HT receptor bearing fly line was lost, and no further studies were performed with it. This study validates the approach of ectopic expression of receptor species in Malpighian tubules, and demonstrates that the physiological consequences of this type of intervention can be assessed.

The receptor used in the following studies is a human 5-HT1D receptor. The human 5-HT1Dβ receptor is coded by an intronless gene encoding a 390 amino acid peptide (Demchyshyn *et al.*, 1991) with considerable homology within the putative transmembrane domains of canine and human 5-HT1D receptors. The pharmacological profile of the receptor expressed in chinese hamster ovary cells is consistent with, but not identical to the profile of other 5-HT1D receptors. Another human 5-HT1D receptor subtype was expressed in mouse Ltk^- cells (Levy *et al.*, 1992), and the effect of the receptor on intracellular adenylate cyclase recorded from total cell homogenates. This study demonstrated that this class of receptor reduces intracellular cAMP level.

As described in Chapter 1, cAMP is thought to be a significant second messenger in *Drosophila* Malpighian tubules; heterologous expression of a cell surface receptor capable of causing a decrease in intracellular cAMP level would therefore be a powerful tool in the molecular and biochemical dissection of Malpighian tubule function.

The following data describe the construction of a UAS-5-HT1Dβ receptor gene construct and its germ-line transformation in *Drosophila*.The receptor sequence is activated under the control of an enhancer trapped P{GAL4} element, and the ability of the expressed receptor to modulate intracellular cAMP level in response to 5-HT is tested.

4.2.1 The 5-HT1Dβ receptor gene

The 5-HT1Dβ receptor gene was supplied (gift, Lidia Demchyshyn) as a 2 kb DNA fragment cloned in the *Sac1* site of the vector pSP73 (Stratagene). The vector was transformed into competent *E.coli*, and prepared by alkaline lysis (see 2.1.2a). Orientation of the receptor gene in the vector was unknown. To establish the orientation of the gene in the vector, DNA sequencing was performed using the the T7 primer.

4.2.1b Sequence data from 5-HT1Dβ

The following sequence of the plasmid was established using the T7 primer

---

GGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCTCTCTCAGGACTATTCTGGATTCTCAGGATCCATT
GCCCTTACCCAGGAGAGAGAGCCTCAGCTGGGACCCAGAAACCGCGAAAGAAGATTCGAGGTACCTGTG
GAACCAGACACAACTTGGTCCCAAA

Figure 4.2.1

Sequence data obtained using the T7 primer and the supplied 5-HT1Dβ receptor gene in pSP73.

Human serotonin 1Dβ receptor (5-HT1Dβ) gene, complete cds.
Length = 1959

Score = 313 (86.5 bits), Expect = 3.4e-40, Sum P(2) = 3.4e-40
Identities = 65/68 (95%), Positives = 65/68 (95%), Strand = Minus / Plus

```
Query:   163 TTTGGGACCAAGTTGTGTCTGGTTCCACAGGTACCTCGAATCTTCTTTCGCGGTTTCTGG 104
             || ||||||||||||||||||||||||||||||  ||||||||||||||||||||||||
Sbjct:  1826 TTGGGGACCAAGTTGTGTCTGGTTCCACAGGTAGGTCGAATCTTCTTTCGCGGTTTCTGG 1885

Query:   103 GTCCCAGC 96
             ||||||||
Sbjct:  1886 GTCCCAGC 1893
```

```
 Score = 312 (86.2 bits), Expect = 3.4e-40, Sum P(2) = 3.4e-40
 Identities = 64/66 (96%), Positives = 64/66 (96%), Strand = Minus / Plus

Query:    94 GAGGCTCTCTCTCCTGGGTAAGGGCAATGGATCCTGAGAATCCAGAATAGTCCTGAGAGA 35
             |||||||||||||||||| |||||||||||||||||| ||||||||||||||||||
Sbjct:  1894 GAGGCTCTCTCTCCTGGGCAAGGGCAATGGATCCTGAGAAGCCAGAATAGTCCTGAGAGA 1953

Query:    34 GAGCTC 29
             ||||||
Sbjct:  1954 GAGCTC 1959
```

Figure 4.2.2

Section of BLAST search report showing alignment of sequence supplied to the 5-HT1Dβ receptor gene cDNA sequence.

This sequence was used to perform a BLAST database search, which generated the output above.

Analysis demonstrates that the sequence is indeed that of the 5-HT1Dβ receptor. The T7 primer generates DNA sequence from the 3' end of the 5-HT1Dβ receptor gene, orienting the gene in the vector as shown below.

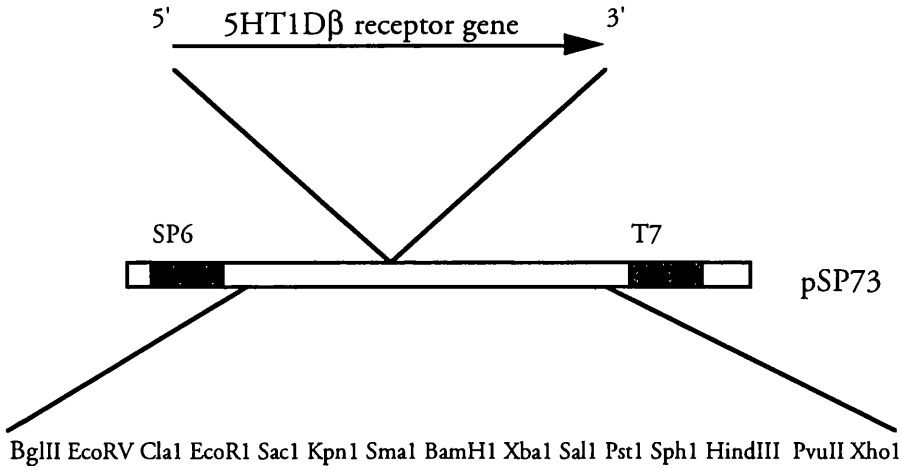


Figure 4.2.3

The orientation of the 5-HT1Dβ receptor gene (cloned in the *SacI* site) relative to the SP6 and T7 promoter sites on either side of the multiple cloning site is shown above the diagram of this section of the vector. The order of restriction enzyme recognition sequences of the multiple cloning site is shown below the diagram of the vector.

4.2.2 Cloning strategy for the 5-HT1Dβ receptor gene

The receptor sequence must be cloned into the vector pUAST in the correct orientation for *Drosophila* germ line transformation and subsequent analysis.

As the multiple cloning site of pUAST does not have a *SacI* site, the 5-HT1Dβ receptor gene must be inserted into the vector using the sites present in the multiple cloning site of pUAST while preserving the orientation of the sense strand. Therefore, a site between the *BglII* and *SacI* site of pSP73 and a site between the *SacI* and *XhoI* sites of pSP73 must be used for the 5' and 3' ends of the receptor gene respectively.

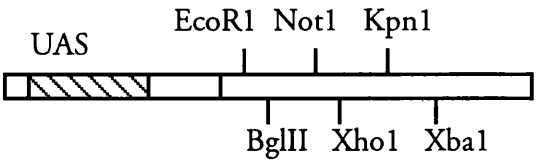


Figure 4.2.4

Detail of the multiple cloning site region of the vector pUAST. 5' of the multiple cloning site are the GAL4 binding sequences followed by the *hsp70* TATA box. Sequences which are to be expressed in sense *in vivo* are cloned with the 5' end of the sense strand adjacent to the upstream activation site.

On the 5' side of 5-HT1Dβ receptor sequence in pSP73 , *BglII* and *EcoR1* are available; on the 3' side *Kpn1*, *BamH1*, *Xba1* and *Xho1* are available. *BamH1* and *BglII* both cut within the gene sequence and cannot be used. Therefore the sequence can be sucessfully cloned into pUAST in the desired orientation using the restriction enzymes *EcoR1*, and *Kpn1*or *Xba1* or *Xho1*.

4.2.3 Cloning of 5-HT1Dβ receptor gene sequence into pUAST

The 5-HT1Dβ sequence was excised from the vector pSP73 using the restriction endonucleases *EcoR1* and *Xba1*. The insert was ligated into pUAST using these same sites, and the pUAST-5-HT1Dβ plasmid was prepared for microinjection into *Drosophila* embryos.

DNA sequencing of this vector was performed with a pUAST specific primer (GAAATCTGCCAAGAAGT). This oligonucleotide specifically primes sequencing reactions across the multiple cloning site of pUAST from the side of the upstream activation sequence (UAS). The sequence data (not shown) confirms that the 5-HT1Dβ receptor gene sequence has be inserted into the vector in the desired orientation.

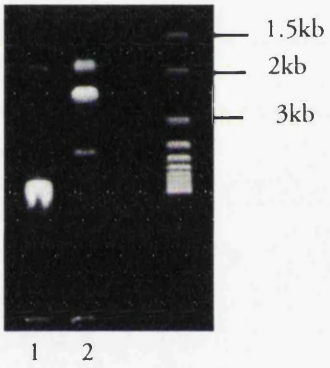


Figure 4.2.5

Agarose gel showing; 1, 5-HT1Dβ receptor gene in pUAST. The molecule is cut with restriction endonucleases *EcoR1* and *Xba1* to release an approximately 2 kb receptor gene; 2, 5-HT1Dβ-pSP73 cut with *EcoR1* and *Xba1*. The released fragment is seen to be the same size as the receptor gene of pSP73-5-HT1Dβ.

4.2.4 Germ line transformation with pUAST-5-HT1Dβ

The plasmid pUAST-5-HT1Dβ was germ line transformed as described in 2.12.2. Three individual flies with revertant phenotype eye colour were obtained. Independent homozygous lines from these flies were generated.

4.2.5 Confirmation of insertion of 5-HT1Dβ sequence in transgenic animals

If the revertant eye colour phenotype of the flies obtained results from the insertion of the vector pUAST-5-HT1Dβ, the 2 kb 5-HT1Dβ receptor gene sequence can be excised from genomic DNA of these flies by digestion with restriction endonucleases *EcoR1* and *Xba1*. The presence of a 2 kb fragment in this genomic digest can be demonstrated by Southern hybridization, using the 5-HT1Dβ receptor sequence as a probe.

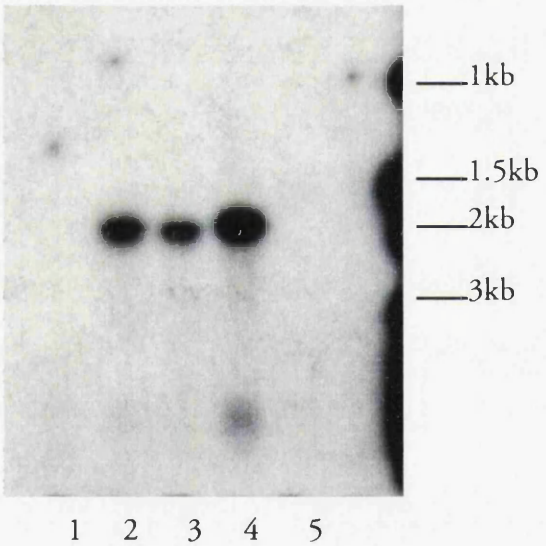


Figure 4.2.6

Southern blot of *Drosophila* genomic DNAs hybridised with the 5-HT1Dβ receptor gene sequence. All DNAs are double cut with *EcoR1* and *Xba1*. Lanes 1and 5 wild type (Oregon-R) genomic DNA; lane 2, 3 and 4, double cut genomic DNA of the three independently maintained transgenic fly lines. The blot was hybridized and washed at 65°C to 0.5xSSC, and exposed over 4 nights to film.

This Southern blot shows that each of the separate lines possess a 2 kb *EcoR1/Xba1* restriction fragment which hybridizes with the 5-HT1Dβ sequence. Neither of the two wild type lanes loaded hybridize with this sequence, and the background hybridization in

all lanes is very low. This indicates that the revertent eye colour phenotype observed in the separately maintained transgenic lines is due to the insertion of the pUAST-5-HT1Dβ plasmid. The human 5-HT1Dβ receptor gene does not specifically hybridize to *Drosophila* sequences, and non specific hybridization is low. These further indicate that the transgenic animals have been transformed with the human serotonin receptor gene.

4.2.6 Are the transgenic lines derived from the same insertion event?

The result shown above, demonstrates that the 5-HT1Dβ receptor gene has been inserted in all of the three lines showing eye colour phenotype reversion following germ line transformation procedures. That experiment however highlights the similarities between these lines, and does not expose differences, if any. The three transgenic lines may be derived from a single insertion in the germ line of a parent, or may be derived from different insertions in the germ line of one or several flies. If these lines are derived from the same insertion event, they will have identical restriction fragment lengths after restriction endonuclease digestion. If derived from different insertion events, it is likely that the restriction fragment profile will differ between the lines. Genomic Southern hybridization using the 5-HT1Dβ receptor gene sequence as probe can be used to distinguish between these possibilities.

The *EcoR1* and *Xba1* sites of the insertion are both unique within the insertion. When genomic DNA from a pUAST-5-HT1Dβ line is digested with either enzyme, the insert will be present on a DNA fragment generated by clevage of the site present on the vector and the nearest wild type genomic site. Therefore the distance between the introduced and nearest wild type sites can be diagnostic for uniqueness of insertion.

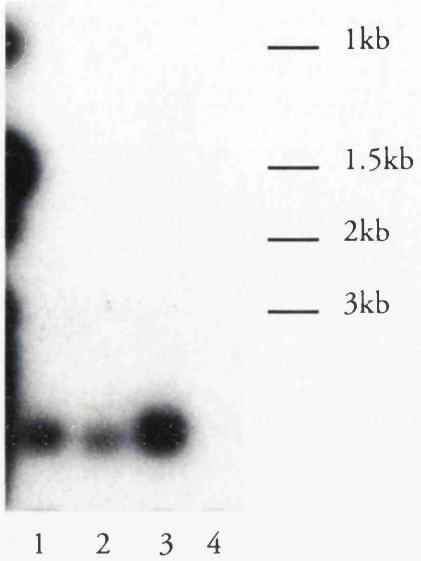


Figure 4.2.7

Southern hybridization of a blot bearing; lane 1, 2, and 3 - DNA from the separate transgenic lines; lane 4 - wild type Oregon R DNA. All DNA is singly digested with *EcoR1*. The blot was hybridized with 5-HT1Dβ receptor gene sequence, washed at 65^{o}C to 0.5xSSC, and exposed to film over four nights. Wild type DNA does not hybridize with the probe, consistent with previous data. The probe hybridizes to a single restriction fragment in all lines as expected, and the fragments in the three of these lines are identical in size, at approximately 9 kb.

From the blot above, it is shown that the three transgenic lines are genetically identical with respect to the pUAST-5-HT1Dβ insertion.

4.3 Expression of the 5-HT1Dβ receptor gene

Section 4.2 detailed the cloning of the human serotonin receptor gene downstream of GAL4 binding sites in a P element transformation vector, and its insertion into the *Drosophila* genome. This section describes the activation of the transgenic receptor gene.

4.3.1 Strategy for expression of the 5-HT1Dβ receptor gene

Expression of the 5-HT1Dβ receptor gene can be activated by providing a tissue specific source of the GAL4 protein. The desired location for heterologous expression in this study are the Malpighian tubules. Several P{GAL4} lines with Malpighian tubule specific expression have been identified and characterized (Sozen *et al.*, in preparation). One of these lines, line C324, expresses throughout the tubule. This line is used in all of the subsequent studies.

To demonstrate that the C324 line is capable of activating expression of a transgene downstream of UAS, this GAL4 construct was crossed into a UAS-*lacZ* reporter background. Activated *lacZ* expression is scored by analysis of β-Gal activity in the tubule in the method described in 2.15.

4.3.2 Crossing scheme of p{GAL4} X UAS-*lacZ*

P{GAL4} X UAS-*lacZ*
P{GAL4} UAS-*lacZ*

UAS-*lacZ* ; P{GAL4}

Flies homozygous for P{GAL4} line C324 and the UAS-*lacZ* construct are mated to generate the UAS-*lacZ*; P{GAL4} genotype

4.3.3 β-Gal localisation in UAS-*lacZ*;P{GAL4} flies

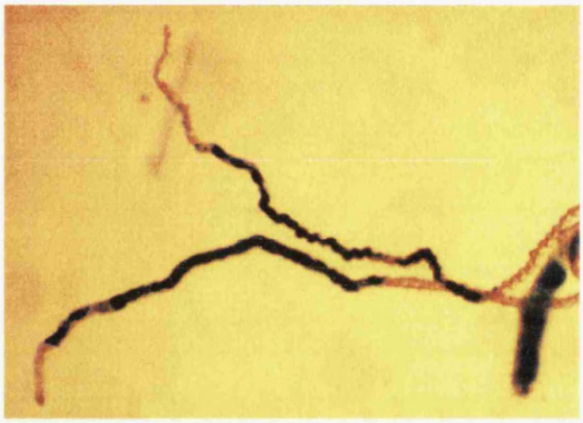


Figure 4.3.1

Photograph of a Malpighian tubule expressing β-Gal protein under the control of an enhancer trapped p{GAL4} line. There is extensive expression throughout the main segment tubule.

This experiment shows that the P{GAL4} line C324 is capable of activating a high level of expression of a gene downstream of the UAS sequence. It is therefore of use in further experimentation.

4.3.4 Activation of the 5-HT1Dβ receptor gene

The 5-HT1Dβ receptor gene is activated by the enhancer trapped C324 p{GAL4} line.

P{GAL4}	X	UAS-5-HT1Dβ
P{GAL4}		UAS-5-HT1Dβ

UAS-5-HT1Dβ ; P{GAL4}

Flies homozygous for UAS and P{GAL4} were mated to generate a fly line bearing both inserts in the genome.

As the chromosomal localisation of the UAS-5-HT1Dβ construct has not been determined, the notation above is given for guidance only, to demonstrate that progeny of the cross carry copies of both the enhancer trapped {GAL4} element and the UAS-5-HT1Dβ receptor gene construct.

4.3.5 Demonstration of activation of transcription of the 5-HT1Dβ receptor

An RT-PCR approach can be used to discover if any sequence is being transcribed in a target tissue. Gene specific oligonucleotides can be used to screen a first strand cDNA library, made specifically for this purpose, for presence or absence of a particular cDNA sequence. Ideally, the gene specific primers should bracket a small intron of the genomic sequence so that genomic contamination of the cDNA library can be assessed.

Oligonucleotides, based on sequences used to design oligonucleotides for PCR by Demchyshyn *et al.* , 1992 (Demchyshyn *et al.*, 1992) , were synthesised. These oligonucleotides (AAAGATGCCTGCTGGTTCCACCT ; ATCCTGAGAAGCCAGAATAGTCCT) will generate a product of 309 bp from both the genomic and cDNA copy of the 5-HT1Dβ gene in a PCR reaction.

cDNA was synthesised as described in 2.8 and used as template in a PCR reaction with the oligonucleotides given above.

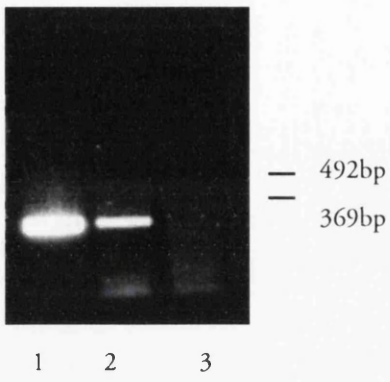


Figure 4.3.2

Agarose gel of RT-PCR product described above. Lane 1, positive control template, pUAST-5-HT1Dβ; this reaction controls firstly the efficiency of the PCR reaction, and also gives the size of the expected RT-PCR product. Lane 2, UAS-5-HT1Dβ/p{GAL4}C324 cDNA. The presence of the correct size PCR product suggests that the 5-HT1Dβ gene is being transcribed in this fly line. Lane 3, negative control, no template; this negative control sucessfully demonstrates that PCR products in lanes 1 and 2 are derived from the specific templates added.

The presence of the expected size band in the RT-PCR product lane (lane 2) demonstrates that there is 5-HT1Dβ template in the cDNA sample. It does not distinguish between cDNA and genomic template, and therefore this experiment does not prove that the receptor gene sequence is being transcribed.

4.3.6 Is the cDNA template contaminated with genomic DNA?

To establish if there is genomic contamination in the cDNA, a pair of oligonucleotides which hybridize to low abundance expressed sequences in Malpighian tubules, and which bracket a small intron in genomic structure can be used to direct RT-PCR from this cDNA sample. Presence of a genomic derived PCR product in a PCR reaction with cDNA as template, would mean that the positive result shown in Figure 4.3.2 would not necessarily demonstrate the presence of 5-HT1Dβ transcript in *UAS-5-HT1Dβ/C324* flies.

The oligonucleotides C155 and G 653 hybridize to sequences coding the 57K subunit of the V-ATPase gene, and bracket a small intron (Davies *et al.*, in press), allowing the genomic contribution to the PCR product to be assessed.

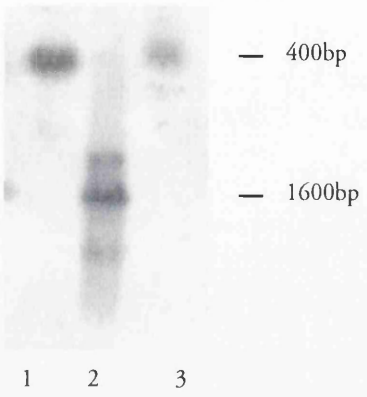


Figure 4.3.3

Southern hybridization of PCR reaction using C155 and G653 and various templates. Lane 1, plasmid template bearing cDNA copy of gene; lane2, wild type genomic template; lane 3, UAS-5-HT1Dβ/C324 cDNA template. A clean negative control lane on this blot showed that the signals are derived from the templates and not PCR contamination (not shown). The blot was hybridized with DIG dUTP labelled cDNA sequence and washed at 65°C to 0.1xSSC. After washing the blot was developed as described 2.2.6b.

This data shows that the cDNA template from Malpighian tubules of flies bearing UAS-5-HT1Dβ/C324 contains a cDNA sequence known to be expressed in Malpighian tubules, and that this cDNA preparation is not at all contaminated with genomic DNA.

Taken together, these data strongly suggest that there is a 5-HT1Dβ receptor gene transcript in the Malpighian tubules of UAS-5-HT1Dβ/C324 flies.

4.4 Analysis of heterologous expression of 5-HT1Dβ receptor in *Drosophila* Malpighian tubule cells

Having shown that a transgenic insertion of a human gene can result in transcription of that gene, the functionality of the transcript is assessed.
As described in 4.1, this class of 5-HT receptor couples to the modulation of cAMP concentration. The following experiments detail the analysis of cAMP concentration *in vivo* of UAS-5-HT1Dβ/C324 Malpighian tubules when challenged with agonists of this receptor.

4.4a The assay

The chosen system for cAMP concentration determination is the Amersham Biotrak cAMP [^{125}I] assay system. This system has a sensitivity of 2-128 fmol/sample. Experiments estimating tubule concentration of cGMP in Malpighian tubules have used a similar assay (Davies *et al.*, 1995), and the 2-128 fmole/sample range was found to be sufficiently sensitive for this system.
The assay is a competitive binding assay between a fixed amount of ^{125}I labelled cAMP, the variable amount of unlabelled cyclic nucleotide present in the tissue , and a fixed amount of anti-cAMP specific antibody. In the assay the amount of cAMP present in a sample is inversely proportional to the quantity of labelled cAMP present after reaction, and the concentration can be determined by interpolation of a standard curve.
The kit provides sufficient reagents for 100 reactions when used according to the manufacturers protocols. In all of the experiments shown, all volumes used are exactly half of those recommended (Coast *et al.*, 1991). This amendment to the protocol does not affect the concentrations of cAMP in the standard curve samples or in any other sample.
As the kit is expensive, and the number of reactions possible from each kit are limited, the experiments which follow are designed to give as much information as possible - using sufficient controls to allow interpretation of data, while minimising the number of controls where possible to maximize data output.
To control the effect of temperature on this tissue, all dissections and stimulations are performed at 18°C. This temperature was chosen, as it is the temperature of the only controlled environment available

4.4.1 Can cAMP be measured in *Drosophila* Malpighian tubules?

Before analysis of the effect of expression of the 5-HT1Dβ receptor on Malpighian tubule cAMP levels, a basic calibration experiment was performed to establish if tubule cAMP levels could indeed be measured, and therefore to discover how many tubules per sample to use in subsequent experiments.

In this experiment 6, 10, 20 and 30 tubules were dissected into separate tubes containing 50 µl of *Drosophila* saline. The strain of *Drosophila* used was w^{1118}, the parental strain of the transgenic flies. Two volumes of ice cold ethanol were added to the tubes containing the dissected tubules. The tissue was macerated in the tube using a mini pestle. Ethanol and water were removed in a rotary evaporator, and the dried tubule derived matter was resuspended in 250µl cAMP assay buffer (Amersham) before proceeding with cAMP level assay.

Quadruplicate samples for each tubule number point were dissected, and cAMP levels were determined in duplicate from each sample; therefore, n=8 in the statistical treatment of the data. Number of counts were determined on an LKB 1282 Compugamma universal gamma counter. Standard error was calculated as a function of the number of counts (i.e. not from derived cAMP levels), and its percentage of the number of counts determined. This percentage value was applied to the cAMP level for each point derived from the standard curve, to give the values shown below. Raw data are given in Appendix 3.1.

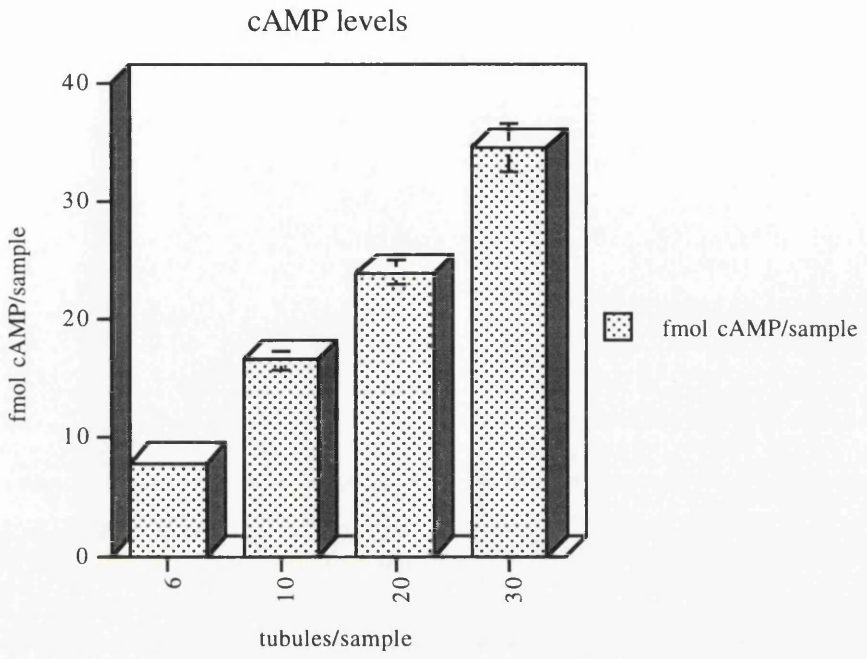


Figure 4.4.1a

Graphical representation of the detected cAMP levels in preparations from 6, 10, 20 and 30 Malpighian tubules using the protocols described above.

no of tubules	fmol cAMP/sample	% standard error
6	7.94	±3.5
10	16.6	±4.9
20	24	±4.2
30	34.6	±5.74

Figue 4.4.1b

Table giving the data used to draw the graph 4.4.1a. cAMP concentration is given as fmol/sample with the percentage standard error calculated from raw counts data (n=8).

The results demonstrate that the protocols used are capable of measuring cAMP levels in Malpighian tubules. There is an almost linear relationship between the number of tubules used per assay and the cAMP level determined. This indicates that the assay is accurately measuring the cAMP concentration in all preparations. Using this data, the tubules from four flies (i.e. 16 tubules) were used per sample in the next experiment. It was anticipated that resting cAMP levels from 16 tubules will be in the mid range of that shown above, therefore small decreases or increases in cAMP level should be measurable. It can be seen (Figure 4.4.1) that the standard error values increase with the number of tubules used per sample, and using a smaller number may limit the errors seen.

4.4.2 Does 5-HT affect tubules expressing the 5-HT1Dβ receptor gene?

The effect of 5-HT on transgenic tubules can be examined by assaying the *in vivo* cAMP concentration at various time points after supply of 5-HT. In the same way the effect of differing concentration of supplied 5-HT can be examined. Naturally, control reactions where cAMP levels in wild type tubules are assessed at each time point and at each 5-HT concentration used are desirable. Not all of these controls can be performed with the resources available, and so a strategy for this experiment was developed.

In the reactions used to generate the data shown below, cAMP concentration of 16 tubules per sample is determined at t = 5, 10, 15, 20 and 30 seconds after stimulation with millimolar, micromolar and nanomolar 5-HT. The serotonin species used is 5-hydroxytryptamine hydrochloride (Sigma, H9523). The data were analysed to establish a timepoint and 5-HT concentration which generate a significant effect on intracellular cAMP level, and further experiments are to be performed using this 5-HT level and the timepoint uncovered using control tubules.

There is a difficulty in assigning a genotype for control tissue. The genotype of test tissue is derived from a w^{1118} background, into which is inserted a p{GAL4} element and a UAS element. There is validity in testing all three separate lines (w^{1118} ; *p{GAL4}, C324*; *UAS-5-HT1Dβ* receptor gene bearing line) as negative controls. To establish if there is a significant difference between the lines an experiment was performed using tubules from w^{1118}, *p{GAL4, C324}, UAS-5-HT1Dβ* fly lines and tubules from the activated p{GAL4}/UAS line. Tubules were dissected into 50µl of a 1:1 mixture of Schneiders medium (Gibco) : *Drosophila* saline, supplemented with 4% glucose, and macerated in 2 volumes of ice cold ethanol/ 200µM IBMX (3-Isobutyl-1-methylxanthine). All subsequent reaction conditions were as described before.

This experiment allowed comparison of the resting concentration of cAMP in the four fly lines tested, and may be useful in assessing differences between the lines. If no significant differences were seen then any of these lines can be used as the negative control in future reactions.

Interestingly, the inclusion of the cAMP phosphodiesterase inhibitor IBMX in the ethanol, along with the use of a richer bathing medium, almost doubles the cAMP concentration observed per tubule.

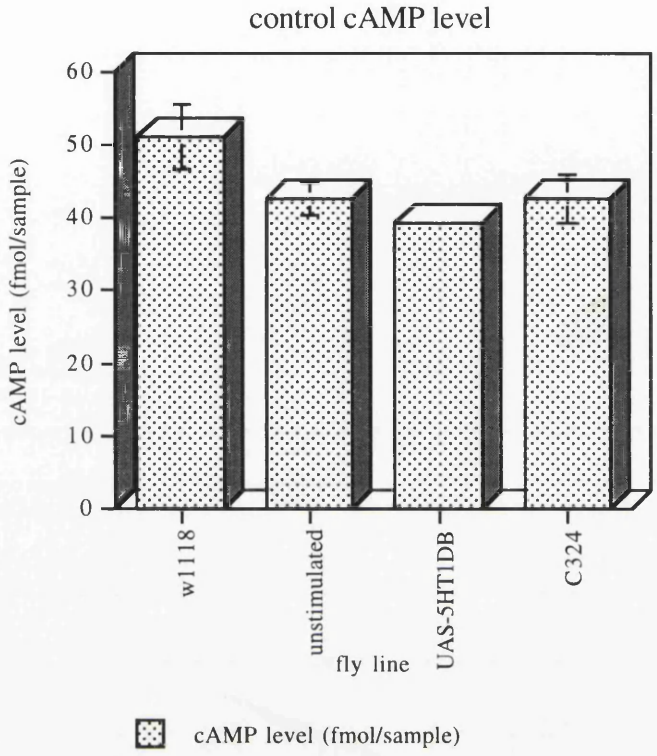


Figure 4.4.2a.

Graphical representation of the cAMP concentration determined from samples of 16 Malpighian tubules from unstimulated w^{1118} *UAS-5-HT1Dβ/C324*, *UAS-5-HT1Dβ* and *C324* fly lines.

fly line	cAMP fmol/sample	%standard error
w^{1118}	51.2	±8.7
unstimulated	42.65	±5.2
UAS-5-HT1D*β*	39.35	±2.2
C324	42.65	±7.9

Figure 4.4.2b

Table showing data used to draw the graph 4.4.2a. cAMP concentration is given in fmol/sample tested with percentage standard error calculated from raw counts data (n=6) (unstimulated, tubules from *UAS-5-HT1Dβ/C324* flies).

There is no significant difference in the resting cAMP level between w^{1118} tubules and receptor expressing tubules (t=1.65; p,0.05 with four degrees of freedom). There is very little difference between the cAMP levels in the tubules of the other lines tested. Most

importantly, the unstimulated UAS/GAL4 line shows almost identical cAMP concentration to the two parent lines (for raw data see Appendix 3.2).

4.4.3 Effect of 5-HT on Malpighian tubules from *UAS-5-HT1Dβ/C324* line

The following figures show graphical representations of the cAMP level from *UAS-5-HT1Dβ/C324* Malpighian tubules at 5, 10, 15, 20 and 30 seconds after stimulation with millimolar, micromolar and nanomolar 5-HT. The 5-HT is solubilised in 1:1 saline:Schneiders' medium supplemented with ~~4%~~ 0.4% glucose (for raw data, see Appendix 3).

4.4.3a Effect of millimolar 5-HT

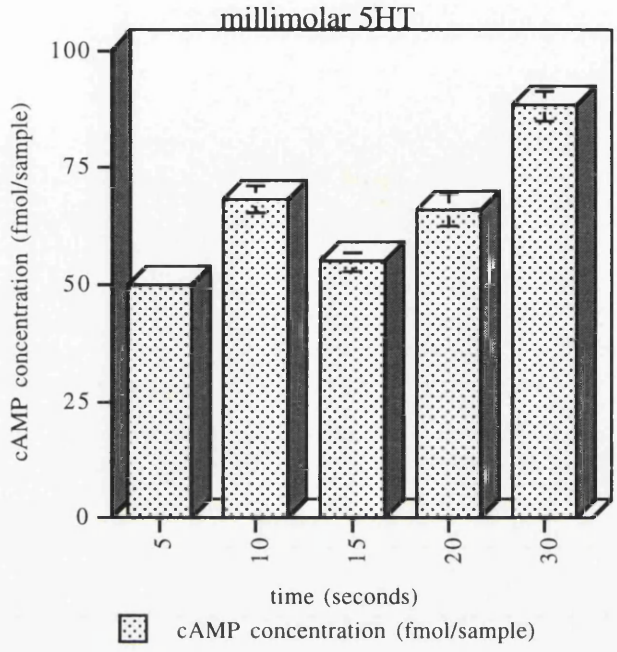


Figure 4.4.3a

Graphical representation of the cAMP concentrations derived from Malpighian tubules from *UAS-5-HT1Dβ/C324* line after stimulation with 1 mM serotonin (final concentration).

time (seconds)	fmol cAMP/sample	percentage standard error
5	50. 11	±3.1
10	68.39	±4.3
15	54.95	±3.38
20	66.1	±4.99
30	88.1	±3.39

Figure 4.4.3b

Table giving numerical data used to draw graph 4.4.2a. cAMP level per sample is given in fmol/sample. Standard errors are expressed as percentages of the mean counts observed (n=6).

4.4.3b Effect of micromolar 5-HT

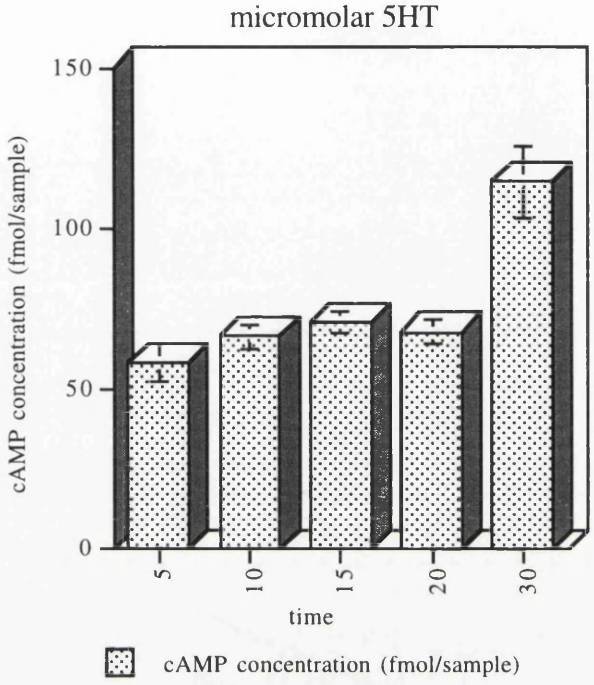


Figure 4.4.4a

Graphical representation of cAMP concentrations of Malpighian tubules of a *UAS-5-HT1Dβ/C324* line after stimulation with 1 μM serotonin (final concentration)

time (seconds)	fmol cAMP/sample	percentage standard error
5	58.21	±10
10	66.1	±6.23
15	70.79	±4.51
20	67.6	±5.93
30	>128	±10.41

Figure 4.4.4b

Numerical data used to generate graph shown 4.4.4a. cAMP concentrations are given in fmol cAMP per sample. Standard errors are expressed as a function of mean counts observed(at t=5, 15 and 30 seconds n=5; t=10 and 20 n=6).

4.4.3c Effect of nanomolar 5-HT

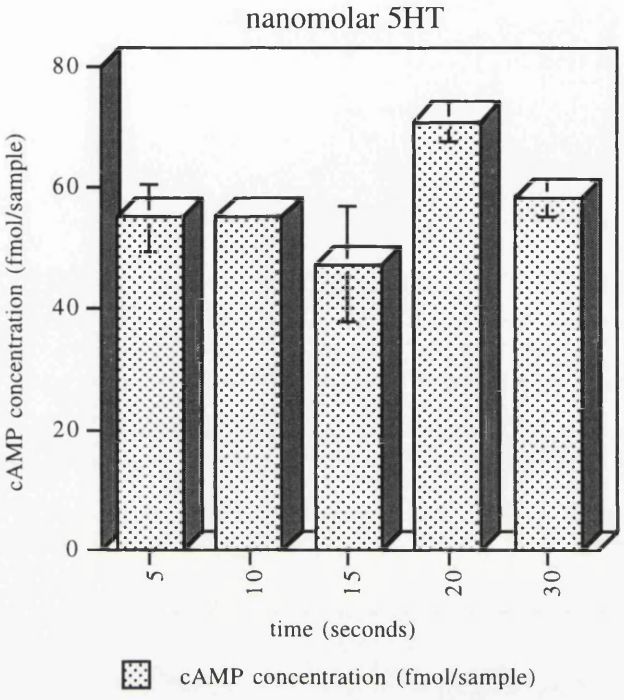


Figure 4.4.5a

Graphical representation of cAMP levels determined from tubules of a *UAS-5-HT1Dβ/C324* line after stimulation with 1 nM serotonin (final concentration)

time (seconds)	fmol cAMP/sample	percentage standard error
5	54.95	±10.1
10	54.95	±2.2
15	47.31	±20.3
20	70.79	±4.9
30	58.21	±5.55

Figure 4.4.5b

Numerical data used to generate graph 4.4.5a. cAMP concentrations are given in fmol/sample assayed. Standard errors are expressed as percentages of the mean counts observed per time point (n=6)

The graphs drawn represent accurately the values determined for each time point in all but one case. At 30 seconds after stimulation with micromolar 5-HT, the cAMP value

indicated by the number of counts was above the highest point of the standard curve (128 fmol). Consequently to establish a value, a linear regression analysis was performed; with the best straight line, a value of 114.28 fmol was calculated. This value is plotted and standard errors are calculated from the percentage error of the genuine value. This figure (114.28 fmol) underestimates the concentration of cAMP and is shown for graphical purposes only.

From the graphs (figures 4.4.3a-4.4.5a) it is seen that stimulation with both millimolar and micromolar serotonin lead to a significant increase (difference assessed between cAMP levels of stimulated *C324;UAS-5-HT1Dβ* and unstimulated tubules of the same genotype) in cAMP concentration observed after 30 seconds (students t test, $t_{millimolar}=5.233$: $t_{micromolar}=10.62$ both with 4 degrees of freedom). The magnitude of the response is greater after stimulation with micromolar 5-HT (>128 fmol/sample ± 10.41%) than after stimulation with millimolar 5-HT (88.1 fmol/sample ± 3.4%). There is a significant increase in cAMP concentration when tubules are stimulated with nanomolar 5-HT (students t test; $t_{nanomolar}=4.06$, however this increase is seen after 20 seconds, and the magnitude (70.98 fmol ± 4.9%) is the lowest peak of those observed.

4.4.4 Further experiments

Having observed a significant increase in cAMP concentration in Malpighian tubules expressing the 5-HT1Dβ receptor gene 30 seconds after stimulation with micromolar 5-HT, a final set of experiments were designed to characterize this response. Given the magnitude of the response to micromolar 5HT , the number of tubules dissected per sample was reduced to 12. Also, each sample was dissected in triplicate, but the cAMP assay was not duplicated because of the dwindling consumable content of the kit. Hence, in the statistical analysis n = 3.

w^{1118} tubules were chosen as the negative control for the response as it is the common genetic background of all the lines used.

Tubules expressing the 5-HT1Dβ receptor under C324 GAL4 control were stimulated with micromolar 5-HT as a positive control, and an unstimulated sample of these tubules was assayed as an internal control.

Furthermore, an attempt was made to characterize the response described before. In Demchyshyn *et al.*, 1992, competitive binding assays with tritiated 5-HT and unlabelled agonists and antagonists were performed to generate K_i values for these effectors. In this study, the 5-HT1Dβ receptor when transfected and expressed in Chinese Hamster Ovary cells gave a K_d of approximately 4 nM. The partial antagonist of this receptor subclass, yohimbine (Levy *et al.*, 1992), in the study with the 5-HT1Dβ receptor gave a K_i of 41.2 nM. Therefore there is a difference of a single order of magnitude in effector concentrations.

The possibility that the cAMP concentration elevation in Malpighian tubules expressing 5-HT1Dβ receptor is due to an interaction between the receptor and the agonist was tested. Coincubation with a partial antagonist should reduce the cAMP level response to below the maximal stimulation, however, this reduction should not bring the cAMP level to that of unstimulated tubules. The maximal effect on cAMP concentration of Malpighian tubules was seen at 1 μM. Thus, in this competition experiment 1 μM 5-HT was supplied along with 10 μM yohimbine (Sigma, Y3125).

The results of these experiments are shown below;

4.4.4a

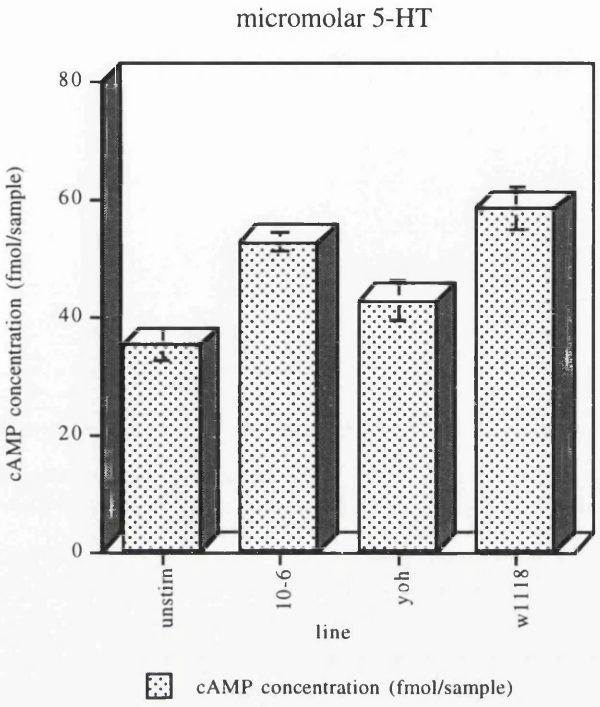


Figure 4.4.6a

Graphical representation of cAMP levels determined from, unstim - unstimulated tubules expressing the 5-HT1Dβ receptor; 10-6 - cAMP level of tubules expressing the 5-HT1Dβ receptor 30 seconds after stimulation with 1 µM 5-HT; yoh - cAMP level at 30 seconds of tubules co-stimulated with 1 µM 5-HT/ 10 µM yohimbine hydrochloride; w1118 - tubules of w^{1118} line 30 seconds after stimulation with 1 µM 5-HT. The data represent cAMP levels from 12 tubules per sample (n=3).

treatment	cAMP fmol/sample	percentage standard error
unstimulated	35.07	± 7.6
10^{-6}	52.48	± 3
yohimbine	42.65	± 8.7
w^{1118}	58.21	± 6.1

Figure 4.4.6b

Numerical data used to generate graph given 4.4.6a. Key; unstimulated, cAMP levels of unstimulated tubules of *C324/UAS-5-HT1Dβ;* 10^{-6}, cAMP levels 30 seconds after stimulation with 1 µM 5-HT of tubules from *C324/UAS-5-HT1Dβ;* yoh, cAMP levels 30 seconds after costimulation with 1 µM 5-HT and 10 µM yohimbine: w^{1118}, cAMP level of tubules from w^{1118} 30 seconds after stimulation with 1 µM 5-HT. cAMP concentration given in fmol/sample, in this experiment 12 tubules per sample were dissected. Standard errors are expressed as percentages of the mean number of counts observed (n=3).

In this experiment the cAMP level per tubule of tubules expressing the 5-HT1Dβ receptor does not reach those seen in the previous experiment. However, there is a rise over the cAMP levels of unstimulated tubules expressing the receptor, and co-incubation of tubules expressing the receptor with yohimbine and 5-HT reduces the cAMP level below that of tubules stimulated with 5-HT alone, but does not reduce the level to that of unstimulated tubules. These results indicate that the rise in cAMP level seen in the tubules is in fact due to the interaction of 5-HT with the expressed receptor.

However, tubules from the strain w^{1118} when stimulated with 1 μM 5-HT and assayed after 30 seconds show an increase in intracellular cAMP concentration, with a level higher than that seen with tubules expressing the 5-HT1Dβ receptor in this assay.

4.5 Discussion

The data presented show that a UAS-5-HT1Dβ receptor gene construct has been inserted into the *Drosophila* genome. Three individuals resulting from a single germ line transformation event were recorded. Under the control of an enhancer trapped p{GAL4} element, the 5-HT1Dβ receptor gene sequence was expressed, presumably as a result of the interaction between the GAL4 protein and the UAS sequence upstream of the receptor gene.

It is shown that femtomolar levels of the cyclic nucleotide, 3', 5' cyclic adenosine monophosphate, could be measured from samples resulting from between 6 and 30 Malpighian tubules. Levels of cAMP in tubules of the p{GAL4} line, tubules of the UAS-5-HT1Dβ line and tubules of a line in which the 5-HT1Dβ receptor had been activated were of a similar magnitude.

Tubules expressing the transgenic receptor gene were stimulated with millimolar, micromolar and nanomolar 5-hydroxytryptamine, and cAMP measurements taken at 5, 10, 15, 20 and 30 seconds after stimulation at 18°C. Peak cAMP levels were observed after 30 seconds with both millimolar and micromolar 5-HT stimulation, the magnitude of the response being the greater upon micromolar 5-HT stimulation. A peak cAMP level was seen after nanomolar stimulation, this peak occurred 20 seconds after supply of 5-HT and was the lowest cAMP peak of those observed.

Co-incubation of Malpighian tubules expressing the transgenic receptor with 5-HT and a partial antagonist of the 5-HT1Dβ receptor reduced the stimulation in cAMP level observed to below that of tubules stimulated with 5-HT alone, yet the cAMP level seen in these tubules was above the level of unstimulated tubules expressing the receptor.

Determination of cAMP level in tubules of w^{1118} flies 30 seconds after stimulation with micromolar 5-HT gave a value of 6.46 ± 6.1% fmol/tubule. This cAMP level is greater than that seen with stimulated tubules expressing the 5-HT1Dβ receptor established in the same assay. It is however lower than the level seen in stimulated 5-HT1Dβ receptor expressing tubules in a previous assay.

The results demonstrate that the Malpighian tubules of *Drosophila melanogaster* are amenable to heterologous expression studies using the GAL4/UAS system, and confirm previous observations (Davies *et al.*, 1995) that cyclic nucleotide levels can be measured in the tissue.

Further interpretation of the data is more difficult. This difficulty highlights the importance of a complete understanding of the pharmacology of Malpighian tubules, and the effect of pharmacological agents on *in vivo* cAMP levels in tubules of wild type flies. For example, the data reported 4.4.4a suggests that the cAMP level stimulation observed in tubules expressing the 5-HT1Dβ receptor after 5-HT supply can be attenuated by co-incubation with a partial antagonist of this class of receptor; this provides some evidence that the observed cAMP level increase upon stimulation with 5-HT is indeed 5-HT1Dβ receptor mediated. However tubules of w^{1118} flies, in the same assay show a similar cAMP level after stimulation with 5-HT to tubules expressing a 5-HT receptor. This single negative control is insufficient, and it is immediately obvious that in a study such as this every timepoint and treatment should be controlled. This would double the workload and expense of further experimentation, and questions the practicality of using Malpighian tubules as an *in vivo* model system for this sort of heterologous expression study.

The elevated cAMP level observed in tubules of w^{1118} flies after 5-HT supply is unexpected on the basis of tubule secretion assay data. Supply of millimolar cAMP to *Drosophila* Oregon R tubules in a tubule secretion assay gives an immediate, significant and sustained rise in fluid secretion level (Dow *et al.*, 1994). However supply of millimolar 5-HT to tubules of w^{1118} flies in a similar assay does not give a significant response (personal communication, S. Maddrell).

The interpretation of this data has been that cAMP is a significant intracellular second messenger in this tissue, and that cAMP supply to tubules in the assay causes elevation in intracellular cAMP level resulting in elevated secretion rate. This interpretation is confirmed by the observation that fluid secretion rate in a tubule secretion assay can be elevated by treatment with forskolin, an activator of adenylate cyclase (Dow *et al.*, 1994), and by the study of Coast *et al.* (Coast *et al.*, 1991) in *Acheta domesticus.*

Result 4.4.3b reports a sudden increase in intracellular cAMP level at t=30 seconds of tubules supplied with micromolar 5-HT. At timepoints before t=30 seconds there is little difference between the cAMP levels recorded. This profile of response is more likely to be derived from a specific receptor mediated effect rather than non specific interaction between agonist and tubule membrane proteins; this latter response would be expected to show a slow and steady increase in intracellular cAMP concentration with time. Also, the magnitude of non specific effects would be expected to be proportional to the agonist concentration supplied. In these studies the greatest response is with micromolar agonist, with a lower cAMP level seen with millimolar agonist.

Taken together, the data appear to suggest that a component of the observed *in vivo* cAMP elevation after 5-HT supply to tubules of flies expressing the 5-HT1Dβ receptor is due to agonist-receptor interaction.

Individual serotonin receptor subtypes exhibit characteristic ligand binding profiles and couple to different intracellular signalling systems. 5-HT1A, 5-HT1B, 5-HT1D and 5-HT4 receptors activate or inhibit adenylate cyclase to alter the levels of cAMP in the cell (DeVivo and Maayani, 1986; Shenker *et al.*, 1987). 5-HT2 and 5-HT1c receptors stimulate phospholipase C-catalysed hydrolysis of phosphatidylinositol bisphosphate to yield the second messengers inositol trisphosphate and diacylglycerol (Conn *et al.*, 1986; de Courcelles *et al.*, 1985). Biochemical and genetic evidence suggests that 5-HT1, 5-HT2 and 5-HT4 receptors belong to the class of neurotransmitter and hormone receptors that transduce extracellular signals by activating GTP-binding proteins (Julius, 1991).

Serotonin receptor subtypes are assigned on the basis of pharmacological profiles obtained in many different biological systems, as well as on the cellular consequence of their activation.

The 5-HT1Dβ receptor is classified on the basis of a pharmacological profile of membrane preparations from chinese hamster ovary cells expressing the receptor which is consistent, but not identical with that of a previously characterized 5-HT1D receptor. No attempt was made to characterize the effect of an activated receptor on adenylate cyclase in this study (Demchyshyn *et al.*, 1992). Another human 5-HT1D type receptor was studied for its affect on adenylate cyclase. When the human S12 serotonin receptor was transfected into mouse L*tk*$^-$ cells, it was able to mediate inhibition of adenylate cyclase in total cell homogenates in response to serotonin. The pharmacological profile of the receptor, which was based on adenylate cyclase assay data and 5-[^{3}H]-HT membrane preparation binding, assigned this protein as a 5-HT1D receptor (Levy *et al.*, 1992).

In the data presented in this study cAMP levels were seen to rise in a tissue expressing the 5-HT1Dβ receptor in response to serotonin. This rise was attenuated in competitive stimulation with the receptor subtype partial antagonist yohimbine. It appears then, that the expressed 5-HT1Dβ receptor is interacting with the host signalling machinery, although this interaction appears to be with a G_s type protein rather than with a G_i species. Furthermore, the K_d for the 5-HT1Dβ receptor was calculated in Demchyshyn *et al.* (1992) at 4 nM. Nanomolar stimulation of Malpighian tubules expressing the 5-HT1Dβ receptor with serotonin results in the lowest peak response seen; the greatest response observed is with tubules challenged with micromolar 5-HT. Therefore, not only is this receptor initiating a different

response to that expected of the receptor subtype, but this response is occurring at a much higher agonist concentration than expected.

In Butkerait *et al.*, (Butkerait *et al.*, 1995), the human 5-HT1A (Koblika *et al.*, 1987; Albert *et al.*, 1990) receptor was expressed in insect cells derived from *Spodoptera frugiperda* (Sf9) cells. In this experiment the receptor demonstrated low affinity binding to the agonist, diagnostic of lack of coupling to cellular G-proteins (Butkerait *et al.*, 1995; Nenonene *et al.*, 1994). Co-expression of mammalian $G\alpha i$ proteins together with various combinations of $\beta 1$ and $\gamma 1$ subunits with the 5-HT1A receptor increased the affinity of the receptor for agonists to that observed for the G-protein coupled form of receptor expressed in mammalian cells (Butkerait *et al.*, 1995). These observations suggest that the high concentration of agonist necessary to obtain maximal response from the 5-HT1Dβ in *Drosophila* Malpighian tubules may be because this receptor is not coupled tightly to host G-proteins *in vivo*.

Reconstitution studies have also been performed with *Drosophila* serotonin receptors in Sf9 cells. In one case (Obosi *et al.*, 1996) two *Drosophila* serotonin receptors were expressed, one, 5-HT*dro*1 (Witz *et al.*, 1990) known to couple to activation of adenylate cyclase, the other, 5-HT*dro*2B (Saudou *et al.*, 1992) thought to cause an inhibition in adenylate cyclase activity. The 5-HT*dro*1 when expressed in Sf9 cells was capable of stimulating an elevation of intracellular cAMP level when challenged with 5-HT. On the other hand, the expression of the 5-HT*dro*2B receptor in the same cells did not result in a decrease in cAMP level on stimulation with 5-HT. Immunoblot blot analysis of endogenous G-proteins revealed that Sf9 cells lack G-protein sybtypes $G\alpha i$ 1-3 but express subtypes $G\alpha s$ and $G\alpha q$.

Cloned G-protein genes in *Drosophila* have been reported to have a restricted spatial and temporal expression patterns. For example, a $G\alpha$ subunit cloned in *Drosophila*, Gfα, is expressed primarily in embryonic, larval and pupal stages, with low levels in adult flies (Quan *et al.*, 1993). The gene in *Drosophila* coding the G-protein gamma subunit, D-Gγ, has a complex expression pattern in embryonic, adult and larval tissues. Of three different sized transcripts coding the subunit, each has a discrete expression pattern which changes through the life cycle, with transcripts concentrated in neural tissues (Ray *et al.*, 1994).

Thus, the presence of a full complement of G-protein subunits cannot be guaranteed in cells of the Malpighian tubule, and these cells may not be the best system for heterologous expression of receptors coupled to inhibition of adenylate cyclase.

In conclusion, heterologous expression of a human 5-HT1Dβ receptor in *Drosophila* Malpighian tubule cells results in a potentially active receptor which appears to be unable to couple to *Drosophila* signalling systems at physiological agonist concentrations. At higher agonist concentrations a cellular response, inappropriate for

the receptor subtype, is seen. This response is thought to be due to non-specific interaction between the activated receptor and cellular signalling pathways.

4.6 Further work

If the Malpighian tubule is to be further investigated as a suitable model system for heterologous expression studies in an *in vivo* context a more comprehensive data base of tubule pharmacology and biochemistry should be developed. As well as investigating fully the effects of treating tubules with various pharmacological agents on intracellular second messenger levels, the presence or absence of proteins of the receptor mediated signalling pathway should be determined. There are several approaches available for these experiments, for example; an RT-PCR based approach with oligonucleotide sequences designed from published *Drosophila* G-protein gene sequences to screen for G-protein transcripts; or immunoblotting using specific anti-G-protein subunit antibodies to screen for the presence of particular G-proteins in the tissue.

Heterologous expression of a receptor demanding a high level of signalling pathway conservation may not yet be viable in this model system.

A more measured approach to the investigation of this tissue as a valid model for heterologous expression studies is advisable. For example, a transmembrane protein with enzymatic activity which can be easily assayed, and is dependent upon an extracellular signal would be an ideal start. This type of study would demonstrate if, like cell culture based systems, a cell surface receptor can be correctly transcribed, processed and targetted in this tissue, and the efficacy of the introduced protein could be assessed independently of components of a signalling pathway which may not be present in the cells used. An epidermal growth factor (EGF) receptor would be a suitable candidate for such studies. Members of this receptor family have an intracellular tyrosine kinase activity upon stimulation with EGF. Tyrosine phosphorylation can be assayed using immunoblotting with an anti-phosphotyrosine antibody. This assay, though not necessarily quicker than the physiological assays used in this study is certainly more cost effective, and a more practical assay for this type of study.

Chapter 5

Results 3

5.1 Introduction

The usual genetic approaches to understanding mechanisms of organ function and development in *Drosophila* rely on the identification of novel mutations with scorable phenotypes. Identification of mutations can lead to the cloning of genes, which when mutated generate the phenotype seen, this in turn allows the heterologous expression type studies discussed in chapter 4.
Scorable mutated phenotypes in *Drosophila* Malpighian tubules could result from a mutation in the the morphogenic pathway, leading to aberrant tubule morphology, or mutation in a protein component of the fluid secretion pathway, this type of mutation could be scored using the tubule secretion assay. The tubule secretion assay, like all assays, relies on repetition for its accuracy, and this therefore makes it impractical in a classical mutagenesis study.
Therefore a genetic dissection of fluid secretion mechanisms of Malpighian tubules of *Drosophila melanogaster* requires a more direct approach, where aspects of tubule physiology disclosed by the tubule secretion assay or other physiological observations are directly investigated. Here we illustrate this with the analysis of a candidate aquaporin (water channel) in Malpighian tubules.

Peak stimulation of fluid secretion from Malpighian tubules has been observed upon stimulation with homogenate of *Drosophila* thoracic ganglion. Typically the rate of fluid production after this stimulation is in the range of 3-4 nl/min, however a flow rate of 6 nl/min has been reported (Dow *et al.*, 1994). These rates imply that each cell of the *Drosophila* tubule is capable of transporting its own volume of fluid in 15 seconds. The actual rate of secretion per cell is likely to be greater than this, as it is suggested that not all cells of the tubule contribute to the production of fluid (O'Donnell and Maddrell, 1995). To sustain these very high rates of secretion, a paracellular route for water and solute flux has been discussed; however, an alternative possibility has recently emerged, where diffusion of water through the plasma membrane of Malpighian tubule cells is facilitated by the presence of water channel proteins (aquaporins) in the membrane .

5.1.1 Aquaporins

Plasma membranes are thought to be major barriers to water flow. The lipid bilayer is somewhat permeable to water by virtue of simple diffusion, however, the inducible

rate of fluid secretion in this and other systems cannot be due to simple diffusion alone. For example, the glomeruli within a single adult human kidney pass approximately 100 litres of fluid per day, and approximately 85% of this volume of fluid is reabsorbed by cells of the proximal nephron and the descending thin limb of the loop of Henle. These segments of the nephron are known to be highly permeable to water and are known to be sites of expression of a water channel protein (Chrispeels and Agre, 1994).

Water channel proteins (aquaporins) are a functionally defined group of water transporting proteins with homology to the major intrinsic protein (MIP) of lens fibre (Yang *et al.*, 1996). Channel forming Intrinsic Protein (CHIP), the first identified water channel protein, was first purified from human erythrocytes and kidney (Denker *et al*, 1988; Preston and Agre, 1991); members of the MIP family share many structural similarities with this protein. Topological prediction using hydropathy analyses and secondary structure predictions predict six bilayer spanning α-helices.

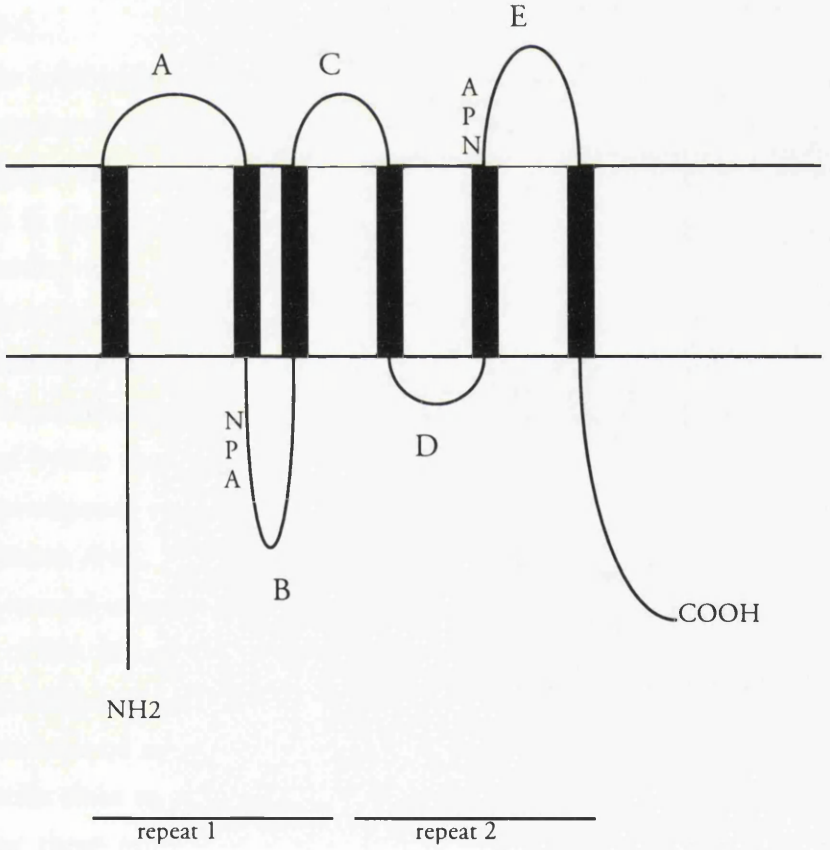


Figure 5.1

Diagram showing predicted membrane topology of Aquaporin CHIP.The N and C termini are cytoplasmic, as are trans- membrane domain connecting loops B and D.

Mutagenesis studies with Aquaporin CHIP using introduced epitope tagged sequence insertion demonstrated that the loops A, C and E are extracytoplasmic, and that the loops B and D are on the cellular side of the membrane (Preston *et al.*, 1994). In the same study the N terminal sequences were shown to reside on the cellular side of the membrane using anti-NH_2-peptide antibody; the C terminal cytoplasmic tail of CHIP could be removed by digestion of inside out red cell membrane vesicles with α-chymotrypsin (Preston *et al.*, 1994). Therefore these data suggest that the membrane topology for Aquaporin CHIP (Figure 5.1) predicted by hydropathy analyses and secondary structure predictions is correct. The two halves of the molecule exhibit substantial sequence similarity to one another but are oriented oppositely in the membrane so that corresponding regions are found on opposite sides of the membrane (Knepper, 1994). Some residues are highly conserved among members of the MIP family; the B and E loops both contain the Asp-Pro-Ala (NPA) sequence that is characteristic of the major intrinsic protein family of proteins (Knepper, 1994); also, a conserved glutamate residue exists in the first and fourth bilayer spans, and a conserved glycine is present in the third and sixth bilayer spans (Chrispeels and Agre, 1994).

Reversible inhibition of membrane water permeability by mercuric chloride and certain organomercurials is a defining feature of most water channels. Fluid transport in Malpighian tubules is very sensitive to mercury. 25μM mercuric acetate supplied to tubules in a tubule secretion assay abolishes secretion; this effect can be reversed by co-incubating the tubules in 1mM β-mercaptoethanol (Dow *et al.*, 1995). Mutagenesis studies with aquaporin-CHIP28 (Preston *et al.*, 1993; Zhang *et al.*, 1993) have demonstrated that one of four cysteines (C189) when mutated to serine confers insensitivity to mercuric ions, otherwise the biology of the protein is unaffected by the mutation. This cysteine is thought to lie at a narrow point in the membrane-channel aperture and is located three residues from the second N-P-A motif (Preston *et al.*, 1993). Not all water channel proteins are sensitive to mercurial ions. Mercurial-insensitive water channel (MIWC), aquaporin 4, was cloned from a rat lung cDNA library. Expression of MIWC cRNA in *Xenopus* oocytes conferred increased membrane water permeability, this increased permeability was not inhibited by the presence of mercuric ions in the bathing medium (Hasegawa *et al.*, 1994). Amino acids close to the NPA motif of this protein were mutated to cysteine and cRNA for these mutant strains injected into *Xenopus* oocytes. Five individual mutations were reported to confer sensitivity to mercuric ions, these mutations were at residues 70 71, 72 and 73, and also at residue 189 (Shi *et al.*, 1996). Thus, cysteine residues adjacent to the NPA motifs confer mercurial sensitivity in this class of protein.

It is possible that some members of the MIP family have a primary function different from the formation of membrane water channels.
Expression of a cRNA for bovine major intrinsic protein of lens (Gorin *et al.*, 1984) in *Xenopus* oocytes resulted in a 4-5 fold increase in osmotic water permeability of the oocytes, whereas expression of a CHIP aquaporin gene resulted in >30 fold increase in osmotic water permeability of *Xenopus* oocytes (Mulders *et al.*, 1995). More detailed studies (Zampighi *et al.*, 1995) expressed major intrinsic protein of lens and CHIP aquaporin separately in *Xenopus* oocytes, and measured the water permeabilities on a single channel basis. The results of this study suggest that the major intrinsic protein of lens is capable of acting as an aquaporin, though at two orders of magnitude lower efficiency than CHIP aquaporin. Thus major intrinsic protein of lens may have a major function other than to allow facilitated diffusion of water.
Mutation of the genomic sequence of major intrinsic protein of lens in mouse results in autosomal recessive cataract (Shiels *et al.*, 1996). This provides evidence for a role for MIP in transparent lens development in mouse; therefore suggesting function of MIP other than to facilitate diffusion of interstitial fluid through the tissue.

In *Drosophila*, a single member of the MIP family has been cloned. *Big brain, bib*, was identified as one of six zygotic neurogenic genes. The other neurogenic genes appear to fit into a cascade defined by genetic interactions; however, *bib* does not show any interaction with them. 40% of amino acids in a segment of the N terminal half of the big brain protein are identical to those of MIP of bovine lens. The C-terminal half of *bib* shows no significant homology with any known proteins (Yi Rao *et al.*, 1990). Low levels of *bib* expression have been recorded in Malpighian tubules, with the protein immunolocalized with α-bib antibody to the apical membrane (Dan Doherty, personal communication).

5.1.2 Do insect tubules use aquaporins?

Considering that the plasma membranes of Malpighian tubule cells of *Drosophila melanogaster* display a high and mercury sensitive water permeability, that a family of water channel proteins have been identified and characterized, and that a member of the MIP family has been identified, the possibility of a dedicated water channel protein active in the Malpighian tubule was discussed.
The prospect of a second *Drosophila* MIP present in Malpighian tubules was investigated. Degenerate oligonucleotides for PCR were designed based on sequences around the conserved NPA motifs of this class of protein (WChL2; GG(A/C/G/T)G(C/G)(ACGT)CAATTCAA(C/T)CC(ACGT)GC(ACGT)GT(AC

GT)AC:WChR2;C(A/G)AGGATCC(G/T)(A/C/G/T)GC(A/C/G/T)GG(A/G)TT(A/C/G/T)AT) and used to screen a first strand cDNA library made from Malpighian tubules.

```
ggattcctga tcgttggtga gatcagcatt ctgaaggctg ccttctacat catcgtccaa
tgcgtgggcg ccattgctgg agcggctgtg ataaaggtgg cactcgatgg agtggctggt
gcgacctgga gtatcctcct tgatccctcc ctgaactgtg cacaggcggt gctgatcgaa
gcgctgatca cctttatttt ggttttcgtg gtcaaggctg tttcggatcc tggacgccag
gatatcaagg gatcagcgcc actggctgtt ggtttggcca tcgccgctgg ccatttgtgt
gcaatcaaac tgagcggtgc cagcacg
```

Figure 5.1.2

327bp sequence from Malpighian tubule RT-PCR as described above.

Sequences producing High-scoring Segment Pairs:		Reading Frame	High Score	Sum Probability P(N)	N
gi|1055326	(U38807) DRIP [Drosophila melanog...	+1	431	1.7e-54	1
gi|1262285	(U51638) water channel [Haematobi...	+1	238	2.8e-32	2
pir||S49304	membrane channel protein - Cicade...	+1	70	8.4e-20	3
gnl|PID|e236599	(X97159) aquaporin [Cicadella vir...	+1	137	3.6e-17	2
gi|404779	(L24754) chip aquaporin [Rana esc...	+1	146	5.8e-16	2
pir||S37639	CHIP28 protein - rat /gi|313804 (...	+1	124	9.3e-13	2
pir||JC1320	water channel protein CHIP28 - rat	+1	123	1.3e-12	2
pir||A44395	proximal tubule water transporter...	+1	123	1.3e-12	2
sp|Q02013|AQP1_MOUSE	AQUAPORIN-CHIP (WATER CHANNEL PRO...	+1	123	1.3e-12	2
sp|P29975|AQP1_RAT	AQUAPORIN-CHIP (WATER CHANNEL PRO...	+1	123	1.3e-12	2
sp|P29972|AQP1_HUMAN	AQUAPORIN-CHIP (WATER CHANNEL PRO...	+1	124	4.6e-12	2
gi|688358	(S73482) uterine water channel, h...	+1	124	4.6e-12	2
gi|841316	(U22658) aquaporin [Bufo marinus]	+1	101	5.9e-12	3
gi|1072053	(U34845) mercurial-insensitive wa...	+1	135	1.2e-11	1
gi|1236246	(D63412) aquaporin [Homo sapiens]	+1	135	1.2e-11	1
gi|1072055	(U34846) mercurial-insensitive wa...	+1	135	1.3e-11	1
gi|1144350	(U33012) mercurial-insensitive wa...	+1	133	2.2e-11	1
gi|1293552	(U48398) mercurial-insensitive wa...	+1	133	2.3e-11	1
sp|P47863|AQP4_RAT	AQUAPORIN 4 (WCH4) (MERCURIAL-INS...	+1	133	2.3e-11	1
gi|1293554	(U48399) mercurial-insensitive wa...	+1	133	2.4e-11	1
gi|1314306	(U41518) channel-like integral me...	+1	124	2.1e-10	1
sp|P47865|AQP1_BOVIN	AQUAPORIN-CHIP (WATER CHANNEL PRO...	+1	124	3.9e-10	1

Figure 5.1.3

Portion of the result of a protein sequence database search. It can be seen that the 327bp sequence has been entered into the database (accession number, U38807), and that the DNA sequence, when translated, is most highly homologous to other water channel protein sequences.

This analysis yielded a product of 327 bp which was sequenced (sequence data shown Figure 5.1.2). Sequence analysis (Figure 5.1.3) confirmed that the fragment derived

by RT-PCR was highly homologous to other members of the major intrinsic protein family, and showed that the product was quite clearly different from *big brain.*
The cytological location of the DNA fragment was localized by *in situ* hybridization to polytene chromosomes of 3rd instar larvae to 47F on the right arm of chromosome 2 (Zongshen Wang, personal communication). Thus, both the sequence of the RT-PCR product and its' genetic location are different to those of *big brain*, which is located at 30F.

Thus it appears that there is a novel water channel protein gene being expressed in the Malpighian tubules of *Drosophila melanogaster.* The descriptions which follow detail the attempt made to clone a cDNA coding a tubule expressed water channel sequence, and a characterization of the genetic resources in and around 47F.

5.2.1 Library screening with 327bp PCR product

An adult *Drosophila* cDNA library was screened with the 327 bp RT-PCR product. The library is made from whole adult male and female Canton-S *Drosophila*, and was both random primed and oligo(dT) primed. This library, acquired from Stratagene, is constructed in λZapII. The library was plated and hybridized as described in chapter 2.9 and 2.2.6; hybridized filters were washed to low stringency, and exposed to film, usually overnight.

A single positive phage plaque was isolated, and the insert excised to the phagemid pBS by the methods of chapter 2.10. The plasmid was prepared by alkaline lysis for subsequent analysis, and will be referred to as pWCh.

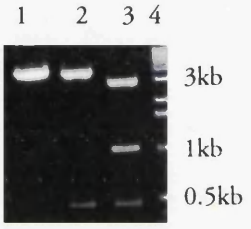


Figure 5.2.1

Restriction endonuclease digestion of plasmid pWCh. Lane 1, *Xho1*; lane 2, *EcoR1*; lane 3, *Xho1* and *EcoR1*; lane 4, 1kb DNA ladder (Gibco).

The cDNA library was constructed to give cDNA molecules cloned directionally using *EcoR1* and *Xho1*, with the *Xho1* site at the 3' end of the clone. *EcoR1* alone excises a DNA fragment of approximately 500 bp, *Xho1* alone linearises the circular molecule.

Double digestion with *EcoR1* and *Xho1* liberates the 500 bp fragment and a 1 kb DNA fragment. This information suggests that there is a second *EcoR1* site within the clone located 500 bp from the 5' *EcoR1* site of the adaptor used and approximately 1 kb from the single *Xho1* site which is introduced with the 3' linker.

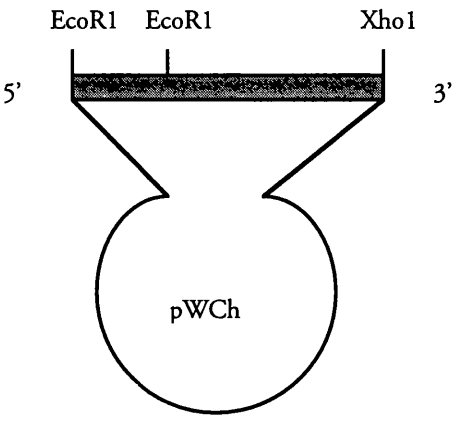


Figure 5.2.2

Structure of the plasmid pWCh predicted from the result shown in Figure 5.2.1.

5.2.2 Sequencing of pWCh

The DNA sequence of the plasmid pWCh was established, and is shown in Figure 5.2.3.

Sequence analysis shows that there is an internal *EcoR1* site 472 bp from the terminal *EcoR1* site. With the clone in the sense orientation dictated by the putative reading frame of the 327 bp PCR product, the terminal *EcoR1* site appears to be at the 3' end of the molecule, with the *Xho1* site at the 5' end. The library construction protocol predicts that a cDNA molecule should be in the opposite orientation.

The 327 bp sequence of *Drosophila* Malpighian tubule RT-PCR product supplied to the database has a single open reading frame, which putative translation gives a protein sequence which is highly homolgous to other MIPs. The homologous sequence of pWCh is interrupted in this region by a 57 bp sequence; this 57 bp sequence is flanked by conserved splice donor/acceptor sites (Lewin, 1994).

Taken together these data appear to suggest that the sequence cloned from the Stratagene *Drosophila* cDNA library is a *Drosophila* genomic DNA contaminant, and is not a *bona fide* cDNA. From the library construction regime it is possible that a genomic DNA fragment could survive to contaminate the library. Genomic contamination of cDNA libraries is a common event; in this case a genomic DNA fragment will not be methylated, *EcoR1* adaptors are ligated onto both ends, and an *Xho1* digestion liberates an *Xho1* site at one terminus to allow directional cloning. A contaminating DNA fragment with a fortituous *Xho1* site and an internal *EcoR1* site can therefore be cloned in this procedure.

The insert of the plasmid pWCh is used in mapping studies discussed later in this chapter, and as a probe to screen another *Drosophila* cDNA library.

5′

TCGAGTGCATCTCACGTCTGACAAATTGCTTGTATCCGCTGGCAGTTCGATATGCAACG
ATATTGTGCAATGGAAAATTCGGAAAATCGCACAATCAGCTTCATGGCAAAGGGCAAAC
TTTGAGCGCAGCTATGGTTAAGATGTTGCGTTGTCAACTCGACATTTGCAAGCGCCGAT
GGAGGTGGAGATAGGCCATTATCTACATTATGTTCGACTGGGCCTTAGCCTCCTTGATT
TGATCTTCATACTTTTTTTCCTCTTTTATTGAGCCTTATAATAAATATATTCTTGAATA
TCCTCTTATTTTGTTCTCTCTCTCAGGGCTTGGGCCACCTCAGTGGCTGTCACATTAAT
CCGGCGGTCACCCTT**GGATTCCTGATCGTTGTTGAGATCAGCATTCTGAAGGCTGCCTT**
CTACATCATCGTCCAATGCGTGGGCGCCATTGCTGGAGCGGCTGTGATAAAGGTGGCAC
TCGATGGAGTGGCTGGTGCGACCTGGAGTATCCTCCTTGATCCCTCCCTGAACTGTGCA
CAGGCGGTGCTGATCGAAGCGCTGATCACCTTTATTTTGGTTTTCGTGGTCAAGGCTGT
TTCGGATCCTGGACGCCAGGATATCAAGGGATCAGCGCCACTGGCTGTTGGTTTGGCCA
TCGCCGCTGGCCATTTGTGTGCA*GTGAGTACGAGGTGATGGAGTTCTCCGACTGGAGGT*
*TAATAAGTGTACCATTTACAG***ATCAAACTGAGCGGTGCCAGCACG**AATCCCGCCGGTCC
TTTGGTCCCGCCGTAGATCAGGGCGTCTGGACCTATCACTGGGTTTACTGGGTGGGTCC
CATTGGCGCGAACTGTTGGCCGGAATCATCTACAGATTCAATCTTCAAGGTACGCAAGG
GCGATGAATGAGACCGACTCGTACGACTTCTAAACAGTGGCCGAATACCATATATGTAT
TTTTGTAAATGTCCGCACCTGCTTTCCATTCCCATCACACATTCGCTTTCCAAAAAAAA
AAAAAAACATCGAATTCGTTTGCCGAAATTTCTCGTAGTTTAGGTTTGCTCCACCATCA
CAACCAAGCTGAATTTTCAACGCAATTTGTACCATTTTATTTTTTAAACAATAAGAAAA
GCAAATGTCTCGTTTAGTTGTACCGCGATGCCCCAGCACTTTAAAATATGCAATATTGT
ATTTGAGTAATTTTAAGCTAAATTATATGCACATCATCATTACGCGAATCGAAGGAAAA
CTGTATTGAAGCTTAACTAAATTGTACTGGGAAATCACTTTCCAAAGCAGCGAACAGGT
GGATTACCTACGCTGTTGATCGCCACTTGCTGAGCACCTGCGAATTGTATTATGACTAT
GTGTAATAAAAAAATATAAAGAAATTACCAATGTTCATCTTGTCTGAATGTCCTGGGGC
TTCATTCATTCATTGCCAATCGTCATCAGTTACTCTTTTTTATTATTTCTATTTTTTAC
CCCGG**GAATTC** 3′

Figue 5.2.3

DNA sequence of pWCh insert. 5' *Xho1* site and 3' *EcoR1* site are underlined. The sequence in bold is the 327 bp sequence of the RT-PCR product from Malpighian tubules. This sequence was used to screen the Stratagene adult *Drosophila* library to isolate this clone.This sequence is interrupted by a 57 bp intron (in italics); splice consensus sequences at the 5' (GT) and 3' (AG) termini of the intron sequence can be seen.

The clone is oriented in sense as established by the putative reading frame of the 327 bp RT-PCR product. In this orientation the 5' and 3' sites are in the opposite orientation to that predicted by the library construction protocol.

5.2.3 Further cDNA library screening

An in-house *Drosophila* adult body library was acquired. In this, cDNA was derived from oligo dT primed mRNA directionally inserted into λNM1149 *via EcoR1* and *HindIII* linkers so that the *EcoR1* is the 5' end and *HindIII* the 3' end (Steven Russell, personal communication).

The library was screened with a probe consisting of both the 1 kb *Xho1* and 472 bp *Xho1/EcoR1* fragments of pWCh. Again, hybridized library lifts were washed to low stringency at 65°C, and exposed to film. Nine individual positive plaques were obtained, and DNA from eight of these was prepared as described chapter 2.11

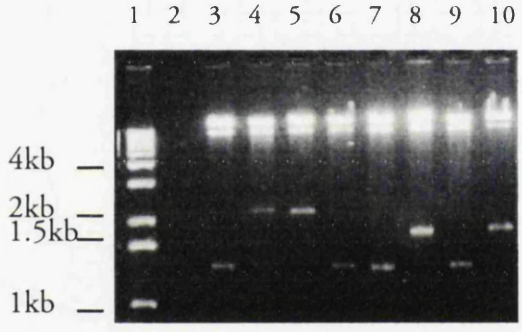


Figure 5.2.4

Agarose gel showing DNA of the eight water channel positive phage clones identified in a library screen as described 5.2.3. Lane 1, 1kb DNA ladder; lanes 3-10, phage DNA excised with *EcoR1* and *HindIII*.

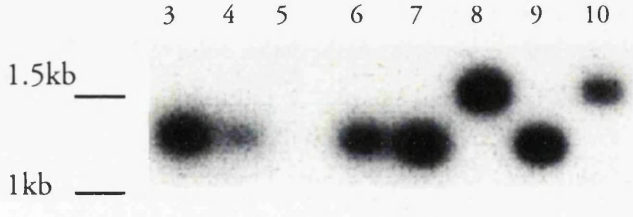


Figure 5.2.5

Southern hybridization of agarose gel shown Figure 5.2.4. The Southern blot of the gel was hybridized with the DNA insert of pWCh, washed at 65°C to 0.1XSSC and exposed to film for 15 minutes.

There are three individual species of DNA fragment excised from these lambda clones. To distinguish between false and genuine positives the gel was blotted and hybridized with the same DNA probe used to screen the cDNA library (Figure 5.2.5).

It can be seen that two of the three DNA size fragments cloned are homologous to water channel DNA. The fragments at 1.4 and 1.6 kb hybridize to the water channel DNA probe, however, the 2.2 kb fragment of lanes 4 and 5 does not hybridize with the probe. Thus, five clones of approximately 1.4 kb and two clones of approximately 1.6 kb have been isolated.
From the Southern hybridization result it is seen that the intensity of hybridization is proportional to the amount of DNA per lane. Thus it appears that the positive clones have identical affinity for the probe, and it is likely that clones of the same size are identical to each other. In future studies the DNA clones shown in lanes 9 and 10 are taken as representative samples of all the cloned fragments. These DNAs will furthermore be referred to as, lane9 - WCh2; lane 10 - WCh3.

The fragments of these plasmids were subcloned into the phagemid pBluescript (Figure 5.2.6)

It can be seen that for each pair, the lambda insert has been cloned into the phagemid. Also, both clones can be excised using only *EcoR1*, and WCh3 DNA can be additionally excised using *HindIII* alone.

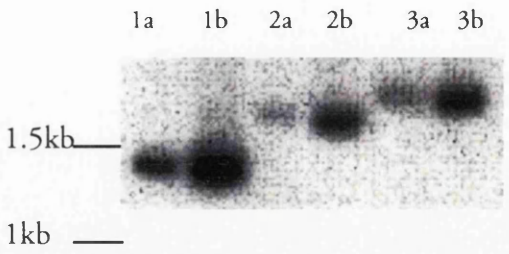


Figure 5.2.6

Southern hybridization of DNA clones isolated as described above. Lanes 1a, 2a and 3a show insert DNA excised from phage clones, and lanes 1b, 2b, and 3b show excised inserts of pBS subclones. Lanes 1a and 1b, DNA of the clone shown in lane 9 (WCh2) of 5.2.4/5.2.5 excised from λNM1149 and cloned into pBS using *EcoR1* alone; lanes 2a and 2b, DNA of the clone shown lane 10 (WCh3) of 5.3.4/5.2.5 above excised from λNM1149 and cloned into pBS using *HindIII* alone; lanes 3a and 3b, DNA of the clone shown lane 10 (WCh3) of 5.3.4/5.2.5 excised from λNM1149 and cloned into pBS using *EcoR1* alone. The blot was hybridized with insert DNA of the plasmid pWCh, and washed at 65^{o}C to 0.1XSSC and exposed to film for 15 minutes.

It can be seen that for each pair, the lambda insert has been cloned into the phagemid. Also, both clones can be excised using only *EcoR1*, and WCh3 DNA can be additionally excised using *HindIII* alone.

5.2.4 Sequencing of newly derived cDNA clone

The largest cDNA clone was chosen for complete sequencing. Thus the insert of pWCh3, cloned by *EcoR1* digestion, was sequenced. From the sequence data a putative open reading frame sequence of 735 bp was generated, and this sequence is shown in Figure 5.2.7a. A search of protein databases using the BLASTX algorithm shows that the putative translation codes a protein which is highly homologous to many members of the major intrinsic protein family (Figure 5.2.7b).

The 735 bp putative open reading frame translates a protein of 244 amino acids(Figure 5.2.7c).

The predicted topology of the putative translation shares features conserved among members of the major intrinsic protein family: There are conserved glutamate residues in the first and fourth putative membrane spanning domains, none of the other putative membrane spanning domains contain glutamate residues; there are glycine residues in the

third and sixth putative membrane spanning domains, however the other putative membrane spanning domains are surprisingly glycine rich; and the predicted B and E loops contain cysteine residues as well as the well conserved Asn-Pro-Ala of major intrinsic protein family members. The putative translation given does not show any consensus sequences for N-glycosylation.

ATGGTCGAGAAAACAGAAATGTCGAAATTCGTTGGCGTTGCCGACATAACCGAGAACAA
GAAAATYTGGCGCATGCTGCTCGGCGAANTGGTGGGCACATTTTTCTTGATCTTCGTCG
GCGTTGGCAGCACGACGAGGGGAAGTGTACCACAAATCGCATTCACCTTTGGCTTGACG
GTGGCCACCATCGCCCAGGGGTTGGGCCACCTCAGTGGCTGTCACATTAATCCGGCGGT
CACCCTTGGATTCCTGATCGTTGGTGAGATCAGCATTCTGAAGGCTGCCTTCTACATCA
TCGTCCAATGCGTGGGCGCCATTGCTGGAGCGGCTGTGATAAAGGTGGCACTCGATGGA
GTGGCTGKKGCGACCTGGAGTATCCTCCTTGATCCCTCCCTGAACTGTGCACAGGCGGT
GCTGATCGAAGCGCTGATCACCTTTATTTTGGTTTTCGTGGTCAAGGCTGTTTCGGATC
CTGGACGCCAGGATATCAAGGGATCAGCGCCACTGGCTGTTGGTTTGGCCATCGCCGCT
GGCCATTTGTGTGCAATCAAACTGAGCGGTGCCAGCATGAATCCCGCCCGGTCCTTTGG
TCCCGCCGTAGTGCAGGGCGTCTGGACCTATCACTGGGTTTACTGGGTGGGTCCCATTG
CCGGNGGCCTGTTGGCCGGAATCATCTACAGATTAATCTTCAAGGTACGCAAGGGCGAT
GATGARACCGACTCGTACGACTTCTAA

Figure 5.2.7a

DNA sequence of the putative open reading frame of the plasmid pWCh3.

Sequences producing High-scoring Segment Pairs:		Reading Frame	High Score	Smallest Sum Probability P(N)	N
gi|1262285	(U51638) water channel [Haematobi...	+1	448	1.3e-109	3
gnl|PID|e236599	(X97159) aquaporin [Cicadella vir...	+1	195	4.4e-75	4
gi|404779	(L24754) chip aquaporin [Rana esc...	+1	267	3.3e-58	3
sp|P29972|AQP1_HUMAN	AQUAPORIN-CHIP (WATER CHANNEL PRO...	+1	229	2.0e-53	3
gi|688358	(S73482) uterine water channel, h...	+1	229	2.0e-53	3
pir||S37639	CHIP28 protein - rat /gi|313804 (...	+1	228	5.2e-53	3
pir||JC1320	water channel protein CHIP28 - rat	+1	227	5.2e-53	3
pir||A44395	proximal tubule water transporter...	+1	227	7.2e-53	3
sp|Q02013|AQP1_MOUSE	AQUAPORIN-CHIP (WATER CHANNEL PRO...	+1	226	7.2e-53	3
gi|1055326	(U38807) DRIP [Drosophila melanog...	+1	419	7.9e-53	1
sp|P47865|AQP1_BOVIN	AQUAPORIN-CHIP (WATER CHANNEL PRO...	+1	224	1.8e-52	3
sp|P29975|AQP1_RAT	AQUAPORIN-CHIP (WATER CHANNEL PRO...	+1	227	1.8e-52	3
sp|P30301|MIP_HUMAN	LENS FIBER MAJOR INTRINSIC PROTEI...	+1	212	3.2e-51	3
gi|1072053	(U34845) mercurial-insensitive wa...	+1	268	1.1e-50	3
sp|Q06019|MIP_RANPI	LENS FIBER MAJOR INTRINSIC PROTEI...	+1	204	3.9e-50	3
pir||JN0557	lens fiber membrane major intrins...	+1	204	4.0e-50	3
sp|P47863|AQP4_RAT	AQUAPORIN 4 (WCH4) (MERCURIAL-INS...	+1	269	7.5e-50	3
pir||A53194	water channel protein MIWC, mercu...	+1	259	2.0e-49	3
gi|841316	(U22658) aquaporin [Bufo marinus]	+1	209	2.4e-49	4
gi|1236246	(D63412) aquaporin [Homo sapiens]	+1	267	5.0e-49	3
sp|P06624|MIP_BOVIN	LENS FIBER MAJOR INTRINSIC PROTEI...	+1	202	6.7e-49	3

Figure 5.2.7b

Portion of output from blastx search of protein databases with the putative open reading from sequence of pWCh3. The putative translation has highest homology with other insect water channels (*Haematobia* - Buffalo fly), and is highly homologous with CHIP aquaporin protein sequences.

```
ATG GTC GAG AAA ACA GAA ATG TCG AAA TTC GTT GGC GTT GCC GAC ATA
Met Val Glu Lys Thr Glu Met Ser Lys Phe Val Gly Val Ala Asp Ile>

50          60          70          80          90

ACC GAG AAC AAG AAA ATY TGG CGC ATG CTG CTC GGC GAA NTG GTG GGC
Thr Glu Asn Lys Lys Ile Trp Arg Met Leu Leu Gly Glu Xxx Val Gly>

  100         110         120         130         140

ACA TTT TTC TTG ATC TTC GTC GGC GTT GGC AGC ACG ACG AGG GGA AGT
Thr Phe Phe Leu Ile Phe Val Gly Val Gly Ser Thr Thr Arg Gly Ser>

    150         160         170         180         190

GTA CCA CAA ATC GCA TTC ACC TTT GGC TTG ACG GTG GCC ACC ATC GCC
Val Pro Gln Ile Ala Phe Thr Phe Gly Leu Thr Val Ala Thr Ile Ala>

       200         210         220         230         240

CAG GGG TTG GGC CAC CTC AGT GGC TGT CAC ATT AAT CCG GCG GTC ACC
Gln Gly Leu Gly His Leu Ser Gly Cys His Ile Asn Pro Ala Val Thr>

          250         260         270         280

CTT GGA TTC CTG ATC GTT GGT GAG ATC AGC ATT CTG AAG GCT GCC TTC
Leu Gly Phe Leu Ile Val Gly Glu Ile Ser Ile Leu Lys Ala Ala Phe>

290         300         310         320         330

TAC ATC ATC GTC CAA TGC GTG GGC GCC ATT GCT GGA GCG GCT GTG ATA
Tyr Ile Ile Val Gln Cys Val Gly Ala Ile Ala Gly Ala Ala Val Ile>

  340         350         360         370         380

AAG GTG GCA CTC GAT GGA GTG GCT GKK GCG ACC TGG AGT ATC CTC CTT
Lys Val Ala Leu Asp Gly Val Ala Xxx Ala Thr Trp Ser Ile Leu Leu>

    390         400         410         420         430

GAT CCC TCC CTG AAC TGT GCA CAG GCG GTG CTG ATC GAA GCG CTG ATC
Asp Pro Ser Leu Asn Cys Ala Gln Ala Val Leu Ile Glu Ala Leu Ile>

       440         450         460         470         480

ACC TTT ATT TTG GTT TTC GTG GTC AAG GCT GTT TCG GAT CCT GGA CGC
Thr Phe Ile Leu Val Phe Val Val Lys Ala Val Ser Asp Pro Gly Arg>

          490         500         510         520

CAG GAT ATC AAG GGA TCA GCG CCA CTG GCT GTT GGT TTG GCC ATC GCC
Gln Asp Ile Lys Gly Ser Ala Pro Leu Ala Val Gly Leu Ala Ile Ala>

530         540         550         560         570

GCT GGC CAT TTG TGT GCA* ATC AAA CTG AGC GGT GCC AGC ATG AAT CCC
Ala Gly His Leu Cys Ala Ile Lys Leu Ser Gly Ala Ser Met Asn Pro>

  580         590         600         610         620

GCC CGG TCC TTT GGT CCC GCC GTA GTG CAG GGC GTC TGG ACC TAT CAC
Ala Arg Ser Phe Gly Pro Ala Val Val Gln Gly Val Trp Thr Tyr His>
```

```
    630           640           650           660           670

TGG GTT TAC TGG GTG GGT CCC ATT GCC GGN GGC CTG TTG GCC GGA ATC
Trp Val Tyr Trp Val Gly Pro Ile Ala Gly Gly Leu Leu Ala Gly Ile>

       680           690           700           710           720

ATC TAC AGA TTA ATC TTC AAG GTA CGC AAG GGC GAT GAT GAR ACC GAC
Ile Tyr Arg Leu Ile Phe Lys Val Arg Lys Gly Asp Asp Glu Thr Asp>

          730

TCG TAC GAC TTC TAA
Ser Tyr Asp Phe>
```

Figure 5.2.7c

Translation of the putative reading frame of pWCh3. The primary sequence and predicted topology of CHIP28 (Preston and Agre, 1991) are used to identify conserved stretches of sequence, and to assign possible topology of the protein sequence in a plasma membrane.

Sequences with sequence homology and spatial similarity to putative membrane spanning regions of CHIP28 are shown in bold. Stretches of amino-acid sequence with close conservation to the CHIP28 sequence are underlined. This analysis predicts a protein with intracellular NH_2 and COOH domains, with 6 membrane spanning domains. Five loops of amino acids link these six membrane spanning domains (see Figure 5.1), A,C and E are extracellular and B and D are intracellular.

The amino acid sequences corresponding to the B and E loops of the translated sequence contain the conserved NPA motifs and each possess a cysteine residue. Close protein sequence conservation between CHIP28 and the putative translation of pWCh3 begins with the B loop and continues through the third putative membrane spanning domain; there is little conservation in the C and D loops and the fourth putative membrane spanning domain between the two sequences; however, sequence conservation begins again in the fifth putative membrane spanning domain and continues to the sixth putative transmembrane sequence. Therefore, the sequences surrounding the putative B and E loops of the protein are the most highly conserved. An asterisk above the DNA sequence between bases 540 and 550 marks the position of the 57bp intron of the genomic sequence shown in Figure 5.2.3.

5.2.5 Analysis of pWCh2

Oligonucleotides were designed(Het Left, AGATCTCAAAATGGTCGAGAAAACAGAAATG; Het Right, AGATCTGGTATTCGGCCACTGTTTAG) from sequences including the putative start and stop codons of the pWCh3 open reading frame, which when used in a PCR reaction with the plasmid pWCh3 will amplify a DNA sequence the length of the putative open reading frame. These oligonucleotides were used in a PCR reaction with the plasmid pWCh2 DNA as template. The PCR product was run on an agarose gel and the result is shown below (Figure 5.2.8).

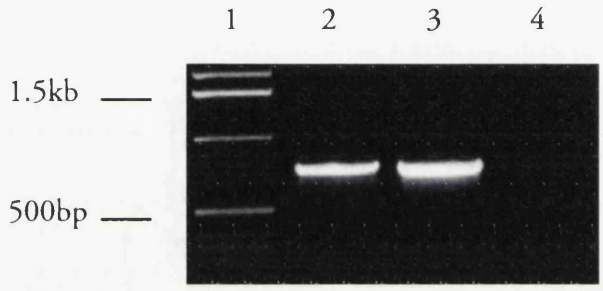


Figure 5.2.8

Agarose gel showing PCR product from pWCh2 template and oligonucleotides Het Left and Het Right. Lane 1, 1kb DNA ladder; lane 2, pWCh3 template (positive control); lane 3, pWCh2 template; lane 4, negative control (no template).

These data show that these oligonucleotides generate identically sized products from both pWCh2 and pWCh3 in PCR reactions. It appears then that the plasmid pWCh2 contains the complete and uninterrupted open reading frame present in pWCh3 even though the insert is not as long as that of pWCh3. Sequence analysis of pWCh2 (not shown) shows that this plasmid lacks a polyadenylation signal, so it is possible that this sized construct is the result of a mis-primed transcript in the cDNA synthesis step of library construction leading to a truncated cDNA sequence.

The full DNA sequence from data which the oligonucleotides WCh Het Left and WCh Het Right are designed are not shown.

5.3 Mapping of putative aquaporin

A novel member of the MIP family has been identified in the Malpighian tubules of *Drosophila melanogaster*. Preliminary studies with the 327 bp RT-PCR fragment localise the gene to 47F on the right arm of chromosome 2. Several P1 clones are mapped to this area, these P1 clones were acquired (gift, Steven Russell, University of Cambridge) and used to both confirm the localization of the gene to this segment of the *Drosophila* genome, and to map the location of the gene more finely within 47F.

P1 clone	localization
DS02304	47F1-7
DS02784	47F1-7
DS00182	47F1-9
DS00210	47E1-F2
DS08732	47F8-16
DS00478	47F8-18
DS02108	47F8-18

Figure 5.3.1

Table listing P1 clones with inserts mapping to 47F.

P1 clones DS00182, DS08732, DS00478 and DS02108 were used in a Southern hybridization analysis to map finely the localisation of a cloned water channel DNA fragment as these overlapping P1 clones completely span 47F. Results shown Figure 5.3.2 show that P1 clone DS00182 does not hybridize with the water channel probe, and that P1 clones DS08732, DS00478 and DS02108 do hybridize with a water channel probe. Therefore assignment of the 327bp RT-PCR product to 47F is correct. Also, this data implies that the clone lies somewhere between the distal ends of DS00182 and DS08732, giving a localization of 47F9-16 (Figure 5.3.3).

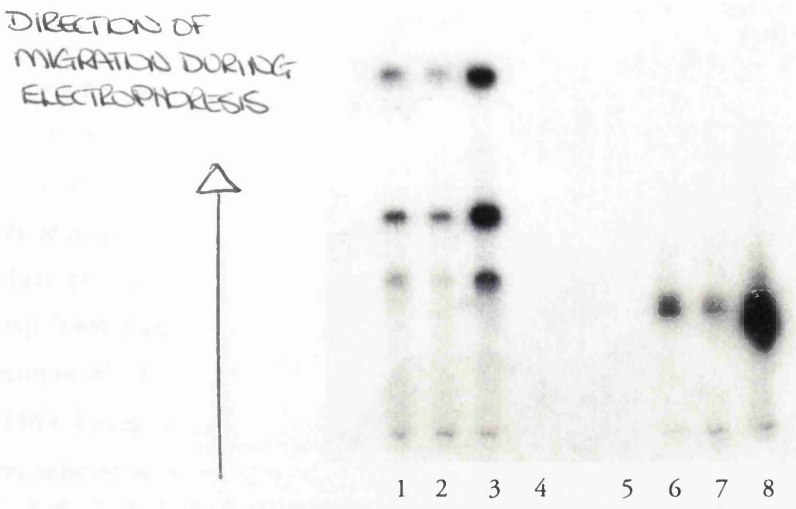


Figure 5.3.2

Southern hybridization of 47F P1 clones with water channel DNA. The blot was hybridized with pWCh3 insert DNA (*EcoR1* ends), washed at 65^{o}C to 0.1XSSC and exposed to film for 30 minutes. Lanes 1-4, P1 clones digested with *EcoR1;* lanes 5-8 P1 clone DNA digested with *BamH1.*

Lanes 1 and 8 DS02108; lanes 2 and 7 DS00478; lanes 3 and 6 DS08732; lanes 4 and 5 DS00182.

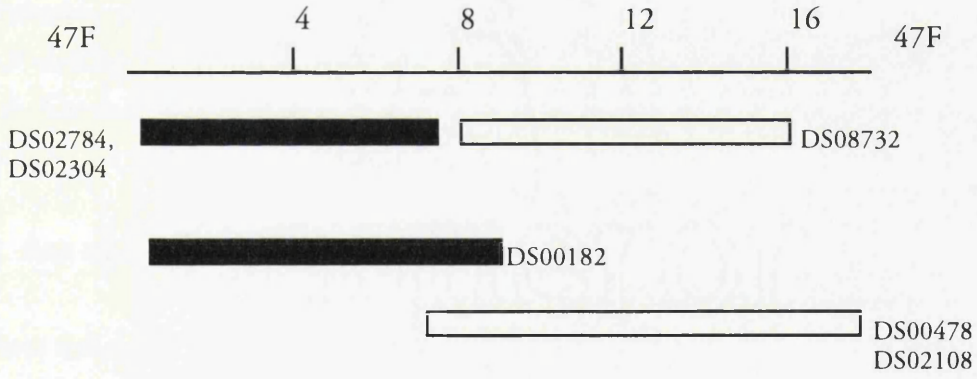


Figure 5.3.3

Schematic representation of P1 clone DNA inserts of 47F. Clones DS02784 and DS02304 span 47F 1-7, DS08732 spans 47F8-16, DS00182 spans 47F1-9, DS00478 and DS02108 span 47F8-18. Open boxes indicate that these P1 clones hybridize to water channel DNA sequences in a Southern hybridization. Therefore the smallest region in which the water channel gene can lie, as defined by hybridization studies with these P1 clones, is 47F9-16.

5.3.2 Aberrations at 47F

Lists of genetic aberrations at any particular cytological location can be generated using the on-line *Drosophila* database - Flybase. This database was searched to disclose a list of available aberrations at 47F. One class of aberration which can be acquired for study are deficiencies, or fly lines with deleted segments stably maintained over a suitable marked balancer chromosome. A flybase search disclosed three such deficiencies (http://www.ebi.ac.uk:7081/), *Df(2R)-enA, Df(2R)-enB, E3363. Df(2R)-enA* is deleted between 47D3 and 48B2-5; *Df(2R)-enB* bears a deletion between 47E3 and 48A4 ; and *E3363* bears a smaller deletion between 47A and 47F. Flies bearing these three deficiencies were acquired (gifts, stock centres at Bloomington and Umeå), and used in the following studies.

The flybase database is continually updated, and more recent searches show that there are now a large number of deficiencies at this locus available, however in the work which follows the three lines described above are used.

Not all of the abberations available to the *Drosophila* research community are listed on Flybase, and a second source of *Drosophila* stocks is the Berkeley *Drosophila* Genome Project (BDGP). The P-element insertions listed in Flybase are not particularly near to 47F, however several P-elements insertions generated by the BDGP are much closer. These P-element lines are K06103, 47F1-2; K17005, 47F4-9; K05103, 47F8-9 ; P1386, 47E1-2. These P-element bearing lines were acquired and used in the studies reported below.

5.3.3 Are the deficiency lines hemizygous for the water channel gene?

The three deficiency lines described above have deletions which are reported to span all, or part of 47F. These lines may therefore be hemizygous for the water channel gene as an intact copy is maintained on the balancer chromosome. A technique used to disclose hemizygosity in these circumstances is quantitative Southern hybridization. Hybridization signal is proportional to the amount of DNA present in a particular sample. If similar amounts of digested control and test genomic DNAs are run on an agarose gel and blotted, the signal intensity of a particular probe after hybridization can be observed. The use of a probe from a non deleted area of the genome will give the relative intensity of signals of control and test DNAs in a fully diploid area. Subsequent use of a DNA probe which is though to represent DNA deleted in the deficiency will show the relative intensity between the control and test DNAs, and any difference in the relative intensity of signal in control and test DNAs can be observed. If the test probe is not from a hemizygous portion of the genome then the ratio of test/control signal

relative intensity of signal in control and test DNAs can be observed. If the test probe is not from a hemizygous portion of the genome then the ratio of test/control signal intensity of test probe:control probe should be 1:1. If however the DNA probe used is hemizygous in the fly line tested this ratio will be 1:2.

In the experiments described next, the three deficiency lines have been subjected to this type of analysis. The DNAs of Oregon-R flies (control) and *Df(2R)-enA*, *Df(2R)-enB* and *E3363* flies (test samples) are digested with *EcoR1* and for each deficiency line a blot for Southern hybridization is made with deficiency and Oregon-R digested DNA. The non deleted control probe used in all samples is the 860 bp urate oxidase promoter fragment cloned and analysed in chapter 3. This DNA originates on chromosome 3, and its copy number should not be altered in any way by the genetic background of the flies used. The test probe used in the analysis is derived from pWCh, either the 1kb *EcoR1/Xho1* fragment or the 472bp *EcoR1* fragment are used; the probe used is described in the figure legends (Figure 5.3.4).

Figure 5.3.4

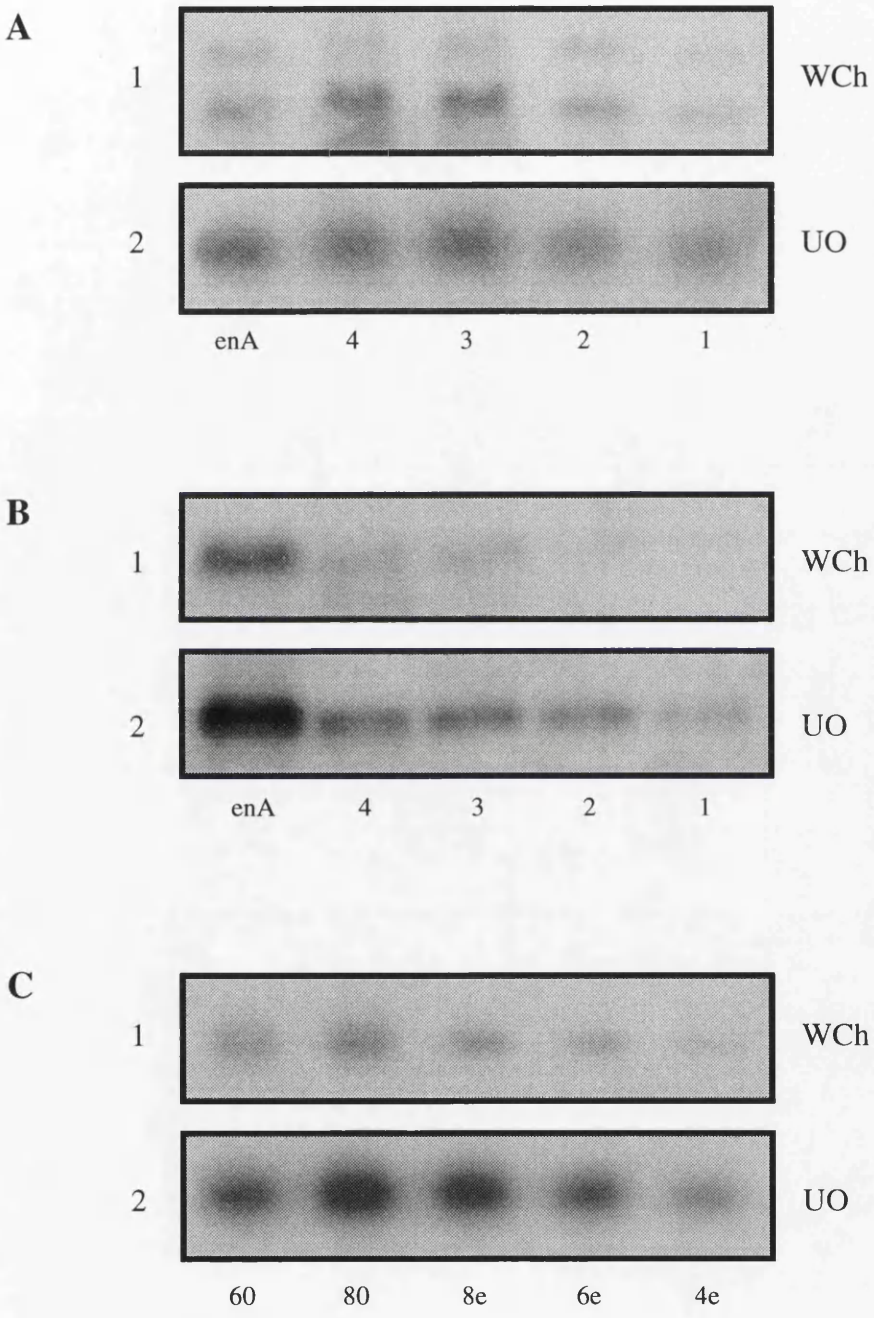


Figure 5.3.4

A:

Southern hybridization of *Df(2R)-enA* and Oregon-R genomic DNAs. Both DNAs are cut with *EcoR1*. Lanes 1,2,3,4; 1,2,3 and 4 μg Oregon-R DNA: lane enA; approximately 4μg of *Df(2R)-enA* DNA. The upper blot (1) shows the signal obtained with the 472 bp *EcoR1* fragment of pWCh is used as probe, the lower blot (2) shows the signal obtained when the 860 bp urate oxidase promoter fragment is used as a probe.

It can be seen that in blot 2, the intensity of the *Df(2R)-enA* signal with the probe is approximately equal to that of lane 3. When water channel sequences are used to probe the same blot the intensity of hybridization of *Df(2R)-enA* DNA is decreased relative to that of the controls, to a level approximately between those seen in lanes 1 and 2. Therefore it appears that the line *Df(2R)-enA* is hemizygous for the water channel DNA sequences.

B:

Southern hybridization of *Df(2R)-enB* and Oregon-R genomic DNAs. Both DNAs are cut with *EcoR1*. Lanes 1, 2, 3, 4; 1, 2, 3 and 4 μg Oregon-R DNA: lane enA; approximately 4μg of *Df(2R)-enB* DNA. The upper blot (1) shows the signal obtained with the 472 bp *EcoR1* fragment of pWCh is used as probe, the lower blot (2) shows the signal obtained when the 860 bp urate oxidase promoter fragment is used as a probe.

It can be seen that in blot 2, the intensity of the *Df(2R)-enB* signal with the probe is much greater than any of the controls used. When water channel sequences are used to probe the blot, the relative intensity of test to contol DNA appears unchanged. Therefore it is likely that the line *Df(2R)-enB* is not hemizygous for water channel DNA sequences.

C:

Southern hybridization of *E3363* and Oregon-R genomic DNAs. Both DNAs are cut with *EcoR1*. Lanes 60 and 80; approximately 6 and 8μg of Oregon R genomic DNA: lanes 4e, 6e and 8e; approximately 4, 6 and 8μg of *E3363* DNA. The upper blot (1) shows the signal obtained with the 1 kb *EcoR1/Xho1* fragment of pWCh is used as probe, the lower blot (2) shows the signal obtained when the 860 bp urate oxidase promoter fragment is used as a probe.

On examination of these signals it can be seen that the signals of 6e and 60 are almost equivalent in intensity when the urate oxidase DNA sequence is used as probe. However in the upper blot of this pair, the intensity of the 6e signal is reduced relative to the signal in lane 60; the band of 6e is fainter than that of 60 with the water channel probe, and the 6e signal covers a much smaller area than that of 60. Therefore, it appears that the line *E3363* is hemizygous for water channel DNA sequences.

The assignment of hemizygosity is based on the primary data of the experiments shown here and others which are not shown.

Blots were made for Southern hybridization with the genomic DNAs of the P-element lines K05103, K06103, K17005 and P1386. The blots were hybridized with water channel DNA species in an effort to establish if the P-element insertions are within or close to the water channel genomic sequence. Insertion of a P-element introduces novel restriction endonuclease recognition sites, and if such an event has occurred within or close to the water channel gene, it may be detected as a restriction fragment length polymorphism (RFLP) on a Southern hybridization.

5.4.1 Screening with WCh3 DNA

The first hybridization shown is of a blot containing *EcoR1* digested DNAs of all the P-element lines used, and hybridized with the *EcoR1* fragment of WCh3. This cDNA fragment should span the entire genomic region of the translated sequences of the gene.

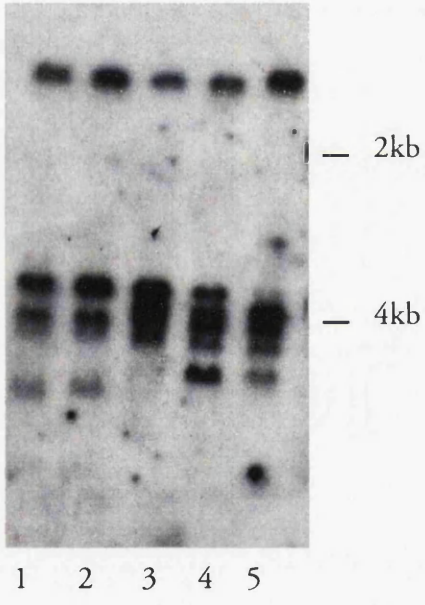


Figure 5.4.1

Genomic Southern hybridization of P-element line genomic DNAs to WCh3 DNA. The probe used is the *EcoR1* ended insert from WCh3. Lane 1, K17005; lane 2, K06103; lane 3, K05103; lane 4, P1386; lane 5, Oregon-R. After hybridization the blot was washed at 65°C to 0.1XSSC and exposed to film for 5 nights.

It can be seen from Figure 5.4.1 that there is no single line with a unique restriction pattern.The restriction fragment profile obtained in both Oregon-R and P-element lines is diagnostic for hybridization of sequences of the probe to repetitive sequences of the genome. In particular, in specific hybridization the intensity of signal observed is

proportional to the length (in base pairs) of the fragment. Not all of the bands shown on this blot follow this generality, and therefore it is concluded that they are the result of less specific hybridization between probe and genomic DNAs This type of observation is thought to be due to hybridization of sequences of the probe to other related gene sequences, or to non-expressed sequences which have some homology with the probe used. Molecular protocols define washing at 65°C in 0.1XSSC as sufficiently stringent to remove non-specific hybridization, therefore the signals in question are due to specific hybridization between probe and genomic DNA fragments.

The practical conclusion of this result is that the cDNA probe cannot be used to screen for restriction fragment length polymorphisms as sequence elements within it are not unique to water channel sequences at 47F.

Therefore, to screen the P-element lines shown on this blot, the 472 bp *EcoR1*and 1 kb *Xho1/EcoR1* fragments of pWCh are in separate hybridizations of this same blot (Figure 5.4.2).

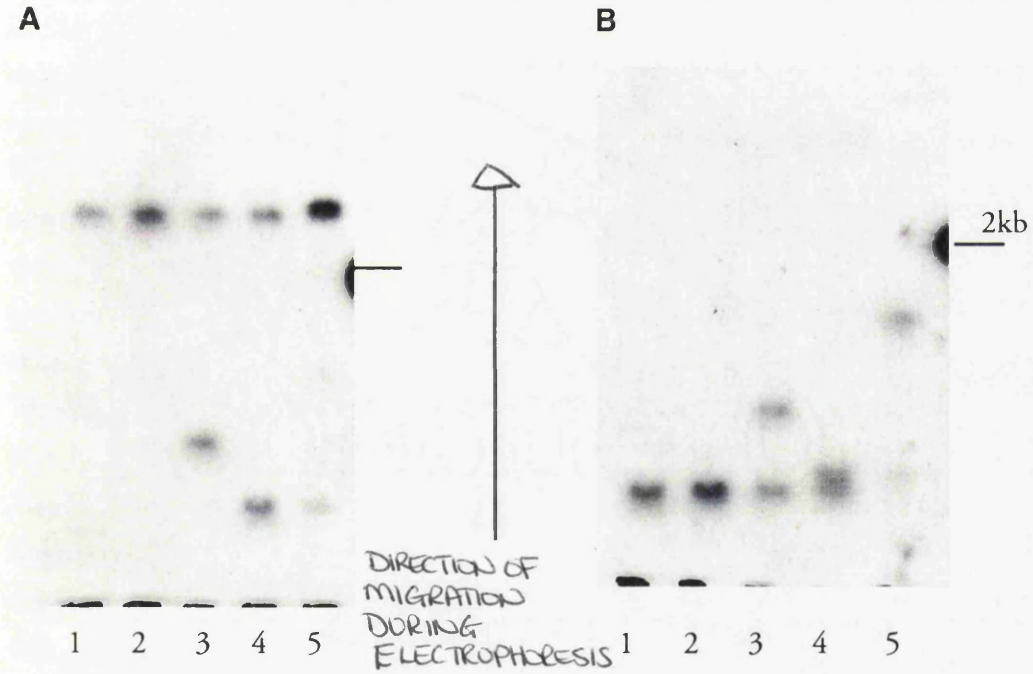


Figure 5.4.2

Southern hybridizations of P-element line genomic DNAs. The blots were hybridized with A, 1 kb *EcoR1/Xho1* pWCh DNA; B, 472 bp *EcoR1* pWCh DNA. Both blots were washed at 65°C to 0.1XSSC and blot A was exposed to film for 1 night, blot B was exposed for 6 nights.

Lane 1, K17005; lane 2, K06103; lane 3, K05103; lane 4, P1386; lane 5, Oregon-R.

It can be seen from these hybridizations that the probes used can hybridize to only a single DNA fragment in some lanes, therefore the sequences hybridizing to a ladder of bands per lane are outwith the sequences of the two probes used in Figure 5.4.2, but are present in the cDNA clone.

In Figure 5.4.2A the restriction fragment profile given by the probe is identical in lines Oregon-R and P1386, this profile is different from the other lines tested. These lines have been obtained from different sources and were not isogenised before analysis. It is thought that this variation is due to variations in genetic background, and not to the presence of P-elements within the water channel genomic sequence.

There is however an additional band observed in the DNA of line K05103. This line has been assigned by BDGP to location 47F8-9, and it is possible that this RFLP is due to insertion of a P-element in the sequence covered by the probe. To test for this possibility this blot was stripped and rehybridized with the 472bp *EcoR1* fragment of pWCh. The blot is made with *EcoR1* digested DNA; if the internal *EcoR1* site of pWCh is conserved in the lines analysed the RFLP detected with one genomic *EcoR1* fragment will not be detected by an adjacent *EcoR1* fragment. Therefore detection of the same RFLP with the second probe provides evidence that the observed RFLP is not due to the presence of the P-element. It can be seen in Figure 5.4.2B that there is an RFLP seen in this DNA sample with this probe. When the autoradiographs are lined up by hand it can be shown that the additional band in this lane is exactly the same size in hybridizations with both probes. Therefore the RFLP is not due to insertion of the P-element in or close to water channel genomic DNA sequences.

The most likely explanation for this observation is that on one chromosome 2 of this pair in K05103 a small mutation, possibly a point mutation, has removed the genomic *EcoR1* site seen in clone pWCh, meaning that both the 472bp fragment and the 1kb fragment of pWCh now lie on the same *EcoR1* fragment of this chromosome. The other copy of chromosome 2 retains this site, and therefore the Southern hybridization with this line gives two fragments rather than the one seen in K17005 and K06103. Further evidence to support this possibility arises from the different restriction fragment lengths seen with the two probes used. The common fragment in all P-element lines with the 1kb probe is less than 2kb, while the common fragment with the 472bp fragment is a much greater size than this. This implies that the internal *EcoR1* site of pWCh is conserved in the P-element lines as the two subcloned DNA fragments do not lie on the same genomic *EcoR1* fragment (except on one chromosome of K05103)

To fully investigate if the RFLP seen with the P-element line K05103 is due to insertion of a P-element within or near sequences of the water channel gene or due to a fortuituous loss of an *EcoR1* site in one chromosome of this line, all P-element line

genomic DNA s were digested with a different restriction endonuclease, *PvuII*, blotted, and the blots hybridized with the 472 bp *EcoR1* and 1kb *Xho1/EcoR1* fragments of pWCh.

5.4.2 Further restriction analysis of P-element line DNA

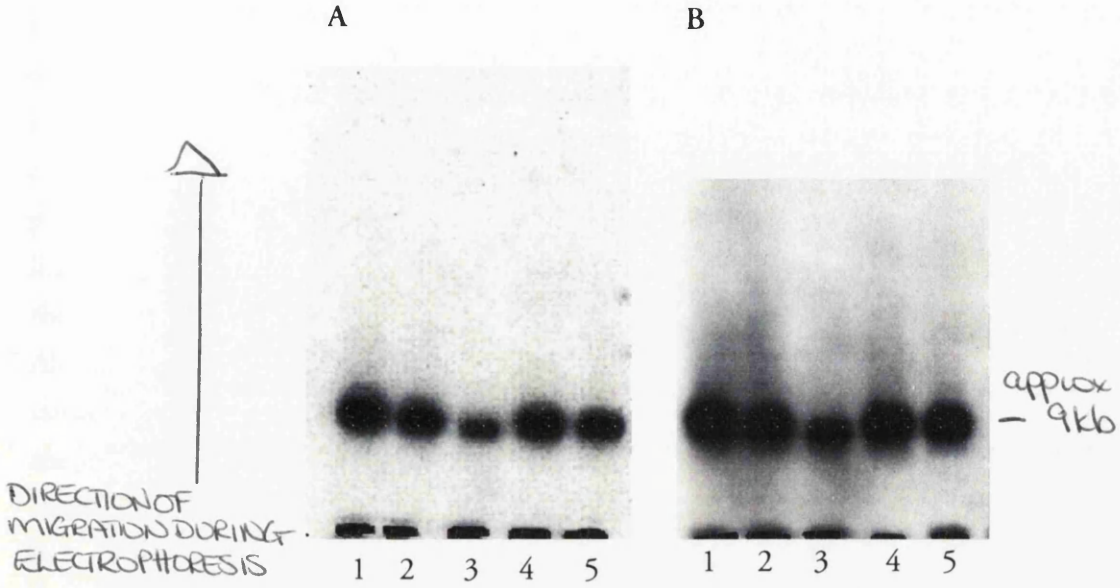


Figure 5.4.3

Southern hybridizations of P-element genomic DNAs digested with *PvuII*. Blot A is hybridized with the 472 bp *EcoR1* pWCh fragment, washed to 0.1XSSC at 65°C and exposed to film for 7 nights. Blot B is hybridized with the 1 kb *EcoR1/Xho1* pWCh fragment, washed to 0.1XSSC and exposed to film overnight. Lanes 1, K06103; lane 2, K17005; lane 3, K05103; lane 4, P1386; lane 5, Oregon-R.

Both probes hybridize to a single *PvuII* restriction fragment. There is no backround signal with either probe, and there appears to be no homology between the probes and multiple fragments of the genomic DNA. This confirms the results seen Figure 5.4.2 for these probes. Although the hybridizations are to different blots, the blots are derived from the same gel (one gel containing two identical sets of restriction digests was run, a single blot made, and the blot cut in half) , and therefore by aligning the blots by the position of the wells the sizes of the bands can be compared. It appears that the two probes hybridize to the same *PvuII* fragment. There are no additional bands in any lane, and there are no differences in the restriction profiles of the lines used.

Thus it is concluded that none of the P-element insertions of the lines tested are sufficiently close to the water channel gene sequences to be detectable by Southern analysis.

5.5 Complementation analysis

Complementation analysis is a powerful tool in genome analysis which allows the effect on lethality of a pair of mutations to be assessed. In *Drosophila*, homozygous lethal mutations are maintained as heterozygotes over balancer chromosomes. These chromosomes suppress recombination, but also are homozygous lethal, meaning that pure breeding lines homozygous for balancer chromosomes are not found. Therefore a line with lethal mutation maintained over a balancer chromosome can be kept without the need for selection.

All of the mutant lines discussed in this chapter are maintained over the same balancer chromosome bearing a mutaion of wing morphology, the presence of this balancer chromosome, *CyO*, can be scored easily by examination of wing morphology.

In the following experiments, the effect of each P-element and each deletion mutation are examined together. In this type of study an individual of a P-element line is mated to an individual bearing a deletion, and the wing morphology of the progeny scored. By simple Mendelian genetics 25% of the progeny will not survive to be scored, as this fraction will be homozygous for the balancer chromosome. 50% of the total possible progeny are predicted to have *CyO* wing phenotype, as this proportion will bear either a P-element or internally deleted chromosome and the balancer. The remaining 25% can only have straight wings as they inherit both P-element and internally deleted chromosome. If however no straight winged flies are seen in progeny from this cross then it is assumed that the two mutations are causing lethality, and therefore that the insertion is in a portion of the chromosome which is deleted on the other chromosome and that the deficiency has 'uncovered' the source of lethality.

Therefore, this technique is employed here as a genome mapping tool. Each deletion mutant is mated to each P-element line, absence of straight winged flies in the progeny implies that the P-element lies within the deletion *i.e.* the P-element fails to complement the deletion. The presence of straight winged flies in the progeny implies that the P-element lies outwith the deletion *i.e.* the P element complements the deletion.

5.5a *Df(2R)-enA*

DF(2R)-enA	K05103	K17005	K06103
straight	8	13	0
curly	23	26	22

Figure 5.5.1

Results of complementation analysis crosses between *Df(2R)-enA* and the P-element lines K05103, K17005 and K06103. Straight, numbers of straight winged flies in the progeny of the cross; curly, number of curly winged flies in the progeny of each cross.

5.5b *Df(2R)-enB*

Df(2R)-enB	K05103	K17005	K06103
straight	14	6	0
curly	40	3	56

Figure 5.5.2

Results of complementation analysis crosses between *Df(2R)-enB* and the P-element lines K05103, K17005, and K06103. Straight, numbers of straight winged flies in the progeny of the cross; curly, number of curly winged flies in the progeny of the cross.

5.5c *E3363*

E3363	K06103	K17005	K06103
straight	30	3	0
curly	65	6	46

Figure 5.5.3

Results of complementation analysis crosses between *E3363* and the P-element lines K05103, K17005, and K06103. Straight, numbers of straight winged flies in the progeny of a cross; curly, number of curly winged flies in the progeny of a cross.

5.6 Staining patterns of the P-element lines

Tubules from the P-element containing lines K05103, K06103, K17005 and P1386 were dissected and stained for β-galactosidase activity. None of the lines K05103, K06103 and K17005 showed any degree of β-galactosidase activity, however line P1386 did show activity of β-galactosidase in tubules.

Figure 5.6.1

Photograph of Malpighian tubules of line P1386 stained for β-galactosidase activity. Nuclear staining can be seen from the ureter, through the lower tubule and into the main segment. Proximal stained nuclei (closer to the ureter) have a greater staining intensity than nuclei more distal. There is a low level of β-galactosidase activity in the remaining cells of the main segment.

Nuclei of the initial segment stain with an intensity intermediate between that of the lower tubule / ureter and the main segment.

Both anterior and posterior tubules of P1386 flies gave the β-galactosidase staining pattern as described Figure 5.6.1. β-galactosidase activity was observed in feeding third instar larvae, but was not seen in wandering third instar larvae or pre-pupae.

Sections of a whole adult *Drosophila* were taken, and these sections stained to disclose β-galactosidase activity. Weak expression was observed in the eye and the ovary, as well as the expected expression pattern in the gut and Malpighian tubules.

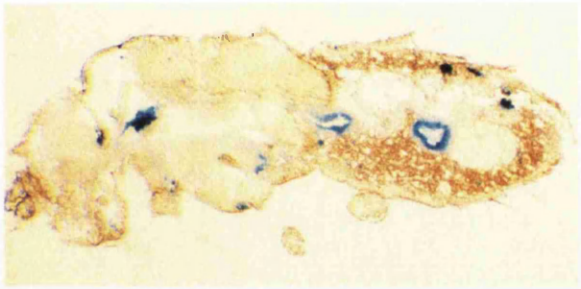


Figure 5.6.2

Section of adult female P1386 *Drosophila* stained for β-galactosidase activity. Strong signal is seen in the gut in both the thorax and abdomen. In the abdomen there is punctate β-galactosidase activity, and these cells are likely to be derived from the Malpighian tubules. The ovaries and eyes show a low level of β-galactoside activity.

Thus, P1386 is the only P-element line of those tested giving Malpighian tubule expression of *lacZ*. Expression from the trapped enhancer is not restricted to the Malpighian tubule; however, the Malpighian tubule expression appears to be developmentally regulated.

5.7 Discussion

An attempt was made to clone a cDNA coding a major intrinsic protein in Malpighian tubules of *Drosophila melanogaster* by screening a commercial adult cDNA library with a previously characterized RT-PCR product. The clone identified from the library was a 1486 bp DNA fragment; the structure of this DNA fragment suggests that it is a contaminant of the library. Also, the homologous sequence of the RT-PCR product is interrupted in the clone by a 57 bp sequence which appears to be an intron. Therefore this clone is thought to result from contamination of the library with genomic DNA sequences during construction.

Another adult body cDNA library was acquired and screened using the clone described above as probe. This analysis revealed two distinct size fragments of 1.4 kb and 1.6 kb which hybridize with the probe product. The fragments were subcloned, and the largest clone sequenced by primer extension analysis. This analysis revealed a DNA sequence with a putative open reading frame (ORF) of 735 bp; this ORF sequence has high homology with other members of the major intrinsic protein family, and appears to be a novel member of the family.

Oligonucleotides were designed to amplify the open reading frame in a PCR reaction; when the 1.4 kb and 1.6 kb DNA clones are used as template in PCR reactions with these oligonucleotides, the PCR products are identical in size. This implies that the smaller clone contains the whole of the open reading frame; sequence analysis shows that this clone lacks a polyadenylation signal, implying that this clone results from a mispriming event in library construction.

Analysis with the 327 bp RT-PCR product maps that DNA to 47F on the right arm of chromosome two. The genomic and cDNA clones derived from the analysis above were used in higher resolution mapping analyses; P1 clone analysis maps the gene to between 47F9-47F16. Four P-element lines were analysed to establish if the P-element insertion had occurred within or near to the cDNA sequence. Southern analysis was unable to detect a P-element insertion within or near to the cDNA sequence in any of the lines tested.

Three deficiency lines were tested for hemizygosity for cDNA sequences by quantitative Southern hybridization. This technique suggests that the lines *Df(2R)-enA* and *E3363* are hemizygous for the water channel gene, but the line *Df(2R)-enB* does not appear to be hemizygous for this DNA.

Complementation analysis with the P-elements and the deficiencies suggest that two of the three P-elements tested do not map in the positions they are assigned, as they complement all of the deficiencies used. The P-element line K06103 fails to complement

any of the deficiency lines used, and appears to be the line with the closest P-element to water channel DNA sequences.
Southern hybridization of the water channel cDNA clone to *Drosophila* genomic DNA results in multiple bands with differing intensities; use of the genomic fragments in similar experiments give less complex signals.
Only the P-element line P1386 shows *lacZ* expression in Malpighian tubules; expression is seen in 3rd instar larvae and adult, though in wandering 3rd instar larvae and pre-pupae this expression is repressed. In the adult, β-galactosidase activity is observed in the ovary, eye and gut, as well as in the Malpighian tubules.

The insert of pWCh when used to probe Southern blots of genomic DNA give a simple signal pattern, consistent with this fragment being derived from genomic DNA. The cDNA sequence however gives a complex signal pattern. This signal pattern suggests that there are sequences in the cDNA clone which hybridize with different levels of specificity to many different sequences in the genome. When the cDNA sequence is used to probe a Southern blot of P1 clones at 47F a relatively simple band pattern is seen, implying that some of the sequences with which the cDNA probe is hybridizing are not present at 47F. The sequences of the cDNA which recognise many different genomic DNA fragments are not present within the genomic clone.
These sequences of the cDNA which are responsible for this Southern hybridization pattern may be repetitive sequences of the genome, or may be highly homologous sequences of other *Drosophila* genes which hybridize to the cDNA probe. This latter possibility is however unlikely; in chapter 4, flies were made homozygous with a human serotonin receptor transgene, Southern hybridization with genomic DNA from transgenic flies using the human receptor DNA as probe did not give a complex signal based on the hybridization of the conserved sequences of heptahelical receptor genes. Thus the complex signal pattern seen with the insert of plasmid pWCh3 is more likely to be due to the presence of repetitive sequences of the genome.
The cDNA clone has proved very difficult to manipulate; certain pairs of oligonucleotides made from sequences of the cDNA clone could not amplify single products from the cDNA clone or from genomic DNA (results not shown), and sequence data was often uninterpretable. These practicalities have limited the possible approaches to study of this sequence and greatly increased the workload necessary to sequence the cDNA. The cDNA sequence given is of the open reading frame alone, and is the best possible contiguous fragment possible with the data generated in this study. It is possible that future sequence analysis may uncover errors in the sequence data given here, however with the resources and technology available the sequence shown is the best obtainable.

The putative protein sequence is most closely related to two other insect major intrinsic proteins, and the sequence of the putative translation of the cDNA cloned in these studies is similar to that of the *H.irritans* major intrinsic protein along most of its length (not shown). The *Drosophila* sequence contains the highly conserved Asn-Pro-Ala motifs of the B and E loops, as well as the conserved cysteines of these loops. Sequence conservation analysis shows that amino acid residues around the B and E loops are conserved in the putative translation of the pWCh3 ORF, Aquaporin CHIP and MIP26 (Preston and Agre, 1991). These areas of the protein are thought to be directly functional in pore formation (Jung *et al.*, 1994), and it is interesting that they are conserved in this sequence.

The A and E loops of the human Aquaporin CHIP amino acid sequence have N-glycosylation consensus sequences, and these sites were used to demonstrate that these loops are extracellular (Preston *et al.*, 1994); the DNA sequence given is incomplete in the putative loops A and C, though a single amino acid insertion in the putative A loop of the translation will not generate a consensus sequence for N-glycosylation. Sequence resolution of 2 bases of the putative C loop of the translation may show an asparagine residue separated by a single amino acid from a threonine - and this would be a consensus site for N-glycosylation; however, it is predicted that the amino acids of this loop are not in contact with the lumenal side of the endoplasmic reticulum, and would therefore not be glycosylated. Six possible membrane spanning domains are given (Figure 5.2.7c). Assignment of the third, fifth and sixth putative membrane spanning domains is relatively simple, as these sequences contain amino acid homology with the membrane spanning domains of human aquaporin CHIP (Preston and Agre, 1991). The B and E loops are also relatively easy to assign for this same reason. The assignment of topological identity to the rest of the protein sequence is more difficult, and is rather arbitrary. There are 16 glycine residues and 5 proline residues in total in the putative membrane spanning regions; neither of these residues favour the formation of α-helical structures, and the presence of so many in regions likely to form membrane spanning domains is puzzling; however some of these residues are highly conserved and are known to be in transmembrane sequences of other major intrinsic proteins. The position of the intron disclosed by the analysis of clone pWCh suggests that it interrupts the cDNA sequence of the final transmembrane segment. The position of this segment has been assigned on the basis that the nearby cysteine may be extracellular; however, the sequence homology with the final transmembrane domain of aquaporin CHIP in the model of Preston and Agre (Preston and Agre, 1991) suggests that the cysteine may be part of the transmembrane domain, and that the last residue of the helix is the adjacent alanine. If this is the case, the E loop of this protein will not possess an extracellular cysteine, and therefore in the assignment of a possible topology, the cysteine is shown to be extracellular.

P1 clone mapping maps the cDNA to 47F9-16, this confirms the earlier *in situ* analysis with the 327 bp RT-PCR product. All of the deficiency lines tested are predicted to be hemizygous for water channel DNA sequences; however, only lines *E3363* and *Df(2R)-enA* appear to be hemizygous for the probes used. All of the deficiency lines used fail to complement the P-element line K06103. Thus, the P-element insertion in K06103 lies within the deleted section of all of the deficiency lines, and the cDNA appears to lie within a common deleted segment between *E3363* and *Df(2R)-enA.*

The deficiency *Df(2R)-enB* is predicted to be hemizygous for sequences in 47F9-16. Its failure to complement the P-element line K06103 suggests that the line supplied does indeed contain a deletion on one chromosome 2R, encompassing a deletion common to *E3363* and *Df(2R)-enA.* The deletion does not cover the cDNA sequences at 47F9-16; one possible explanation for this anomaly is that this line bears two deletions separated by an island of 47F DNA containing water channel sequences.

Drosophila genome analysis has until now concentrated on the identification of gene sequences whose mutation results in a lethal phenotype. It is possible that mutation of the cDNA cloned in this study does not result in lethality; firstly, this area of the genome has been extenisvely studied(flybase, http://www.ebi.ac.uk:7081/), and no lethal alleles have been described at 47F9-16 which are likely to represent the water channel cDNA; it has been shown that other tissues, known to pass high volumes of water, do not express major intrinsic protein sequences (Yano *et al.*, 1996); human patients with null mutation of the ubiquitously expressed CHIP aquaporin sequence do not suffer clinical consequence from the lack of this protein (Preston *et al.*, 1994a) although, human patients deficient in aquaporin 2 present with nephrogenic diabetes insipidus (vanLieburg *et al.*, 1994).

The P-elements lines K05103, K06103 and K17005 do not show β-galactosidase activity in adult Malpighian tubules. Line P1386 shows a developmentally regulated β-galactosidase activity in Malpighian tubules with activity detected in third instar larvae and adults, and no activity seen in pre-pupae and wandering instar larvae, as well as expression in the ovary and eye of the adult. This temporal pattern of expression in tubules is similar to the temporal expression pattern of the urate oxidase gene, an ecdysone regulated gene (Wallrath *et al.*, 1990). If the P-element reporter of P1386 is disclosing the activity of a water channel enhancer in tubules then it is interesting to speculate that genes essential to the tubules excretory function are repressed during pupariation by a single signal - ecdysone.

The P-element insert of P1386 is at 47E1-2, this is approximately 280 kb from the site of the water channel gene, and is possible that it the P-element expression pattern is uncovering the expression pattern of a water channel enhancer. The high level of expression of the reporter in the lower tubule suggests that if the reporter is uncovering the expression pattern of a water channel gene, then the protein is facilitating the

reabsorbtion of water from the tubule into the haemolymph, as it has been shown that the lower tubule is a site of water reabsorbtion (O'Donnell and Maddrell, 1992). The expression of the reporter in upper tubule (initial segment) argues against a role for the trapped enhancer in water channel gene expression as this segment is thought not to be involved in fluid secretion (O'Donnell and Maddrell, 1992).

In conclusion (Figure 5.7), a cDNA sequence has been cloned which has high homology with members of the major intrinsic protein family from the Malpighian tubules of *Drosophila melanogaster*. This cDNA sequence is derived from genomic sequences at 47F9-16. The deficiencies *E3363* and *Df(2R)-enA* are hemizygous for cDNA sequences, and none of the P-element lines P1386, K05103, K06103 or K17005 have P-element insertions within the water channel gene or sufficiently close to it to allow detection by Southern hybridization analysis.
The deficiency *Df(2R)-enB* is not hemizygous for water channel sequences, though it, and both the other deficiencies used fail to complement the P-element K06103. None of the deficiencies fail to complement any of the other P-element lines tested.

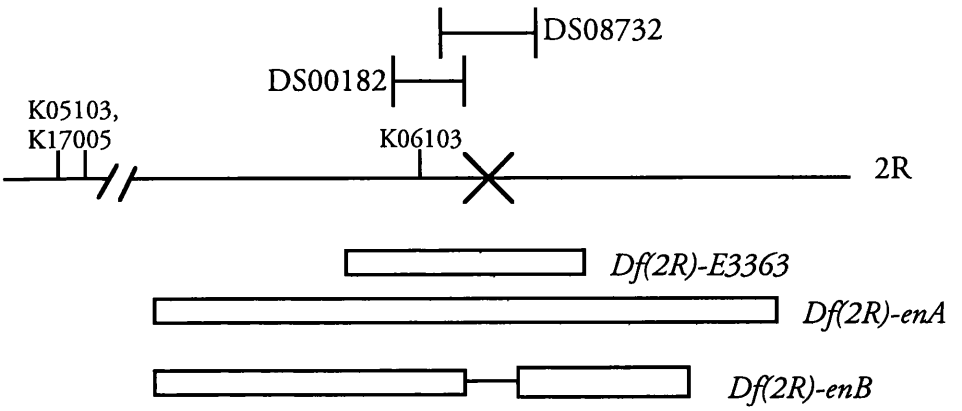


Figure 5.7

Diagrammatic summary of mapping data presented. A length of chromosome 2R is represented, with the position of the gene sequence cloned given by an X. Above this representation, the position of the P1 clones DS00182 and DS00832 are indicated. DS00182 maps to 47F1-9, and DS08732 maps to 47F8-16. As DS08732 contains the putative water channel gene sequences and DS00182 does not, the gene is mapped in this analysis to between 47F9 and 47F16.

Below the representation of the wild type chromosome are schematic representations of the areas deleted in the deficiency lines *Df(2R)-enA*, *Df(2R)-enB* and *Df(2R)-E3363* (hemizygous areas marked by open boxes). The lines *Df(2R)-enA* and *Df(2R)-E3363* are both hemizygous for the region of chromosome 2R bearing the putative water channel gene. *Df(2R)-enB* is not hemizygous for this area, however this line does, in common with the other two deficiencies, fail to complement the P element line K06103, and therefore bears a common deficiency.

The P-element insertions in lines K17005 and K05103 lie outwith the deleted areas of 2R in all of the deficiency lines tested.

5.8 Further Work

The work presented has concentrated on the cloning, sequencing and mapping of a cDNA clone which may encode a water channel protein. DNA sequence identity studies suggest that a protein encoded by the gene may be an aquaporin-type protein, however this study presents no data to support this functional assignment of the gene product.

Evidence for the presence of water channel proteins in Malpighian tubules comes from the documented sensitivity of tubules to mercury, and RT-PCR data which shows that a novel fragment with considerable homology to members of the aquaporin gene family can be amplified using degenerate oligonucleotides based on conserved sequences of aquaporin genes. The genetic evidence strongly suggests that an mRNA coding an aquaporin-type gene is being expressed in Malpighian tubules, however the sensitivity of tubules to mercury does not demonstrate a reversible interaction between the metal ion and a possible water channel protein. Water facilitators of this class can have a reversible interaction with mercury (Chrispeels and Agre, 1994). Co-incubation of tubules with mercuric compounds and β-mercaptoethanol relieves the effect of the metal on the tissue (Dow *et al.*, 1995), however, β-mercaptoethanol strongly binds mercury - and so a possible interpretatation of the data from this experiment is that the tissue is simply never interacting with the mercury in the saline.

Thus the tubule secretion assay can be adapted with pre-incubation of the tissue tubule with mercury, then removal of unbound mercury from saline, followed by analysis of the effect of β-mercaptoethanol on mercury affected tubules. If in these circumstances the tubules can be shown to be affected by mercury, and this effect can be reversed by β-mercaptoethanol, then the tubule secretion assay will have provided strong evidence that there are functional water channels in the tubules of *Drosophila melanogaster.*

The cDNA cloned has considerable sequence homology with aquaporin sequences, but this does not prove that the cDNA codes a water channel protein. If synthetic mRNA can be made from the cDNA and injected into *Xenopus* oocytes for translation, then the effect of the protein coded by the cDNA on water permeability of oocyte membranes can be assessed.

Assignment of membrane topology for the putative translation of the open reading frame of pWCh3 is based on its homology with other major intrinsic protein sequences, and on their putative topology. There is some uncertainty concerning the assignment of the E loop, and whether there is an extracellular cysteine of this protein. As this residue of this loop is thought to participate critically in the function of the molecule, effort should be taken to resolve if this *Drosophila* major intrinsic protein does indeed possess an extracellular cysteine.

Finally, the P-element line P1386 gives a strong tubule-selective and developmentally-regulated *lacZ* reporter gene expression pattern. Even if the expression pattern of the reporter is not an interesting candidate for water channel expression patterns, developmental regulation of tubule expressed genes is important in the understanding of the genetics of epithelial function in *Drosophila*. Thus, cloning and characterization of regulatory sequences around P1386 may help in the dissection of DNA sequences directing developmentally regulated expression in Malpighian tubules of *Drosophila*.

Chapter 6

Conclusion

6 Conclusion

In general, *Drosophila* has lost some favour as a model system, and today transgenic mouse technology has become the preferred approach to ectopic expression studies. Indeed, the mouse transgenic approach does have advantages over *Drosophila* in helping to elucidate the genetic basis of disease; for example, whole vertebrate animal transgenesis is naturally more relevant to understanding human genetic aberrations than study of a small invertebrate.

The benefit obtained from mouse model systems must be weighed against the investment of time and money required to generate a single transgenic animal. Insertion mutation in mice relies on targetted homlogous recombination in embryonic stem cells, followed by difficult manipulations of mouse embryos. Generation of a transgenic mouse is usually a 2-3 year process, which is typically undertaken by postdoctoral staff rather than by postgraduate students.

By contrast, transgenesis in *Drosophila* is a relatively simple procedure, which harnesses available transposon technology. Single insertion events can be generated in months, and P-element technology can be harnessed to produce a series of different insertions with the same construct, thus allowing the effect of genomic positional effect to be subtracted. Transgenic technology in *Drosophila* also makes available sophisticated control mechanisms for activation of transgenic insertions; the possibility of targetted expression of transgenic sequences allows animals to be propagated even if expression of the transgene is deleterious to viability, and expression of the gene can be studied in isolation. These techniques are not available for mouse, and sequences for expression targetting in mouse must be transformed along with suitable DNA control elements. To achieve the same level of precision in targetted expression of transgenes in mouse as is available in *Drosophila* would demand an exhaustive knowledge of DNA control sequences of mouse; in *Drosophila*, promoters can be harnessed, though the details of promoter structure need not be known.

Thus, although *Drosophila* lacks some of the funding potential of the more fashionable vertebrate models, the accessibility and adaptability of transgenic technology makes it the superior choice for use as a model system in studies like those presented in this volume.

In addition to the possibilities of *Drosophila* transgenic technology, the understanding of *Drosophila* genetics is possibly the most advanced for any higher eukaryote. Genomic mapping studies in mouse, human and nematodes have generated sophisticated maps of their respective genomes, but in *Drosophila*,

genomic mapping has almost been completed, and the downstream consequences of genomic organization are beginning to be understood.

A genetic approach to understanding epithelial fluid transport demands a model system for study, methods of genetical intervention and ideally, a repertoire of knowledge of the physiology and genetics of the model system.

The chosen system in this study is the Malpighian tubule of *Drosophila melanogaster.* The approach taken to further understanding of epithelial fluid transport in this model is twofold;

1. to design and implement targetted gene expression systems, and to use recently developed technology to ectopically express a membrane bound receptor.
2. to use more traditional methods of genetic analysis to clone a gene which may be directly involved in water flux in the tissue.

The data presented in this thesis have shown that ectopic gene expression can be targetted to Malpighian tubules of *Drosophila melanogaster,* and that the traditional methods of genetic analysis can be applied to the Malpighian tubule.

The variety and complexity of the work presented here could not be envisaged with any other whole animal model system. On this basis, the Malpighian tubule is a suitable model for studies of epithelial function, and it has been demonstrated that *Drosophila* provides both the genetical resources and technological possibilities to perform these type of sophisticated studies.

The biochemistry, physiology and genetics of Malpighian tubule function in *Drosophila* are beginning to become better understood, and the work presented here advances the possibilities for the complete and integrated understanding of tubule function, and validate the use of the tubule as a model for studies of epithelial function.

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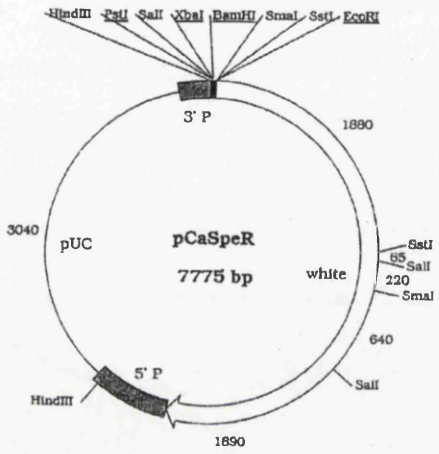
Appendix 1

This appendix contains the maps of the pCaSpeR vectors available. These vectors can be obtained on request from Carl Thummel at the University of Utah, USA.(carl.thummel@genetics.utah.edu), and the maps presented here are reproductions of those provided by his laboratory.

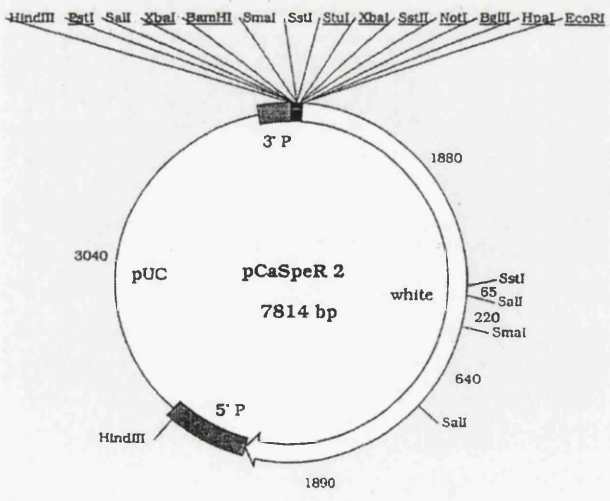
The vector maps listed are, pCaSpeR, pCaSpeR2, pCaSpeR3, pCaSpeR4, pCaSpeR-hs, and pCaSpeR-hs-act.

pCaSpeR



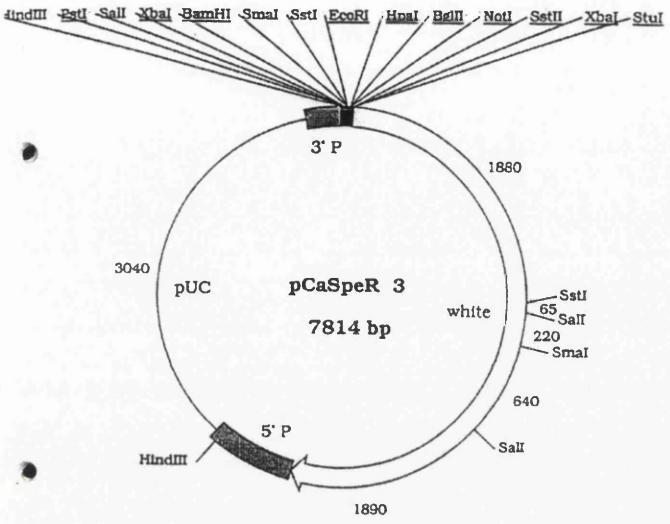
underlined sites in polylinker are unique

pCaSpeR 2



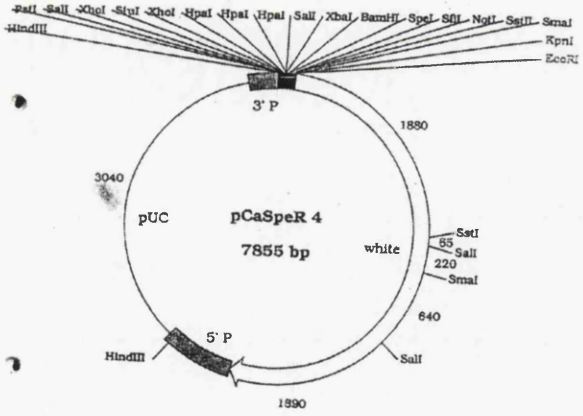
underlined sites in polylinker can be used for cloning

pCaSpeR 3



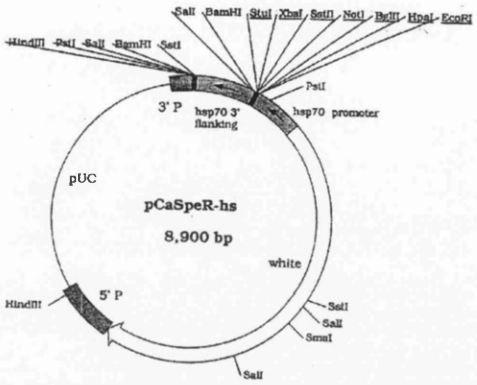
underlined sites in polylinker can be used for cloning

pCaSpeR 4



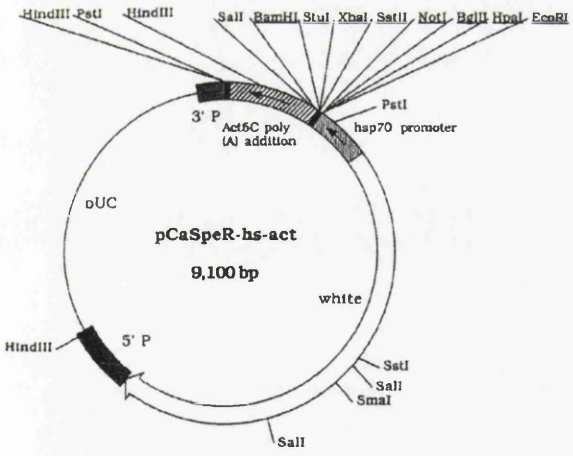
All sites in polylinker except Sma1, Sal1, HindIII can be used for cloning

pCaSpeR-hs



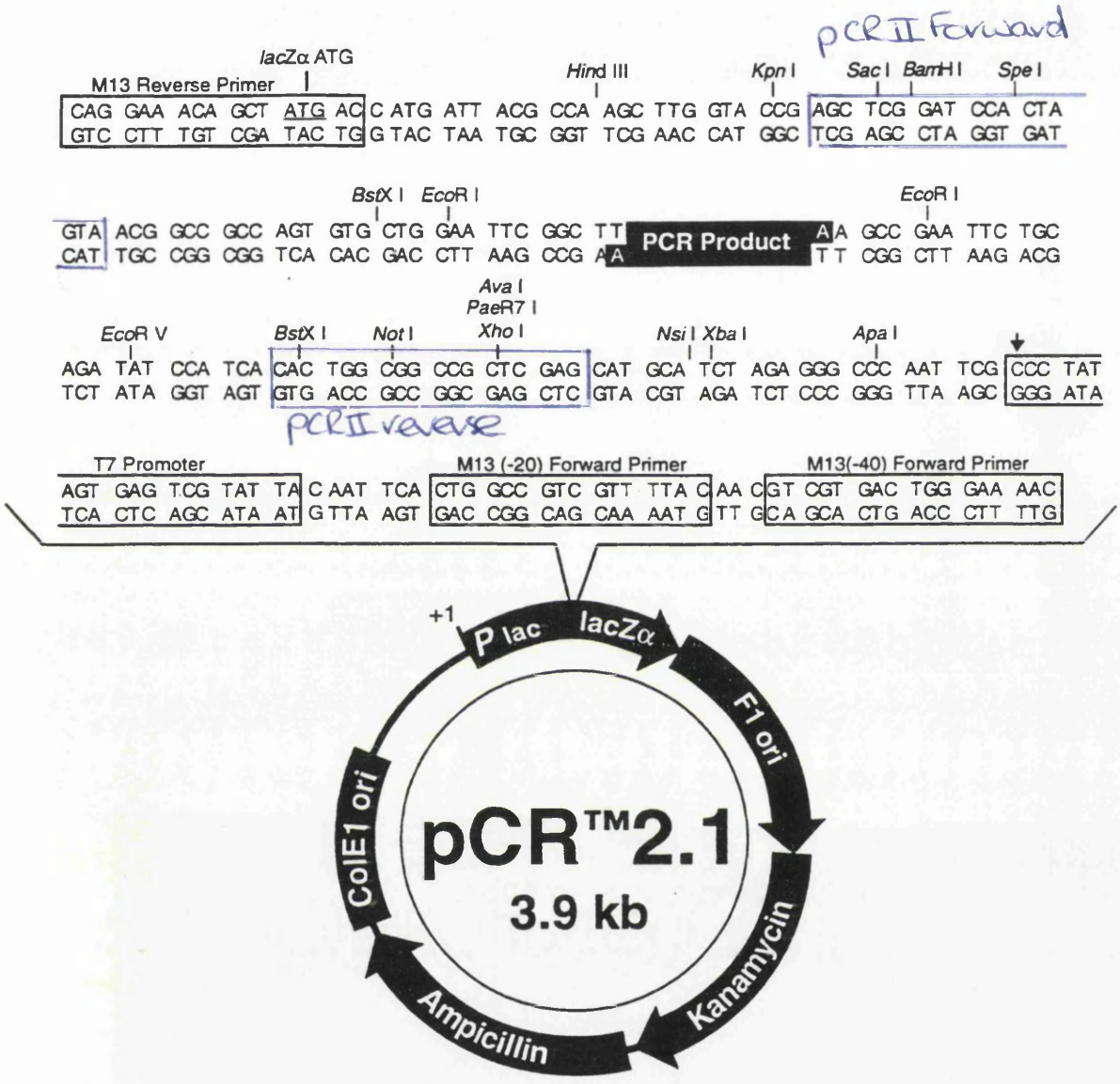
7 unique (underlined) sites in polylinker can be used to i insert an open reading frame for expression

pCaSpeR-hs-act



8 unique and underlined sites can be used for in-frame insertion and expression

Appendix 2



Appendix 2

Detail from instruction manual supplied with TA Cloning Kit (Invitrogen) of the vector pCRII. The insertion site of PCR product is flanked by *EcoR1* sites.

DNA sequences used to design specific oligonucleotides for sequencing across the multiple cloning site are boxed and annotated as pCRII Forward and pCRII Reverse.

UO Right	CGGGATCCTGTGACTGCAACTACTCT
UO Left	GGGGTACCTGCAGTTGCTATG CCA
UOOR	GTTATCCAGGTGTTCTGATA
pUAST seq	GAAATCTGCCAAGAAGT
pCRII forward primer	AGCTCGGATCCACTAGTA
pCRII reverse primer	CTCGAGCGGCCGCCAGTG
5HT1DbFor	AAAGATGCCTGCTGGTTCCAC CT
5HT1DbRev	ATCCTGAGAAGCCAGAATAGT CCT
WCh HetLeft	AGATCTCAAAATGGTCGAGAAACAGAAATG
WCh HetRight	AGATCTGGTATTCGGCCACTGTTTAG

Sequence of novel oligonucleotides used in the generation of the novel data presented in this thesis. Detailed description of the oligonucleotides (as well as their sequences) are given on pages shown: UO Right, UO Left, UOOR, page 55; pUAST seq, page 78; pCRII forward and reverse primers, appendix 2, page 171; 5HT1Db For and Rev, page 84; WCh Het Left and Right, page 125.

Appendix 3

All of the cAMP concentrations presented in chapter 4 are calculated from counts per minute of radiolabelled ^{125}I. With each experiment are a set of controls from which a standard curve can be drawn. Counts per sample standard are expressed as a percentage of the perfect bound (Bo, counts precipitated in a sample lacking cAMP), and plotted against the amount of cAMP in the standard.
Counts derived from assay samples are expressed as a percentage of Bo, and the cAMP value determined by interpolation of the standard curve

A 3.1.1 Standard curve

cAMP fmol/sample	number of counts	number of counts duplicate
2	5435	5070
4	4659	4673
8	3276	3829
16	2636	2728
32	1743	1828
64	1158	1232
128	926	709
perfect bound	5693	5772
total counts	10049	10310

A3.1.2 Counts observed per sample

6 tubules	10 tubules	20 tubules	30 tubules
4052	3252	2129	1490
3821	2946	2076	2250
3685	2770	2025	1850
3784	2812	2592	1813
3125	2326	2495	1524
3323	2274	1824	1586
3337	2231	1999	1623
3090	2550	2138	1388

A3.1.1 and A3.1.2 show raw data used to generate the data shown in Figures 4.4.1a and 4.4.1b on page 89

A3.2a shows the standard curve data for the experiments whose results are shown in A3.2b, A3.2c, A3.2d and A3.2e. The raw counts data shown in A3.2b is used to generate the data shown by Figures 4.4.2a and 4.4.2b on page 91.

A.3.2a Standard curve

cAMP level fmol/sample	number of counts	number of counts duplicate
2	3582	3616
4	3132	2961
8	2495	2400
16	1729	1587
32	1219	1253
64	1005	867
128	577	474
total counts	8655	8996
perfect bound	3984	4281

A3.2b Resting cAMP levels

w^{1118}	unstimulated	UAS-5HT1Db	C324
980	1374	1106	1033
1305	1035	1066	1494
824	1131	1160	1170
1137	983	1088	1110
896	1098	1162	916
738	1011	1235	919

Data shown A3.2c, A3.2d and A3.2e are used to generate the data shown by Figures 4.4.3a/b (page 94), Figures 4.4.4a/b (page 95) and Figures 4.4.5a/b (page 96).

A3.2c stimulation with millimolar 5HT

5 seconds	10 seconds	15 seconds	20 seconds	30 seconds
1152	869	1068	979	768
1046	1003	1040	1087	736
1054	898	1051	888	661
1015	827	1049	951	715
1018	1012	855	792	742
909	775	952	814	848

A3.2d micromolar stimulation with 5HT

5 seconds	10 seconds	15 seconds	20 seconds	30 seconds
1232	1113	832	699	452
631	804	814	744	425
1029	794	924	993	421
994	906	784	961	588
955	1037	996	967	286
	792		950	

A3.2e stimulation with nanomolar 5HT

5 seconds	10 seconds	15 seconds	20 seconds	30 seconds
822	967	855	870	778
860	924	1151	1032	929
1491	965	1385	852	922
949	977	820	878	1105
934	1059	1008	722	954
930	1051	1203	924	1137

The data shown in A3.3a and A3.3b are used to generate the data shown by Figures 4.4.6a/b on page 99.

A3.3a Standard curve

cAMP fmol/sample	number of counts
2	3173
4	2612
8	2094
16	1613
32	1013
64	806
128	504
total counts	8405
perfect bound	3940

A3.3b

unstimulated	10^{-6}	yohimbine	w^{1118}
1247	865	935	694
958	805	841	848
1087	895	1129	826

Appendix 4

Standard fly food recipe

In 1 litre:

10g	Agar
15g	Sucrose
30g	Glucose
35g	Dried yeast
15g	Maize meal
10g	Wheat germ
30g	Treacle
1tablespoon	Soya flour