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A P-glycoprotein homologue in *Manduca sexta*: steps toward cDNA cloning

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Table of Contents

Table of Contents	ii
Abstract	vi
Résumé	viii
Acknowledgements	xi
List of Tables	xii
List of Figures	xiii
List of Abbreviations	xv
Chapter 1: General Introduction	1
A) <i>Manduca sexta</i>, a nicotine resistant insect	1
i) Rapid excretion of unmetabolized nicotine	1
ii) A blood-brain barrier to nicotine	3
a) A physical barrier: the perineurium	4
b) A metabolic barrier: detoxifying enzymes	4
c) A nicotine transport system?	5
B) P-glycoprotein biochemistry	6
i) Structure and Function	8
a) Topology	8
b) ATP-binding sites	8
c) Drug-binding sites	9
ii) Models of P-glycoprotein function	10
iii) Nicotine as a possible substrate for Pgp	11
C) The genetics of P-glycoprotein	13
i) Gene families and nomenclature of P-glycoprotein in different species	13
ii) Structure of the P-glycoprotein genes	16
iii) Gene expression and regulation	19
D) Possible “normal” functions of Pgp	23
i) Transport of hydrophobic cellular products	23
a) Non-MDR P-glycoprotein transports phospholipids	23
b) Evidence for a hormone transport function	25
ii) Protection from xenobiotics	25
a) In aquatic organisms	25
b) In mammals	26
c) In lepidopterans	27
I) <i>Heliothis virescens</i>	27
II) <i>Manduca sexta</i>	29
E) Research Plan	29
Chapter 2: Construction and screening of a cDNA library of <i>Manduca Malpighian tubules</i>	44
Introduction	44
A) Overview of the cloning procedure	44

B) DNA probes	46
i) msspl I	46
ii) vacuolar H ⁺ -ATPase	46
iii) CHP1	47
iv) mdr 49, mdr 65	47
v) DM13, SP01, TU15, TU19	48
Methods	48
A) Rearing and dissection of <i>Manduca sexta</i>	48
B) Construction of a cDNA library from <i>Manduca sexta</i> Malpighian tubule tissue.	49
i) Tissue homogenization and total RNA extraction	49
a) Special buffers	49
b) Other preparations	50
c) Procedure	50
d) Notes	51
ii) mRNA extraction	52
a) Special buffers	52
b) Other preparations	53
c) Procedure	53
d) Notes	54
iii) cDNA synthesis and insertion into λZAP II vector	54
a) First strand cDNA synthesis	55
b) Second strand synthesis	55
c) Blunting the cDNA termini	56
d) Ligation of EcoRI adaptors	57
e) Phosphorylating the EcoRI ends	57
f) XhoI digestion	57
g) Size fractionation of cDNA	58
h) Ligation of cDNA into Uni-ZAP vector arms	58
iv) Packaging of Uni-ZAP vector into phage particles, and plating/titering	59
a) Media and Buffers	59
b) Host bacteria	60
c) Packaging procedure	61
d) Plating and titering	61
v) Amplification of the primary library	62
C) DNA Screening of cDNA library, Round 1	62
i) Phage plating and nytran lifts	62
ii) Probe labeling procedure	64
iii) Probing with CHP1	65
iv) Probing with msspl I	66
iii) Probing with V-ATPase	67
iv) Probing with mdr49, mdr65	67
D) DNA screening of cDNA library, Round 2	68
i) Probing with a DNA probe cocktail: msspl I, DM13, SP01, TU15, TU19	68
ii) Probing with a second cocktail: CHP1A, mdr49, mdr65, DM13, SP01	68
Results	70
A) Construction of a λZAP cDNA library from <i>Manduca</i> Malpighian tubule tissue	70
B) cDNA library screening, Round 1	71
i) Probing with CHP1	71
ii) Probing with msspl I	71
iii) Probing with V-ATPase	71
iv) Probing with mdr49 and mdr65	72
C) cDNA library screening, Round 2	72

i) Probing with DNA probe cocktail: mssp11, DM13, SP01, TU15, TU19	72
ii) Probing with a second probe cocktail: CHP1A, mdr49, mdr65, SP01, DM13	72
Discussion	73
Chapter 3: Troubleshooting the cDNA cloning process	88
Introduction	88
A) Is cross-species DNA hybridization feasible?	88
B) What is the optimal plaque density for cDNA library screening?	88
C) Can the probes detect Pgp genes on Southern blots of genomic DNA?	89
D) Can the probes detect Pgp transcripts on Northern blots of <i>Manduca</i> RNA?	89
E) Is polymerase chain reaction (PCR) feasible as an alternative method of cloning?	89
Methods	90
A) Dot blot cross-hybridization experiments	90
i) Probing with CHP1, mssp11, mdr49, mdr65	90
ii) Probing with mdr49, mdr 65, DM13 and SP01	91
ii) Probing with mssp11	91
iv) Stripping	91
B) Optimizing plaque density using a positive construct, CHP1A-ZAP	92
i) Preparation of the λ ZAP-CHP1A construct	92
ii) Plating and lifts	92
iii) Probing	93
a) Trial 1	93
b) Trial 2	93
c) mssp11	93
C) Southern Blot analyses	94
i) Preparation of Southern Blot	94
ii) Preparation of radiolabeled DNA probes	95
iii) Probing with mssp11 and CHP1	95
a) Standard procedure	95
b) Multiple sandwich procedure	96
iv) Probing with V-ATPase	97
v) Probing with ADP-ATP translocase (AAT) cDNA clone	97
D) Northern Blot analyses	97
i) Northern Blot of total RNA from <i>Manduca</i> Malpighian tubules, mouse brain and mouse liver	97
a) Probing with mssp11 and V-ATPase	98
b) Probing with CHP1 and mdr49/mdr65	99
c) Probing with ubiquitin	99
ii) Northern Blot of mRNA from 4 th and 5 th instar <i>Manduca</i> larvae	99
a) Probing with mssp11	100
I) Standard hybridization	100
II) Multiple sandwich hybridization	101
b) Probing with AAT	101
E) PCR of <i>Manduca</i> genomic DNA with primers to mssp11	101
Results	103
A) Dot blot cross-hybridization experiments	103
i) First series	103
ii) Second series	103
B) Optimizing plaque density using a positive construct, CHP1A-ZAP	104
i) Trial 1	104
ii) Trial 2	105
iii) Probing CHP1A positive control plaques with mssp11	105

C) Southern blot analyses	105
i) Probing with msspl I and CHP I	106
a) Standard hybridization	106
b) Multiple sandwich hybridization procedure	106
ii) Probing with V-ATPase	106
D) Northern blot analyses	107
i) Northern blot of total RNA from <i>Manduca</i> Malpighian tubules, mouse brain and mouse liver	107
a) Probing with msspl I and V-ATPase	107
b) Probing with CHP I and mdr49/mdr65	107
c) Probing with ubiquitin	108
ii) Northern Blot of mRNA from 4 th and 5 th instar <i>Manduca</i> larvae	108
a) Probing with msspl I	108
E) PCR of <i>Manduca</i> genomic DNA with primers to msspl I	109
Discussion	109
A) Dot-blot hybridizations	109
B) Plaque density experiments	111
C) Southern blot analyses	112
D) Northern blot analyses	114
E) PCR as an alternative cloning method	115
Chapter 4: A cDNA clone of the <i>Manduca sexta</i> ADP/ATP translocase	134
Introduction	134
Methods	134
A) Sequencing of ADP/ATP translocase clone	134
B) Southern blot analysis	135
C) Northern blot analysis	135
Results	136
A) Sequencing of AAT clone	136
B) Southern blot analysis	136
C) Northern blot analysis	137
Discussion	137
Chapter 5: General Discussion	144
References	150
Appendix A - Wheat germ based diet for <i>Manduca sexta</i>	163

Abstract

The tobacco hornworm, *Manduca sexta*, is able to feed on the tobacco plant in spite of neurotoxic levels of nicotine that deter other insects. The hornworm's nicotine resistance is due in part to rapid excretion of alkaloids by an active transport mechanism in the Malpighian tubules. Evidence has accumulated that suggests this nicotine transport is mediated by a P-glycoprotein (Pgp) homologue. Pgp is an active transport protein responsible for the increased efflux of anticancer alkaloids seen in mammalian multidrug-resistant (MDR) tumours and cell lines.

One of the proposed natural functions of P-glycoprotein is to provide protection against xenobiotics; this could be accomplished by expression of the efflux pump in excretory and blood-organ barrier tissues to prevent the penetration of foreign toxins to sensitive organs. Involvement of Pgp in the mouse blood-brain barrier to the pesticide ivermectin has been convincingly demonstrated (Schinkel *et al.*, 1994). Demonstrating that a P-glycoprotein homologue is a factor in the nicotine resistance of *Manduca sexta* would provide substantial support for the xenobiotic theory. It would also be the first demonstration of the involvement of Pgp in a natural ecological relationship; thus far Pgp has only been implicated in "unnatural" situations where there is a man-made toxic insult with pollutants, pesticides or cytotoxic drugs.

Ongoing work in our laboratory has shown that alkaloid transport by *Manduca* Malpighian tubules behaves pharmacologically like Pgp drug transport. Corroboration of this physiological work by molecular cloning of the P-glycoprotein homologue from *Manduca* is the long-term goal of this project. As a first step toward this goal, a cDNA

library was prepared from larval *Manduca* Malpighian tubule tissue, in the λ ZAPII vector. This library was screened by DNA plaque hybridization using a variety of Pgp DNA probes, including fragments of Pgp genes and cDNAs from hamster, *Drosophila melanogaster*, *Spodoptera frugiperda* and *Tetranicus urticae*. A PCR product representing a fragment of a putative *Manduca* Pgp homologue was also employed as a DNA probe. None of these probes proved successful in isolating a cDNA clone of *Manduca* Pgp from the library.

Subsequent experiments were intended to identify the problems associated with the cloning project. Southern blot hybridization experiments demonstrated that some of the probes used in the project had very poor sensitivity compared to other non-Pgp probes used as controls. This sensitivity problem was confirmed with plaque density experiments which showed that the hamster Pgp probe CHP1 would only produce strong positive signals when the phage plaques were large. Crowding of the plaques in high density screenings produced smaller plaques (containing less DNA) which were no longer detectable by CHP1. By contrast, one of the non-Pgp control probes (V-ATPase) could detect strong positive signals at a high plaque density. The reason for this difference in sensitivity is unclear, but may be due to the particular radioactive labeling process used in the experiments.

As an offshoot of attempts to clone the *Manduca* Pgp homologue, a partial cDNA clone of the *Manduca* ADP/ATP translocase (AAT), a mitochondrial enzyme involved in oxidative phosphorylation, was isolated from the cDNA library and sequenced. Southern blot analysis indicated that AAT may be represented by a single gene in *Manduca*, in

contrast to other species which have more than one AAT gene. Two major AAT transcripts were detected on a Northern blot of *Manduca* gut tissue.

Résumé

Manduca sexta peut se nourrir sur le tabac, malgré la présence d'une concentration élevée de nicotine, qui repousse d'autres insectes. Cette résistance est partiellement déterminée par un mécanisme d'excrétion de la nicotine dans les tubules de Malpighi. Il est possible que la P-glycoprotéine (Pgp) soit impliquée dans ce transport actif. La Pgp est déjà impliquée dans la résistance pléiotropique rencontrée dans les tumeurs de mammifères.

Nous proposons que la fonction naturelle de la Pgp soit la protection contre les xénotoxines. Ceci pourrait être accompli par l'expression de cette pompe d'efflux au niveau des tissus d'excrétion et des barrières hémato-organiques qui arrêterait la pénétration des toxines étrangères dans les sites sensibles. La participation de la Pgp dans la barrière hémato-céphalique à l'ivermectine chez la souris a été démontrée de manière convainquante (Schinkel *et al.*, 1994). La démonstration que la P-glycoprotéine contribue à la résistance à la nicotine chez *Manduca sexta* pourrait appuyer la théorie de la protection contre les xénotoxines. Ce serait aussi la première démonstration de l'implication de la Pgp dans une association écologique naturelle; jusqu'à maintenant la Pgp a été impliquée seulement dans les situations "non-naturelles", protégeant contre la toxicité derivant de l'activité humaine: les polluants, les pesticides et les drogues cytotoxiques .

Notre laboratoire a déjà démontré que le transport des drogues par les tubules de Malpighi chez *Manduca* présente certaines caractéristiques pharmacologiques communes avec le transport des drogues par la P-glycoprotéine. L'objectif à long terme de ce projet est l'identification d'homologues de la Pgp chez *Manduca* par les méthodes de la biologie moléculaire, afin de confirmer les résultats physiologiques. Tout d'abord, nous avons construit une banque d'ADN complémentaire (ADNc) des tubules de Malpighi de larves de *Manduca*, dans le vecteur λ ZAPII. Cette banque a été criblée avec des sondes homologues de Pgp provenant de diverses espèces, notamment du hamster, de *Drosophila melanogaster*, de *Spodoptera frugiperda*, et de *Tetranicus urticae*. Un produit d'une réaction en chaîne de la polymérase (PCR) qui représente une petite région d'un homologue de la Pgp de *Manduca* a été aussi utilisé comme sonde. Aucune de ces sondes n'a pu identifier de clone de ADNc de la banque.

Pour cerner les problèmes associés à ce projet de clonage, d'autres expériences ont été entreprises. Un transfert de type Southern a démontré que les sondes utilisées dans ce projet étaient peu sensibles lorsque comparées aux contrôles. Ce problème de sensibilité a pu être confirmé grâce à un dépistage des plaques de densité différente. La sonde Pgp de hamster, CHP1, n'émet de signaux suffisamment fort que lorsque les plaques de phage sont assez grandes. Lorsque la densité des plaques est forte, les plaques produites sont plus petites, contiennent moins d'ADN et ne sont plus détectables par CHP1. Par contre, l'une des sondes contrôles non-Pgp, V-ATPase, a permis d'identifier un fort signal sur les plaques de hautes densités. La cause de cette différence de

sensibilité n'est pas claire, mais pourrait être due à la méthode de marquage radioactive utilisée ici dans l'expérience.

Nous avons isolé un clone partiel de ADNc de la translocase ADP/ATP (AAT) de *Manduca*, un enzyme mitochondrial impliqué dans la phosphorylation oxidative, à partir de la banque de ADNc. Les résultats d'un transfert de type Southern suggèrent que l'AAT n'est représenté chez *Manduca* que par un seul gène, alors que chez d'autres espèces on retrouve plutôt une famille de deux ou trois gènes. Deux transcriptions principales de l'AAT ont pu être mises en évidence dans les tissus viscéraux de *Manduca*, grâce à un transfert de type Northern.

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"The weariest nights, the longest days, sooner or later must perforce come to an end."

-Baroness Orczy, *The Scarlet Pimpernel*

List of Tables

Table 1.1: Classification of the known mammalian P-glycoprotein genes	33
Table 1.2: Tissue expression of P-glycoprotein-like genes in a variety of organisms assayed by various methods	34
Table 1.3: Results of a search of the Entrez nucleotide database for 9 organisms	33
Table 2.1: Probe labeling and radioactivity data for CHP1	75
Table 2.2: Probe labeling and radioactivity data for mssp11	75
Table 2.3: Probe labeling and radioactivity data for V-ATPase	75
Table 2.4: Probe labeling and radioactivity data for mdr49 and mdr65	76
Table 2.5: Probe labeling and radioactivity data for mssp11, DM13, SP01, TU19, TU15	76
Table 2.6: Probe labeling and radioactivity data for CHP1A, mdr49, mdr65, DM13, SP01	76
Table 2.7: Probe labeling and radioactivity data for CHP1A, mdr49, mdr65, DM13, SP01 (secondary screening)	77
Table 3.1: Probe labeling and radioactivity data for mssp11, CHP1, mdr49 and mdr65	117
Table 3.2: Probe labeling and radioactivity data for DM13, SP01, mdr49, mdr65	117
Table 3.3: Probe labeling and radioactivity data for mssp11	117
Table 3.4: Results of dot blot experiments	118
Table 3.5: Probe labeling and radioactivity data for CHP1A (Trial 1)	118
Table 3.6: Probe labeling and radioactivity data for CHP1A (Trial 2)	118
Table 3.7: Probe labeling and radioactivity data for mssp11 and CHP1 (standard hybridization)	119
Table 3.8: Probe labeling and radioactivity data for mssp11 and CHP1 (sandwich hybridization)	119
Table 3.9: Probe labeling and radioactivity data for V-ATPase	119
Table 3.10: Probe labeling and radioactivity data for mssp11 and V-ATPase	120
Table 3.11: Probe labeling and radioactivity data for mdr49 and 65 (combined labeling) and CHP1	120
Table 3.12: Probe labeling and radioactivity data for <i>Manduca</i> ubiquitin	120
Table 3.13: Probe labeling and radioactivity data for mssp11	121
Table 4.1: Probe labeling and radioactivity data for AAT	139

List of Figures

Figure 1.1: Protonation reaction for nicotine	35
Figure 1.2: Predicted topology of P-glycoprotein in the cell membrane	36
Figure 1.3: Structures of Pgp substrates vinblastine, colchicine and actinomycin D	37
Figure 1.4: Structures of nicotine, morphine and the synthetic opiate meperidine	38
Figure 1.5: A tree diagram of the evolutionary relationships of members of the P-glycoprotein family	39
Figure 1.6: Conservation of sequence across species and between gene family members in P-glycoprotein	40
Figure 1.7: Conservation of sequence and intron position in the two homologous halves of the human <i>MDR1</i> gene	41
Figure 1.8: Comparison of intron positions in human, <i>C. elegans</i> and <i>Arabidopsis thaliana</i> Pgp genes	42
Figure 2.1: Flow chart illustrating the cDNA library construction and screening procedures	78
Figure 2.2: Complete sequence of mssp11	79
Figure 2.3: Comparison of the predicted amino acid sequence of mssp11 with that of <i>D. melanogaster mdr49</i> using the BLASTX search algorithm	80
Figure 2.4: Cartoon depicting the positions of the DNA probes relative to a generic P-glycoprotein cDNA sequence	81
Figure 2.5: Flow chart of the procedure for producing directional <i>XhoI/EcoRI</i> -linked cDNA from mRNA	82
Figure 2.6: Size analysis of first and second strand cDNA reactions	83
Figure 2.7: Size distribution of 35 clones isolated from the <i>Manduca</i> Malpighian tubule library	84
Figure 2.8: Autoradiograph of a nytran lift of the cDNA library probed with V-ATPase	85
Figure 2.9: Autoradiograph of a nytran lift of the cDNA library probed with CHP1A, mdr49, mdr65, DM13 and SP01	86
Figure 2.10: Gel analysis of insert sizes in 12 clones picked from secondary screening	87
Figure 3.1: Preparation of CHP1A- λ ZAPII construct	122
Figure 3.2: Placement of PCR oligonucleotide primers P1 and P2 in the sequence of mssp11	123
Figure 3.3: Phosphoimager record of the first dot blot hybridization experiment	124
Figure 3.4: Autoradiographs of second series of dot blots	125
Figure 3.5: Phosphoimager record (enhanced) of first plaque density experiment	127
Figure 3.6: Autoradiograph of second plaque density experiment	128
Figure 3.7: Photograph of 0.8% agarose gel prior to Southern transfer	129
Figure 3.8: Southern blot of <i>Manduca</i> and mouse genomic DNA probed with CHP1	130

Figure 3.9: Southern blot of <i>Manduca</i> genomic DNA probed with <i>Manduca</i> V-ATPase probe	131
Figure 3.10: Autoradiographs of Northern blot of total RNA from <i>Manduca</i> Malpighian tubules, mouse brain and mouse liver	132
Figure 3.11: PCR of <i>Manduca</i> genomic DNA with primers specific to msspl I	133
Figure 4.1: Complete sequence of a partial cDNA of ADP/ATP translocase from <i>Manduca</i> Malpighian tubules	140
Figure 4.2: Cartoon comparing the <i>Manduca</i> AAT cDNA clone to the <i>Drosophila</i> cDNA	141
Figure 4.3: Southern blot of <i>Manduca</i> genomic DNA probed with a <i>Manduca</i> AAT probe	142
Figure 4.4: Northern blot of mRNA from gut tissue of 4th and 5th instar <i>Manduca</i> larvae probed with <i>Manduca</i> AAT	143

List of Abbreviations

ABC	ATP-binding cassette
Ach	acetylcholine
(d)ATP	(deoxy)adenosine triphosphate
BSA	bovine serum albumin
cDNA	complementary DNA
CFTR	cystic fibrosis transmembrane conductance regulator
CI	chloroform : isoamyl alcohol (24:1)
CNS	central nervous system
(d)CTP	(deoxy)cytidine triphosphate
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
(d)GTP	(deoxy)guanosine triphosphate
GuSCN	guanidinium thiocyanate
MDR	multiple drug resistance/resistant
M-MuLVRT	Moloney-murine leukaemia virus reverse transcriptase
mRNA	messenger RNA
MXR	multiple xenobiotic resistance
PCI	phenol : chloroform : isoamyl alcohol (25:24:1)
PCR	polymerase chain reaction
pfu	plaque-forming unit
Pgp	P-glycoprotein
PSMO	polysubstrate monooxygenases
RNA	ribonucleic acid
SDS	sodium dodecylsulfate
SER	smooth endoplasmic reticulum
TAP	antigen transport protein
Tris	tris[hydroxymethyl]aminomethane
(d)TTP	(deoxy)thymidine triphosphate
UV	ultraviolet
V-ATPase	vacuolar H ⁺ -ATPase

Chapter 1: General Introduction

Higher plants practice a sophisticated form of chemical warfare to avoid being consumed by herbivorous insects (Ehrlich and Raven, 1964). They produce secondary metabolites (or allelochemicals) which are toxic, and therefore deter feeding by these insects. In response, many insects have evolved a variety of mechanisms of overcoming these chemical defenses.

A) *Manduca sexta*, a nicotine resistant insect

Manduca sexta L. (Lepidoptera: sphingidae), the tobacco hornworm, is an example of such an insect. The larval form of this lepidopteran feeds on the leaves of the tobacco plant, *Nicotiana tabacum*, as well as other solanaceous plants. *Manduca sexta* thrives on tobacco despite high levels of nicotine, a neurotoxic alkaloid that can be up to 8% of the plant's dry weight (*The Merck Index*, 1989). Nicotine acts on the acetylcholine (Ach) receptors in the insect central nervous system (CNS); at low doses it stimulates neurons post-synaptically, while at very high concentrations it can block synaptic transmission (Michaelson and Zeimal, 1973; Jones *et al.*, 1978, as cited in Morris, 1984). Investigation of this phenomenon of nicotine tolerance has demonstrated that it consists of a number of physiological and biochemical mechanisms.

i) Rapid excretion of unmetabolized nicotine

Self *et al.* (1964a) first demonstrated that the tobacco hornworm rapidly excretes most of the nicotine it consumes. *Manduca* larvae fed ¹⁴C-nicotine-treated tomato leaves passed 90% of the label into the faeces four hours after ingestion. After twenty-four hours all traces of label had been eliminated from larvae. Hornworms also excreted

injected nicotine; after 2 hours blood-nicotine was reduced from 1.5 mM to 0.3 mM, and no nicotine was found in the blood (also called hemolymph) after 8 hours. Chromatographic analysis indicated that there was little, if any, metabolism of the excreted nicotine. Two other tobacco-feeding lepidopterans, the tobacco budworm (*Heliothis virescens*) and the cabbage looper (*Trichoplusia ni*) also excrete nicotine unmetabolized, but two non-lepidopteran tobacco pests do metabolize nicotine, primarily to the 5'-oxo derivative, cotinine (Self *et al.*, 1964a). Non-tobacco-feeding insects, as well as human smokers, also metabolize nicotine extensively (Self *et al.*, 1964b; Benowitz, 1987).

Maddrell and Gardiner (1976) demonstrated transport of nicotine by isolated Malpighian tubules (the renal organs in insects) from larval *Manduca sexta*. Fluid collected from the lumen of the tubules contained nicotine at fourteen times the concentration in the bathing medium. The rate of appearance of nicotine in the luminal secretions was determined to be 3.3 nanomoles per minute. This nicotine transport could be demonstrated regardless of whether the diet of the larvae was nicotine-laden or nicotine-free. The tubules of the adult *Manduca*, by contrast, did not transport nicotine at a rate higher than that expected by passive diffusion.

Unexpectedly, Maddrell and Gardiner (1976) also showed that nicotine and other alkaloids were also transported by the Malpighian tubules of the blood-sucking insect *Rhodnius prolixus*. This nicotine transport had saturation kinetics, with a maximal rate of around 700 picomoles per minute, and half-saturation at 2-3 mM nicotine in the bathing solution. *Rhodnius* tubules also transported the alkaloids atropine and morphine. All three alkaloids were transported in an unmetabolized form. Competition between these

alkaloids was also demonstrated; atropine suppressed transport of nicotine and morphine, nicotine suppressed transport of morphine but not atropine, and morphine did not suppress transport of atropine or nicotine. This competition suggests a common mechanism for transport of these alkaloids in *Rhodnius* tubules. Suppression of nicotine transport by atropine has since been shown in *Manduca* tubules as well (Murray, 1996).

ii) A blood-brain barrier to nicotine

Rapid excretion alone cannot explain *Manduca*'s resistance to the effects of nicotine. Despite this excretion, hemolymph levels of nicotine in continuously feeding larvae are around 23 μM (Self *et al.*, 1964a). In the injection experiments discussed above, even substantially higher blood-nicotine levels produced only temporary effects on feeding behaviour (Self *et al.*, 1964a). These levels of nicotine produce massive neuroactivation in the CNS of the adult cockroach, *Periplaneta americana* (Morris, 1984). Clearly the CNS of *Manduca* must be protected by other, inherent mechanisms. Self *et al.* (1964a) suggest that the low pH of the *Manduca* hemolymph (pH 6.6) may be a factor. Nicotine is an amphipathic molecule (Fig. 1.1), and only 2% would be in the lipid-soluble free base form at this pH. However, *Manduca* larvae feed almost continuously from the moment the eggs hatch to the beginning of pupation, a period lasting several weeks. This is plenty of time for nicotine to equilibrate between the CNS and hemolymph compartments. In addition, uptake studies show nicotine penetrates the nerve cords of *Manduca* and the nicotine-sensitive cockroach at approximately equal rates (Morris, 1983a).

a) A physical barrier: the perineurium

Physically separating the CNS (also called the *neuropile*) from the hemolymph is a cortical layer of cells known as the *perineurium*. This layer is composed of interdigitated epithelial-like cells, joined at the base by tight junctions, and is effective at preventing small ionic solutes from invading the extracellular space of the CNS (Morris and Harrison, 1984). This barrier is necessary because the hemolymph has an ionic composition that is hostile to the normal function of nervous tissue (i.e., high in potassium, low in sodium). As previously mentioned, 98% of nicotine is in the cationic hydrophilic form at hemolymph pH. This charged nicotine can breach the perineurium only by diffusion along the tortuous extracellular path created by the interdigitations of the cortical layer cells. This long winding circuit for charged nicotine increases the chances that molecules of nicotine will deprotonate and diffuse laterally into the cortical cells before arrival at the tight junctions. The innermost layer of the perineurium is composed of relatively few cells. Consequently, there are few cell-cell junctions through which nicotine might gain access to the extra-cellular space of the CNS (Morris and Harrison, 1984). These physical structures collude to ensure that the predominant path of nicotine penetration is transcellular rather than extracellular.

b) A metabolic barrier: detoxifying enzymes

Nicotine diffusing into the cells of the perineurium is subjected to a second line of defense: metabolic enzymes, which have a major protective effect in the *Manduca* CNS. After a 30 minute incubation of isolated nerve cords in 100 μM ^{14}C -nicotine, 85% of the radioactivity is recovered as metabolites of nicotine. In comparison, only 43% is

recovered as metabolites with cockroach nerve cords (Morris, 1983a; Murray *et al.*, 1994). Polysubstrate monooxygenases (PSMOs, also known as mixed-function oxidases and cytochrome P450 oxidases) initiate this metabolism in insects and most other animals (Hodgson, 1985, as cited in Murray *et al.*, 1994). These detoxifying enzymes are intracellular and associated with the smooth endoplasmic reticulum (SER). SER is abundant in cells of the perineurium of the *Manduca* CNS (Morris and Harrison, 1984) suggesting that these cells form a metabolic barrier that prevents penetration of nicotine to the central neuropile. This scenario is confirmed by autoradiography; a barrier to radioactive nicotine co-localizing with the perineurium was abolished by treatment with piperonyl butoxide, an inhibitor of PSMOs (Murray *et al.*, 1994).

c) A nicotine transport system?

The physical and biochemical protective mechanisms mentioned above cannot fully explain the insensitivity of the *Manduca* CNS to nicotine. The PSMO enzyme system is saturable. For a 30 min incubation of a nerve cord in 1 mM nicotine, 68% is recovered unmetabolized (Morris, 1983a). This level of nicotine (0.7 mM) is pharmacologically catastrophic, as judged by its effects on the cockroach nerve cord; yet 1 mM nicotine has only minor electrophysiological effects on the *Manduca* CNS (Morris, 1984). A reduced population of nicotinic acetylcholine receptors might provide this sort of protective effect, but this hypothesis seems unlikely given that the nicotinic agonist lobeline (which in vertebrates has a lower potency than nicotine) stimulates intact *Manduca* nerve cords at 10 μ M, and that experimentally breaching the *Manduca* blood-brain barrier enables 50 μ M nicotine to be neurotoxic (Morris, 1984).

Physiological data on the efflux of nicotine and its metabolites from insect CNS tissue demonstrates that *Manduca* has a simpler efflux pattern than the cockroach, *Periplaneta americana* (Morris, 1984b). Whereas three exponential components were resolved for the cockroach, only two were resolved for *Manduca*. This simpler efflux pattern is not the result of species-specific tissue geometry; both species display remarkably similar tripartite efflux patterns for the amino acid leucine (Morris, 1983c). The differences, then, would seem to reflect differences in the way each species deals physiologically with nicotine. Both atropine and unlabeled nicotine alter the kinetics of the efflux patterns in *Manduca* CNS (Morris, 1983b), suggesting that a nicotine transport system paralleling the phenomenon seen in *Manduca* Malpighian tubules (Madrell and Gardiner, 1976; Murray, 1996) may be a contributing factor.

In recent years, evidence has accumulated which suggests a membrane-bound active transport protein with broad substrate specificity is implicated in the nicotine transport system in both the Malpighian tubules and the CNS of *Manduca*. This protein is known as P-glycoprotein.

B) P-glycoprotein biochemistry

In the mid-1980's, P-glycoprotein (Pgp) was revealed to be the molecular culprit in a pernicious problem encountered in cancer chemotherapy: multiple drug resistance (MDR). MDR is evident when a tumour being treated with an anticancer drug becomes resistant not only to the drug used in the therapy, but to a wide range of other drugs that are not similar in structure or function. MDR thus severely curtails the therapeutic options for a patient. The list of chemotherapeutic drugs rendered ineffective by MDR includes vinblastine, daunomycin, and actinomycin D (Juranka *et al.*, 1989). Not only

are these drugs structurally dissimilar, but they have different intracellular targets. Traits that the drugs have in common are that they are large, hydrophobic, aromatic, nitrogenous bases, and they are isolated from natural products. Because of the obvious clinical relevance of the phenomenon, MDR and Pgp have been intensively studied in the last 10 to 15 years.

Laboratory studies of the MDR phenomenon were facilitated by the development of cell lines displaying the MDR phenotype (Gottesman and Pastan, 1988). Kinetic experiments with these cells indicated that an energy-dependent efflux mechanism maintains intracellular concentrations of the drugs at non-toxic levels. Molecular analysis indicated that these cells were consistently overexpressing a 170-kD glycosylated protein in the plasma membrane (Endicott and Ling, 1989). This protein was dubbed P-glycoprotein (P for its perceived ability to alter the *permeability* of drugs). The expression of this integral protein correlated positively with degree of drug resistance, and negatively with intracellular drug accumulation (Endicott and Ling, 1989). Transfection and expression of the Pgp cDNA were shown to be sufficient to cause drug sensitive cells to become drug resistant (Gros *et al.*, 1986). Genetic analysis of human (Roninson *et al.*, 1986) and hamster (Roninson *et al.*, 1984) resistant cell lines showed that MDR was associated with amplification of one or more genes. The amplified fragments were shown to contain genes that coded for P-glycoprotein (Riordan *et al.*, 1985; Chen *et al.*, 1986).

i) Structure and Function

a) Topology

P-glycoprotein is a tandemly duplicated integral membrane protein of approximately 1300 amino acids. Each half of the molecule contains a large hydrophobic region with six membrane-spanning α -helices predicted by hydropathy analysis. In addition, each half has a hydrophilic cytoplasmic domain containing a highly conserved ATP-binding site (Figure 1.2; Juranka *et al.*, 1989). The predicted topology shown in Figure 1.2 is supported by antibody binding studies: one placing the ATP-binding domains in the cytoplasm (Kartner *et al.*, 1985), and another confirming that the loops between helices 1 and 2, and 7 and 8, are extracellular (Georges *et al.*, 1993). Loo and Clarke (1995), using site-directed mutagenesis, confirmed the orientation of the third, fourth and sixth predicted extracellular loops and the first, second, fourth and fifth predicted intracellular loops.

b) ATP-binding sites

This basic structure is common to many other membrane transport proteins, including the cystic fibrosis transmembrane conductance regulator (CFTR; Riordan *et al.*, 1989) and antigen transport proteins of the major histocompatibility complex (TAP; Hill and Ploegh, 1995). These proteins form a large superfamily known as the ABC transporters (for ATP-Binding Cassette; Higgins, 1992). The ATP-binding domains contain the Walker motif: two short sequences (denoted the A and B sites; Figure 1.2) which are common to the nucleotide-binding regions of many completely different ATP-requiring enzymes (Walker *et al.*, 1982). It is thought that a positively-charged lysine

residue in the A site interacts with the negatively-charged phosphate group of ATP, while the B site provides a hydrophobic pocket for the adenine moiety (Beaudet and Gros, 1995).

Binding of ATP analogs to Pgp has been demonstrated (Cornwell *et al.*, 1987), and the ATPase activity of Pgp has been characterized (Doige *et al.*, 1992, and other studies cited in Shapiro and Ling, 1994). Drug transport is dependent on the hydrolysis of ATP; non-hydrolyzable ATP analogs can block drug binding (Endicott and Ling, 1989). Discrete mutations introduced into the A site of either the N-terminal or C-terminal ATP-binding domains abolish the multidrug resistance phenotype (Azzaria *et al.*, 1989). This suggests that both nucleotide binding sites are required for drug transport. Chimeric experiments interchanging homologous segments of the nucleotide binding domains between the two halves of the molecule indicate that most of these segments can be switched with no effect. However, switching certain segments near the Walker B site results in a dramatically altered pattern of drug resistance (Beaudet and Gros, 1995).

c) Drug-binding sites

Changes in the pattern of drug resistance are also seen in experiments attempting to define the substrate-binding regions of the protein. For example, a serine-to-phenylalanine mutation introduced into the eleventh transmembrane helix of a mouse P-glycoprotein resulted in the loss of the ability to confer resistance to adriamycin and colchicine, but retention of the ability to protect against vinblastine (Gros *et al.*, 1991). The decreases in drug transport seen in the mutant result from a decrease in initial drug binding to the protein (Kajiji *et al.*, 1993).

A spontaneous mutation discovered in a human MDR cell line modifies drug resistance in a different manner. The valine-to-glycine mutation in the first intracellular loop (position 185 in Figure 1.2) results in an increase in resistance to colchicine, VP16, and adriamycin and a decrease in resistance to vinblastine, vincristine and actinomycin D, compared to the wild-type protein (Choi *et al.*, 1988). This same mutation also affects the potency of Pgp modulators: agents known to reverse multidrug resistance, such as verapamil (Cardarelli *et al.*, 1995). Some other regions that appear to be involved in substrate recognition include transmembrane segments 6 and 12, and the extracellular loop between transmembrane segments 11 and 12 (Loo and Clarke, 1993; Devine *et al.*, 1992; Zhang *et al.*, 1995). Direct binding of the MDR reversing agent azidopine to two sites, one in the amino-terminal half, and one in the carboxy-terminal half, suggests that the two halves of the molecule may come together to form a single binding site (Bruggemann *et al.*, 1992). However, it is likely that Pgp has more than one, and possibly several, binding sites for drugs.

ii) Models of P-glycoprotein function

Studies of substrate binding have produced varied results, which is not surprising considering the very broad substrate specificity of P-glycoprotein. Indeed, the term "specificity" seems almost inappropriate in the discussion of Pgp: consider the structures of three drugs it is known to transport (Figure 1.3). Studies of the interactions of Pgp with chemically altered variants of colchicine (Tang-Wai *et al.*, 1993), and the MDR inhibitor reserpine (Pearce *et al.*, 1989) indicate that the relative disposition of the basic nitrogen atom and the aromatic rings is an important consideration. The unique characteristics of P-glycoprotein-mediated drug transport have led Higgins and

Gottesman (1992) to propose that Pgp may function in a manner quite different from most transporters. They suggest that Pgp acts as a "flippase", interacting with drugs intercalated with the phospholipid membrane so as to "flip" the drug from the inner to the outer leaflet of the lipid bilayer. This would have the net effect of reducing the concentration of drug in the cell. Others suggest that aromatic amino acids in the transmembrane regions associate to form a hydrophobic core through which the drug can travel (Pawagi *et al.*, 1994). Another theory of drug efflux is a "proton trap" model in which a pool of protons is generated in an aqueous core in Pgp. Since the drugs tend to be amphipathic nitrogenous bases, molecules in their lipophilic form coming into contact with Pgp would be protonated and thus "trapped" in the protein. The protein could then release the drug into the extracellular space (Gottesman *et al.*, 1994).

Thus far, the most convincing evidence is that which supports the "flippase" model. Ruetz and Gros (1994) have demonstrated that this model applies to mouse *mdr2*, a member of the P-glycoprotein family which does not cause multi-drug resistance, but acts as a phosphatidylcholine transporter in the liver. As well, van Helvoort *et al.* (1996) have shown that mouse *mdr1a* and human MDR1 (see discussion of nomenclature below) also function as lipid transporters of the "flippase" type, which suggests the model may hold for the drug transport function as well (see sub-section **D-i-a**).

iii) Nicotine as a possible substrate for Pgp

As a possible substrate for Pgp, nicotine satisfies two important criteria: it has an aromatic/hydrophobic structure, and it has a basic nitrogen group that ascribes amphipathic properties (Figure 1.4; only the nitrogen of the pyridine moiety will protonate at physiological pH, *The Merck Index*, 1989). However, it is a small molecule

compared to most of the Pgp substrates identified so far. It has been suggested that two aromatic rings are necessary for interaction with Pgp (Tang-Wai *et al.*, 1993). This is countered by Callighan and Riordan (1993), who report that the synthetic opiate meperidine (Figure 1.4; note the single aromatic ring *and* the structural similarity to nicotine) is able to inhibit extrusion of vinblastine in drug-resistant cells. This study also reports the important finding that morphine (Figure 1.4) binds to mammalian Pgp with a greater affinity than does the known Pgp substrate daunomycin. Given that nicotine and morphine appear to be transported by a common mechanism in insect Malpighian tubules (Maddrell and Gardiner, 1976), this suggests nicotine may be a Pgp substrate as well. It has been shown recently that nicotine does stimulate the ATPase activity of purified mammalian Pgp preparations at concentrations in excess of 500 μ M (H. McDiarmid and F. Sharom, preliminary observations).

Thus far, there has been little study of nicotine as a possible substrate for the multidrug transporter. This is not entirely surprising considering that most of the substrates studied to date have been anticancer drugs and other cytotoxic agents which act intracellularly. Nicotine is not cytotoxic; it acts at the cell surface as an agonist of acetylcholine receptors in nerve cells. If Pgp is regarded primarily as a defensive mechanism for the expressing cell, then extrusion of surface-acting compounds would seem to be counterproductive. However, there are other ways in which Pgp might function. If it is indeed acting in the Malpighian tubules of insects, it could be expressed at the luminal surface. Nicotine in the hemolymph would diffuse passively through the basal membrane and then be actively transported into the lumen of the tubule. In a

similar manner, Pgp expressed in the apical membrane of the epithelial cells of the perineurium would act to clear nicotine from the extracellular space contiguous to the neural surface. Pgp has recently been shown to protect against a blood-borne neurotoxin at the mammalian blood-brain barrier (Schinkel *et al.*, 1994)

C) The genetics of P-glycoprotein

i) Gene families and nomenclature of P-glycoprotein in different species

The P-glycoproteins are a small multi-gene family, consisting of two genes in humans, three genes in rodents, and various multiples in other organisms. The most comprehensive work on P-glycoprotein genetics has been done on the human and rodent genes. The two human genes are most commonly referred to as *MDR1* and *MDR3*. Only the former confers the multidrug-resistance phenotype; the function of the latter gene was unknown until recently, when it was shown to function as a phosphatidylcholine translocase (van Helvoort *et al.*, 1996). Unfortunately, the naming of these genes has been inconsistent and often results in confusion. The rodents have two MDR-conferring genes and a phospholipid transporter, respectively named *pgp1*, *pgp2* and *pgp3* in hamster, and *mdr1a*, *mdr1b* and *mdr2* in mouse and rat. To make matters worse, the original nomenclature of the mouse genes, *mdr3*, *mdr1* and *mdr2* is still in use by some researchers, and the human phospholipid transporter is sometimes referred to as *MDR2*. A simplified nomenclature used by Ling and colleagues (Childs and Ling, 1996) referring to class I, II and III P-glycoprotein genes is an adequate compromise for the purposes of this discussion (Table 1.1; see end of Chapter 1).

The class I and II P-glycoproteins both exhibit drug transporting ability and share greater sequence similarity with each other than either shares with the class III gene. It is

likely that these two classes arose in rodents from a gene duplication of a common precursor. Cells individually expressing class I or class II genes have been shown to have distinguishable patterns of cross-resistance to drugs. For example, Tang-Wai *et al.* (1995) show that cells expressing mouse class II Pgp are only slightly resistant to actinomycin D (4 times more resistant than drug-sensitive control cells) while cells expressing the class I Pgp are highly resistant to actinomycin D (>100 times more resistant than drug-sensitive control cells). Similarly, differences can also be discerned in the sensitivity of the cells to Pgp modulators.

In other mammalian lineages, the size of the gene family can vary. Cows are predicted by DNA restriction analysis to have three genes while pigs (from the same order Artiodactyla) have five. Monkeys are predicted to have two genes, as are rabbits; dogs are predicted to have four (Ling *et al.*, 1992 as cited in Childs and Ling, 1996). Childs and Ling (1996) show that four of the five pig genes are variations of the class I genes, with no class II equivalent (Table 1.1). It may be that, among mammals, there is a minimum of two P-glycoprotein genes, one class III phospholipid transporter and at least one of the class I or II types of drug transporters.

Beyond Class Mammalia, the classification system loses its usefulness. The cDNA clone from *Xenopus laevis*, for example, does not exhibit significantly greater homology to one of the mammalian isoforms over the other (Castillo *et al.*, 1995; unfortunately, this group reports no Southern blot data which would predict the size of the gene family in *Xenopus*). A dendrogram from Castillo *et al.* (1995) shows that the divergence of the class III and class I/II genes appears to have happened after the divergence of mammals and amphibians (Figure 1.5). Chan *et al.* (1992) report the

isolation of two genomic Pgp clones from winter flounder and note that these two genes also defy classification according to the mammalian model.

Regardless of the origins of the gene families, it is clear that the P-glycoprotein gene is quite ancient. Homologues of the gene have been found in all organisms studied, including yeast (McGrath and Varshavsky, 1989) and plants (*Arabidopsis thaliana*, Dudler and Hertig, 1992; maize, M. Ell, unpublished). Even prokaryotes have single-domain ABC-type transporters which display striking similarity to Pgp; in fact, homology between Pgp and an *Eschericia coli* haemolysin transport protein was what first suggested the drug transport model for P-glycoprotein (Gerlach *et al.*, 1986). The protozoan parasites *Leishmania* spp. and *Plasmodium falciparum* also have homologues (Ouellette *et al.*, 1990; Henderson *et al.*, 1992., Wilson *et al.*, 1989), as does the nematode *Caenorhabditis elegans* (Lincke *et al.*, 1992). Multiple genes have been found in many of these organisms, however the physiological significance of this is not yet clear. Broeks *et al.* (1995) have shown that *C. elegans* deletion mutants missing the *pgp3* gene become sensitive to colchicine and chloroquine, whereas those missing the *pgp1* gene do not. However, it is unclear whether this is due to a difference in gene function, or the fact that the *pgp3* gene is more highly expressed in the wildtype organism.

The only insect P-glycoproteins that have been cloned are from *Drosophila melanogaster*. These genes are designated *mdr49*, *mdr65* (Wu *et al.*, 1991) and *mdr50* (Gerrard *et al.*, 1993) based on their chromosomal location. It is not known how many Pgp gene family members exist, and no physiological role for these genes in *D. melanogaster* has been demonstrated. Although a fly strain with a partial deletion of *mdr49* showed a sensitivity to the Pgp substrate colchicine, curiously, a second strain that

had a full deletion of the gene did not show colchicine sensitivity (Wu *et al.*, 1991). Given the powerful genetic tools available to researchers working on *Drosophila*, it is somewhat surprising that there has been almost no work reported on these genes in the 4-6 years since the Wu and Gerrard papers.

ii) Structure of the P-glycoprotein genes

The P-glycoprotein genes are very highly conserved in their coding sequence, both across species and among members of the gene family (Figure 1.6; Lincke *et al.*, 1992). Conserved regions of sequence generally indicate functionally important domains of a gene. In P-glycoprotein, the most highly conserved regions are the Walker motifs of the nucleotide binding sites (regions 1a/b, 2a/b in Figure 1.6). The transmembrane domains are also fairly well conserved across species, while the least conserved region is the linker sequence between the two homologous halves of the protein.

The complete exon/intron structures of seven P-glycoprotein genes have been determined. These genes are: human *MDR1* (Chen *et al.*, 1990) and *MDR3* (Lincke *et al.*, 1991), mouse *mdr1b* (Raymond and Gros, 1989), *C. elegans pgp1* and *pgp3* (Lincke *et al.*, 1992), *atpgp1* from *Arabidopsis thaliana* (Dudler and Hertig, 1992), and *mdr50* from *Drosophila melanogaster* (Gerrard *et al.*, 1993).

The structures of the three mammalian genes are very conserved. The three genes have 28 exons, all of which have conserved intron splice positions, and 22 of which (exons 6-27) have exactly the same size. Size discrepancies in six exons can be accounted for by minor insertions and deletions in the first five exons and a variable 3'-untranslated region in the final exon. Partial genomic sequences of the three hamster Pgp genes also agree with this mammalian model (Ng *et al.*, 1989). By contrast, the two

homologous halves of the gene have more differences in the intron positions than might be expected given their high degree of sequence similarity (see, for example, *MDR1*, Figure 1.7; Chen *et al.*, 1990). The differences are mostly seen in the transmembrane regions; the two introns which interrupt the ATP-binding site are in similar positions. These differences have led to some debate about the evolutionary origins of P-glycoprotein. Raymond and Gros (1989) propose that the similarities in some of the intron positions, along with the conservation of sequence, indicate that the current structure of P-glycoprotein is the result of the duplication of an intron-containing ancestral gene. However, Chen *et al.* (1990) believe the intron differences (of both position and type) indicate that two related, but independently-evolved transmembrane proteins each fused with an ATP-binding protein, and then fused with each other to form the current four-part structure.

The two *C. elegans* genes, *pgp1* and *pgp3*, have 14 and 13 exons, respectively. In contrast to their mammalian counterparts, these genes share only one conserved intron position with each other. They do, however, respectively share four and five conserved intron positions with mammals (Figure 1.8A; Lincke *et al.*, 1992). This indicates that the *C. elegans* genes have independently lost introns that were once present in the common ancestor of humans and nematodes. The *Arabidopsis* gene has also lost introns. It has only nine introns, but seven of them are conserved within a few nucleotides to homologous intron positions in mammals (Figure 1.8B; Dudler and Hertig, 1992). The *Drosophila mdr50* gene sequence is interrupted by seven introns, six of which are in positions homologous to the mammalian sequence (Gerrard *et al.*, 1993).

It is interesting to note the distribution of the introns between the two halves of the genes. The *Arabidopsis* gene has most of the introns clustered in the N-terminal half of the gene (8 of 9; Dudler and Hertig, 1992), while the *Drosophila* gene has the most introns in its C-terminal half (5 of 7; Gerrard *et al.*, 1993). In the mammalian genes and the *C. elegans* genes the distribution is approximately equal, although all but one of the nine introns position-conserved between mammals and nematodes is located in the N-terminal half (Lincke *et al.*, 1992; Figure 1.8A).

In mammals, the gene family members are closely linked. The human *MDR3* gene is located about 30 kbp downstream of the *MDR1* gene on the long arm of chromosome 7, region 7q21-q31 (Lincke *et al.*, 1991; Trent and Witkowski, 1987). The mouse genes are localized in a cluster on chromosome 5 in the order *mdr1a*, *mdr1b*, *mdr2*, with the latter gene in an inverted orientation (Martinsson and Levan, 1987; Raymond *et al.*, 1990). The *Drosophila* Pgp genes, on the other hand, are located on two chromosomes: *mdr49* and *mdr50* on chromosome 2, and *mdr65* on chromosome 3 (Wu *et al.*, 1991; Gerrard *et al.*, 1993). In *C. elegans*, *pgp1* is on chromosome IV, *pgp2* is on chromosome I, and *pgp3* and *pgp4* are on chromosome X (Lincke *et al.*, 1992).

The promoter regions of some of the mammalian genes have been characterized. Madden *et al.* (1993) show that the human *MDR1* promoter region does not contain a TATA box, which is often required for accurate transcription initiation by RNA polymerase II. However, *MDR1* does contain a consensus sequence for an “initiator” element which is found in other TATA-less promoters like the HIV-1 viral promoter (Madden *et al.*, 1993). Smit *et al.* (1995) show that transcription of the human *MDR3* gene initiates at multiple sites. This is attributed to the fact that the promoter region is

also TATA-less, but has no “initiator” element to compensate, like *MDR1*. Transcription in this gene may be aided by the presence of multiple putative binding sites for the ubiquitous transcription factor SP1. In line with the predominant expression of this Pgp isoform in liver, sites for the liver-specific transcription factors C/EBP and HNF-5 were also found in the promoter region (Smit *et al.*, 1995). Neither the class I nor class II genes in hamster contains a TATA box nor an “initiator” element in the promoter regions. Both contain other regulatory sequences, including a CCAAT box and steroid-responsive sites. As well, the promoter region was found to have a region with silencer activity (*i.e.* a position- and orientation-independent negative transcription regulation element; Zastawny and Ling, 1993). Except for the silencer activity, similar findings were discovered in the mouse class I and II promoter regions (reviewed in Chin *et al.*, 1993).

In the mammalian genes, the translation initiation codon is located within the second exon, while in non-mammalian genes it is located in the first exon. The entire 3'-untranslated region is encoded by the last exon in all species tested; these regions of the transcript are well-conserved across mammalian species and distinct in each of the gene family members (Childs and Ling, 1996; Deuchars *et al.*, 1992). The 3'-UTR in the class III genes is only about 40 bp, while the 3'-UTR in the other two classes is considerably longer (250-450 bp; the hamster genes have short and long versions of both, generated by alternate polyadenylation; Deuchars *et al.*, 1992).

iii) Gene expression and regulation

Table 1.2 gives an overview of the tissue expression of P-glycoprotein in many different organisms. Although the methods used are different, and results for low levels

of expression are often inconsistent, certain results do stand out: 1) Class III Pgp is always seen in the liver, 2) Class I is almost always seen in the brain (when tested; Noonan *et al.*, 1990 and Buschman *et al.*, 1992 did not test brain), 3) Class I and Class II have different expression patterns in rodents, but Class I alone covers most of the same tissues in humans, 4) Class I is often seen in “barrier” tissues like brain capillary endothelium, and testis endothelium. Interestingly, Cordon-Cardo *et al.* (1989) do not detect Pgp in the choroid plexus, a part of the brain that lacks a blood-brain barrier.

There are a number of other interesting points to be taken from Table 1.2. Class I and class II expression in the adrenal gland and the pregnant uterus suggest a possible role in steroid transport (see section D). In the frog and *C. elegans*, expression of the known Pgp genes has only been shown in the intestine, whereas in *Manduca sexta*, immunostaining with the Pgp monoclonal antibody C219 and the polyclonal antibody mdr Ab-1 was seen in Malpighian tubules, the midgut, the fat body and the perineurium. Western blots of *Heliothis virescens* (a lepidopteran like *Manduca*) tissues, using monoclonal antibody c494, demonstrated the presence of a Pgp-like protein in the cuticle and fat body but showed little or no expression in the midgut. *Atpgp1* mRNA was seen in all tissues tested in *Arabidopsis thaliana*, with particular concentration in the peduncle, the portion of the stem bearing an inflorescence. So it appears, based on these expression studies, that different organisms have made use of P-glycoprotein in different ways. These data have implications for the deduction of the natural function of P-glycoprotein which are discussed in the next section.

Studies of regulation of P-glycoprotein have mainly concentrated on cell lines and tumours. Experiments suggest that Pgp expression in cells may be regulated by Pgp

substrates, heat shock, arsenite, cadmium, protein kinases A and C, retinoic acid, and c-Fos and c-Jun transcription factors (reviewed in Chin *et al.*, 1993). But results of these experiments are often conflicting and the exact mechanism of these putative regulatory effects remains to be determined. The MDR1 promoter has been shown to be a target for the product of the RAS oncogene and p53 tumour suppressor gene product (Chin *et al.*, 1993; Nguyen *et al.*, 1994). Progesterone has also been shown to regulate transcription of the murine *mdr1b* gene via the progesterone A receptor action on a site located within the first untranslated exon (Piekarz *et al.*, 1993). This result is in line with the different levels of expression of the class II gene in the pregnant versus non-pregnant uterus (Table 1.2).

P-glycoprotein expression and regulation is of tremendous medical importance, and a detailed understanding could lead to improvements in treatment of human cancers. Cancers derived from tissues that normally express P-glycoprotein (*e.g.* cancers of the liver, colon, kidney, pancreas and adrenal gland) are all generally drug resistant and have a poor response to chemotherapy (Baldini, 1997). In tumours from tissues that normally do not express significant amounts of P-glycoprotein, such as the breast, P-glycoprotein may or may not be expressed. In these cases, expression of Pgp often predicts a poor therapeutic outcome (Bradley and Ling, 1994). Bargou *et al.* (1997) have recently demonstrated the association of a Y-box transcription factor protein, YB-1, with P-glycoprotein immunostaining in primary human breast tumours. A Y-box is present in the promoter of the human *MDR1* gene in a region shown to be necessary for basal expression of *MDR1* in tissue culture (Goldsmith *et al.*, 1993). Bargou *et al.* (1997) examined 27 breast tumours which had not been treated with chemotherapy. Control

normal breast tissue showed no immunostaining of either YB-1 or P-glycoprotein. All 27 tumour samples showed cytoplasmic staining for YB-1. Nine of these samples also showed YB-1 immunostaining in the nucleus, and in a perfect correlation, only these nine tumours showed P-glycoprotein immunostaining. This group also shows that transfection of YB-1 into drug-sensitive breast epithelial cells results in *MDR1* expression and multidrug resistance, further suggesting an involvement of this transcription factor in *MDR1* regulation.

Developmental regulation of P-glycoprotein has not been studied as well as other aspects of the gene. However, van Kalken *et al.* (1992) undertook an extensive study of P-glycoprotein expression in the human fetus. Their results show that the human embryo is expressing *MDR1* mRNA after 7 weeks of gestation (the earliest stage tested). As well, there were distinct differences between adult and fetal expression in a variety of tissues. The definitive zone of the adrenal cortex showed increasing expression throughout gestation. The bronchi of the lung as well as the pharynx were positive for Pgp, unlike in the adult where no staining is seen. Staining of brain capillaries was not seen until the 28th week of gestation, while staining in the kidney and liver was present in early stages of the development of these organs.

Lincke *et al.* (1993) studied stage-specific expression of three Pgp genes in *C. elegans*. They showed by RNase protection assay that *pgp1*, *pgp2* and *pgp3* were detectable throughout the life cycle of the nematode. *Pgp1* level was lowest in eggs, peaked in midlife and declined in late stages. *Pgp2* was low in eggs and higher in the other two stages, while *pgp3* was relatively stable at high levels throughout.

Developmental regulation was also seen in *Manduca sexta*. Murray (1996) showed that the nerve cord of the tobacco hornworm was immunopositive for P-glycoprotein in the three major developmental stages of this insect: larvae, pupae and adult moth (only the larvae feed on noxious plants). Staining in the larval sections was associated with the perineurial layer, as well as with the glial cell region immediately adjacent to the neuropile. In the pupae the CNS is undergoing a fundamental reorganization; this is reflected in the somewhat scattered Pgp staining seen in the perineurium and neural lamella. Staining in adult nerve cords was seen in a thin perineurial layer and was no longer associated with the glial cell region.

Lanning *et al.* (1996a) show developmental regulation of a Pgp-like protein in *Heliothis virescens*, with expression levels increasing in the later developmental stages (instars) of the larvae.

D) Possible “normal” functions of Pgp

The genetic structure and expression data on P-glycoprotein clearly show that the gene did not suddenly appear out of nowhere to frustrate the medical community's attempts to kill tumours. Pgp obviously has a natural role to play, but until recently that role was somewhat of a mystery. Evidence is mounting that suggests Pgp gene products have at least two important functions: transport of compounds produced by the organism, and protection from potentially dangerous foreign compounds.

i) Transport of hydrophobic cellular products

a) Non-MDR P-glycoprotein transports phospholipids

Croop *et al.* (1989) demonstrated that the class III P-glycoprotein gene was the major Pgp gene expressed in mouse liver, spleen and muscle. Using antibodies specific

for the *mdr2* protein, Buschman *et al.* (1992) showed that this protein was expressed in different membrane fractions in liver cells. Fractions derived from the canalicular membrane (the membrane contiguous with the bile canal) were immunopositive for *mdr2* on a Western blot, but fractions derived from the basolateral membrane were not. These studies suggested that *mdr2* has some specialized metabolic function. A study of gene-knockout mice with an *mdr2* gene disruption (Smit *et al.*, 1993) linked *mdr2* to phospholipid secretion into the bile. Mice homozygous for the disruption developed a liver disease caused by the complete absence of phosphatidylcholine in the bile. Heterozygous mice were healthy, but had half the normal level of phosphatidylcholine in the bile. The investigators postulated that *mdr2* could function as a phosphatidylcholine translocase, which flips the phospholipid from the inner leaflet to the outer leaflet of the cell membrane in the same way as the “flippase” theorized by Higgins and Gottesman (1992). This “flippase” transporter function was demonstrated by Ruetz and Gros (1994) in yeast secretory vesicles expressing *mdr2*. They also showed that this phosphatidylcholine translocase ability was specific to *mdr2* Pgp, was ATP-dependant, and was inhibited by the Pgp modulator verapamil.

Recently, van Helvoort *et al.* (1996) demonstrated in a mammalian cell assay that the human equivalent, *MDR3*, is a specific flippase transporter of phosphatidylcholine (it would not transport other phospholipids). Unexpectedly, they also showed that *MDR1* and mouse *mdr1a* could transport a number of different short-chain lipids, including phosphatidylcholine. This led them to speculate that Class I and II P-glycoproteins may have a role as broad specificity translocases of endogenous lipids. This work also lends support to the flippase theory of drug transport proposed by Higgins and Gottesman

(1992). However, the exact nature of this lipid translocase function of *mdr1a* and *MDR1* remains unclear, as it was not active in the isolated yeast secretory vesicle system of Ruetz and Gros (1994) and it apparently was unable to rescue phosphatidylcholine transport in *mdr2*-knockout mice (Smit *et al.*, 1993).

b) Evidence for a hormone transport function

The first Pgp homologue from a eukaryote to be ascribed a normal function was the yeast *STE6* gene product (McGrath and Varshavsky, 1989). This protein is responsible for the secretion of a hydrophobic lipopeptide known as the **a**-factor pheromone, which is necessary for mating in *MATa*-type *Saccharomyces cerevisiae* cells. Raymond *et al.* (1992) demonstrated that transfection of the mouse *mdr1a* gene (referred to as *mdr3* in the study) restored mating capability to *STE6*-deficient sterile yeast. This suggests a role for Pgp in transport of hormones.

P-glycoprotein has also been implicated in steroid hormone transport. Studies have identified Pgp expression in the adrenal gland, the testes, the placenta and the secretory epithelium of the pregnant uterus of mammals (Croop *et al.*, 1989; Arceci *et al.*, 1988). Ueda *et al.* (1992) have specifically shown that the corticosteroids cortisol, aldosterone and dexamethasone are transported by human *MDR1*. Although progesterone was not transported, it did bind to Pgp, and it was shown to inhibit transport of cortisol.

ii) Protection from xenobiotics

a) In aquatic organisms

A number of studies have found that aquatic organisms have a mechanism of protection from natural and man-made toxins that is mediated by a Pgp-like transport

protein. Kurelec (1992) reviews studies of freshwater and marine mussels, marine sponges and freshwater fish and coins the term “multixenobiotic resistance” (MXR) to describe this phenomenon. All of these organisms expressed mRNA for an *mdr1*-like gene, and a protein product recognized by Pgp polyclonal antiserum. The studies also demonstrated that accumulation of xenobiotics, including the known Pgp substrate vincristine, was enhanced in these organisms by the presence of the MDR reversal agent verapamil.

Further evidence of this phenomenon is provided by Toomey and Epel (1993) who demonstrate MXR in embryos of the marine worm *Urechis caupo*. This worm lives and reproduces in sediments that contain potentially toxic xenobiotics. The cell membranes of these embryos express a 145-kDa Pgp-like protein, identified by both monoclonal and polyclonal Pgp antibodies. The embryos also demonstrated efflux of the known Pgp substrate rhodamine (a fluorescent dye). Addition of verapamil inhibited efflux and increased accumulation of the dye in the embryos. By contrast, embryos of the sea urchin *Strongylocentrotus purpuratus*, collected from a comparatively pristine environment, accumulate rhodamine regardless of the presence or absence of verapamil.

b) In mammals

Croop *et al.* (1989) identified *mdr* expression in a number of mouse tissues associated with excretion and/or protection from toxins, including the liver, kidney, spleen, brain, intestine and placenta. Pgp on the canalicular membrane of hepatocytes has been shown to transport antitumour agents into the bile (Kamimoto *et al.*, 1989, as cited in Tatsuta *et al.*, 1992). However, the most convincing evidence for a protective role for Pgp in mammals comes from its observed activity at the blood-brain barrier

(BBB). Several studies have localized Pgp to capillary endothelial cells of the mammalian brain (mouse: Tatsuta *et al.*, 1992; Hegmann *et al.*, 1992; Jetté *et al.*, 1995; Schinkel *et al.*, 1994; rat: Lechardeur *et al.*, 1995; Beaulieu *et al.*, 1995; cow: Tsuji *et al.*, 1992; Beaulieu *et al.*, 1995; pig: Hegmann *et al.*, 1992; human: Cordon-Cardo *et al.*, 1989). Functional characterization of mammalian BBB Pgp revealed that it actively transported vincristine and rhodamine across the apical membrane and that this transport was inhibited by Pgp modulators verapamil and cyclosporin A (Tsuji *et al.*, 1992; Hegmann *et al.*, 1992). Tatsuta *et al.* (1992) demonstrated *in vitro* transepithelial transport of vincristine from the basal side to the apical side of the cells, suggesting that these cells act to “pump back” blood-borne drugs that attempt to penetrate to the neural tissue. Schinkel *et al.* (1994) created gene-knockout mice deficient in the *mdr1a* gene. The mice homozygous for the gene disruption displayed a 100-fold increased sensitivity to the neurotoxic pesticide ivermectin compared to the control mice. These mice were shown to be expressing very little if any Pgp at the capillary endothelial cells of the BBB, and accumulated radiolabeled ivermectin to levels 90-fold that seen in normal mice, in agreement with the toxicity data. The level of ivermectin in other tissues was found to be only 3-4 fold higher than control mice, indicating that the brain is especially dependent on Pgp to protect against potentially dangerous compounds.

c) In lepidopterans

I) *Heliothis virescens*

A P-glycoprotein-like mechanism has recently been shown to be involved in pesticide resistance in the tobacco budworm, *Heliothis virescens* (Lanning *et al.*, 1996a, 1996b). This organism has developed resistance to nearly every pesticide, and,

paralleling the MDR phenomenon, resistance to one pesticide will often result in resistance to other structurally dissimilar pesticides. Lanning *et al.* (1996a) demonstrated by Western blots (using three Pgp antibodies) that a glycosylated Pgp-like protein of approximately 150 kDa is expressed in both pesticide-susceptible and -resistant populations of *H. virescens*, with a 4-6 fold increase in the resistant populations compared to the susceptible populations. In both resistant and susceptible populations, the Pgp-like protein was seen in the cuticle (including epidermis) and fat body, with little expression in the midgut (the Malpighian tubules and CNS were not tested). The resistant populations showed an increase in Pgp-like protein in the later developmental stages (instars) of the larvae. The resistant populations were also able to feed on vinblastine-treated cotton leaves with significantly less mortality than susceptible populations. This vinblastine resistance was decreased by pretreatment of the larvae with quinidine and quinine, two Pgp inhibitors. In the pesticide-resistant populations quinidine also strongly decreased the resistance to the pesticide thiodicarb; a twelvefold decrease in the concentration of thiodicarb required to kill half the test organisms was noted. Control studies showed that quinidine had no significant effect on feeding behaviour or cytochrome P-450 detoxification activity. Lanning *et al.* (1996b) also showed in a separate report that quinidine pre-treatment results in a 2- to 3-fold increase in [¹⁴C]thiodicarb accumulation in budworms after a topical application of the pesticide, compared to budworms without quinidine pre-treatment. These results suggest a role for P-glycoprotein in pesticide resistance, especially pesticides that penetrate through the cuticle. Since there was no detection of the Pgp-like protein in the budworm midgut, it is

unclear what role Pgp might play (if any) in protection against pesticides ingested by this organism.

II) *Manduca sexta*

As mentioned in an earlier section, the Malpighian tubules of *Manduca sexta* larvae have a physiologically described nicotine pump (Maddrell and Gardiner, 1976; Murray, 1996) and there is indirect evidence for the presence of such a pump in the nerve cord (Morris, 1983a-c, 1984). The possibility that these pumping activities are mediated by a Pgp-like transport protein was investigated by Murray *et al.* (1994).

Autoradiographs of *Manduca* ganglia incubated for 1 hour in 100 μ M radioactive nicotine indicated that the blood-brain barrier is effective at excluding nicotine from the sensitive neuropile. Immunohistochemical staining of ganglia with the monoclonal Pgp antibody C219 showed that a Pgp-like protein is expressed in the cortical region of ganglia, correlating with the position of the metabolic barrier to nicotine. Pgp-immunoreactive staining was also visible in the Malpighian tubules, fat body and (unlike *Heliothis*, see above) midgut. Further evidence that Pgp is active in the Malpighian tubules was provided when it was demonstrated that isolated tubules, in addition to pumping nicotine, will pump the Pgp substrate vinblastine; as well, both vinblastine and nicotine transport was shown to be inhibited by verapamil (Murray, 1996; L. Gaertner, personal communication).

E) Research Plan

The long-term goal of the current research is to use molecular methods to identify P-glycoprotein homologues in *Manduca sexta* and to determine whether they are

involved in the nicotine resistance mechanisms that have been physiologically described in this organism.

The body of molecular work on the Lepidoptera is relatively small (Table 1.3), especially when compared to humans and mice. The most work on insects has, of course, been done on *Drosophila melanogaster*, a veritable workhorse in the field of genetics. The commercial importance of the silkworm (*Bombyx mori*) is responsible for its status as the most intensely studied lepidopteran on a molecular level. The molecular work that has been done on *Manduca* is likely a reflection of its importance as a model organism in insect physiology and endocrinology.

Some examples of genes cloned from *Manduca* include the vacuolar H⁺-ATPase, a multiheteromeric proton transporter (Dow *et al.*, 1992), a GABA neurotransmitter transporter (Mbungu *et al.*, 1995), and diuretic hormone (Digan *et al.*, 1992). There have not been any published reports or GenBank submissions of ABC-type transporters cloned from *Manduca*.

Some preliminary work on the P-glycoprotein gene in *Manduca* has been done by Michael Ell (personal communication). Ell probed a Southern blot of *Manduca* genomic DNA at high stringency with a DNA probe, CHP1, from the class I hamster P-glycoprotein, and with probes from the *Drosophila* genes *mdr49* and *mdr65*. The hamster probe identified a single band on the Southern, while the *Drosophila* probes identified two or three bands each. The appearance of multiple bands on a Southern may indicate the presence of more than one Pgp gene in *Manduca*, which would be consistent with other organisms tested. Ell also performed PCR on genomic DNA from *Manduca* using degenerate oligonucleotide primers corresponding to the highly conserved A and B

consensus regions of the nucleotide binding domains of P-glycoprotein. Using this method, Ell produced a clone, mssp11, which was shown to be highly similar to known P-glycoproteins when the sequence was analyzed by the BLAST Genbank search software (Gish and States, 1993; Altschul *et al.*, 1990). The sequence shows that mssp11 corresponds to the region between the A and B sites of the nucleotide binding domain, but it is not known whether it is the N-terminal or C-terminal domain (see following chapter for more description of mssp11).

On the basis of this work and the Pgp immunoreactivity and physiology work of Murray (1996), it was decided that further work on *Manduca* P-glycoprotein was warranted, and that is the purview of this project. Since the two tissues of greatest relevance to the issue of nicotine tolerance are the Malpighian tubules and the CNS, these tissues were dissected from fifth-instar larvae. The following chapters describe molecular work that was done on the former tissue. Chapter 2 describes the construction of a cDNA library from the Malpighian tubules, and the screening of this library with a variety of DNA probes, including mssp11, CHP1, mdr49, and mdr65. Chapter 3 describes an investigation of some of the problems associated with this technique, and troubleshooting of various parts of the procedure. Chapter 4 is a short report of a clone of the previously undescribed *Manduca* ADP-ATP translocase which was incidentally isolated from the cDNA library. Finally, Chapter 5 summarizes the present work and reflects on its consequences for further work on the characterization of P-glycoprotein homologues in *Manduca sexta*.

Table 1.1: Classification of known mammalian P-glycoprotein genes¹.

Class	Species-specific gene names			
	Human ²	Mouse/Rat	Hamster	Pig
I	<i>MDR1</i>	<i>mdr1a(mdr3)</i> ³	<i>pgp1</i>	<i>pgp1A-D</i> ⁴
II		<i>mdr1b(mdr1)</i>	<i>pgp2</i>	
III	<i>MDR3(MDR2)</i>	<i>mdr2</i>	<i>pgp3</i>	<i>pgp3</i>

¹Gene names in parentheses are alternative names for the same gene that are still in use by some researchers.

²In most, but not all, reports, the human genes are spelled in capital letters.

³The class I Pgp gene from rat has yet to be fully cloned, but it is known that rat has three Pgp genes (Silverman *et al.*, 1991; Deuchars *et al.*, 1992).

⁴Preliminary characterization of the Pgp genes in pig has shown that four of the five genes are most similar to the class I genes of other mammals (Childs and Ling, 1996); it may be that the class II genes are restricted to the rodent lineage.

Table 1.2: overleaf**Table 1.3: Results of a search of the Entrez nucleotide database for 9 organisms. Included are three members of the order Lepidoptera: *M. sexta*, *B. mori* and *S. frugiperda*.**

Organism	Number of sequence submissions ¹
<i>Homo sapiens</i> (human)	649 491
<i>Mus musculus</i> (mouse)	139 346
<i>Drosophila melanogaster</i> (fruit fly)	8 785
<i>Xenopus laevis</i> (frog)	1 763
<i>Nicotiana</i> spp. (tobacco plant)	986
<i>Bombyx mori</i> (silkworm)	336
<i>Manduca sexta</i> (tobacco hornworm)	127
<i>Musca domestica</i> (house fly)	60
<i>Spodoptera frugiperda</i> (army worm)	22

¹Submissions retrieved as of 3/1/97; a submission is not necessarily a full gene or cDNA sequence.

Table 1.2: Tissue expression of P-glycoprotein-like genes in a variety of organisms assayed by various methods. The most comprehensive work has been done on humans and rodents. Where there is a distinguishable difference in the level of expression, this is indicated by font style: **bold > italic** > normal. No tissue-specific expression data is available for *Drosophila melanogaster*.

Organism	Gene Product	Tissues	Method	References
human	class I mRNA	adrenal gland, kidney, intestine, liver, brain, prostate	Northern blot, slot blot	Fojo <i>et al.</i> , 1987
	class III mRNA	adrenal gland, kidney, intestine, liver	RT-PCR	Noonan <i>et al.</i> , 1990
	class I protein	liver, adrenal gland, skeletal and heart muscle, tonsil, spleen	RNase protection	Smit <i>et al.</i> , 1994
		capillary endothelial cells of brain, spinal cord, testes, papillary dermis	Immunohistochemistry	Thiebaut <i>et al.</i> , 1987, 1989
		brain, testes, uterus (sub-cell. local.)	Immunohistochemistry	Cordon-Cardo <i>et al.</i> , 1989
		pregnant uterus (secretory epithelium)	<i>In situ</i> hybridization	Stewart <i>et al.</i> , 1996
	class III protein	liver	Immunohistochemistry	Arecci <i>et al.</i> , 1988
				Smit <i>et al.</i> , 1994
mouse	class I mRNA	<i>intestine, heart, lung, CNS, testes, stomach, kidney, liver, spleen, muscle</i>	Northern blot	Croop <i>et al.</i> , 1989
	class II mRNA	pregnant uterus, adrenal gland, placenta, heart, kidney, uterus, liver, muscle, spleen	Northern blot	Croop <i>et al.</i> , 1989
	class III mRNA	<i>adrenal gland, liver, muscle, spleen, heart</i>	Northern blot	Croop <i>et al.</i> , 1989
	class I protein	liver, intestine	Western blot	Buschman <i>et al.</i> , 1992
	class II protein	pregnant uterus	Western blot	Buschman <i>et al.</i> , 1992
	class III protein	liver	Western blot	Buschman <i>et al.</i> , 1992
rat	class II mRNA	lung, liver, kidney, small intestine, spleen	RNase protection	Brown <i>et al.</i> , 1993
	class III mRNA	liver, lung, brain	RNase protection	Brown <i>et al.</i> , 1993
hamster	class I protein	intestine, liver, endothelial cells of testis, brain, female reproductive tract	Immunohistochemistry	Bradley <i>et al.</i> , 1990
	class II protein	adrenal cortex (sex-dependent), lining of pregnant uterus	Immunohistochemistry	Bradley <i>et al.</i> , 1990
	class III mRNA	liver	cDNA cloning	Endicott <i>et al.</i> , 1991
pig	class I (A) mRNA	capillary endothelial cells of brain	RT-PCR	Childs and Ling, 1996
	class III mRNA	liver	RT-PCR	Childs and Ling, 1996
frog	Xe-mdr mRNA	intestinal epithelial cells (highest in terminal ileum)	Northern blot, <i>in situ</i> hybridization	Castillo <i>et al.</i> , 1995
	pgp-like protein	intestine	Western blot	Castillo <i>et al.</i> , 1995
<i>Manduca sexta</i>	pgp-like protein	nerve cord, Malpighian tubules, fat body, midgut	Immunohistochemistry	Murray <i>et al.</i> , 1994; Murray, 1996
<i>Periplaneta americana</i>	pgp-like protein	nerve cord	Immunohistochemistry	Murray <i>et al.</i> , 1994; Murray, 1996
<i>Rhodnius prolixus</i>	pgp-like protein	Malpighian tubules	Immunohistochemistry	Murray <i>et al.</i> , 1994; Murray, 1996
<i>Heliothis virescens</i>	pgp-like protein	<i>cuticle</i> , fat body	Western blot	Lanning <i>et al.</i> , 1996a
<i>Caenorhabditis elegans</i>	pgp1,2,3 mRNA	whole animal, three developmental stages	RNase protection	Lincke <i>et al.</i> , 1993
	pgp1, pgp3	intestinal cells	beta-galactosidase staining of transgenic nematodes expressing pgp-lacZ fusion proteins	Lincke <i>et al.</i> , 1993
<i>Arabidopsis thaliana</i>	<i>atpgp1</i> mRNA	peduncle, leaves, flowers, roots	Northern blot	Dudler and Hertig, 1992

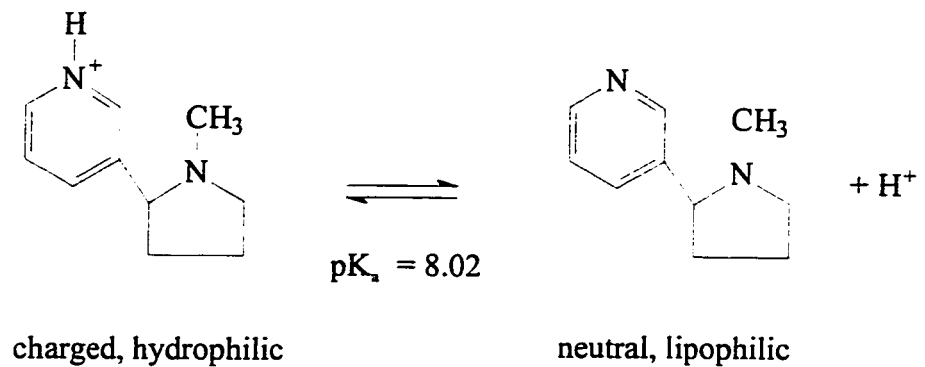


Figure 1.1: Deprotonation reaction for nicotine. The neutral, lipophilic form of nicotine is in equilibrium with the charged, hydrophilic form. At pH = 6.6, the pH of *Manduca* hemolymph, this reaction favors the charged form of nicotine; 98% of nicotine will be protonated. Protonation in the aliphatic ring is negligible at this pH.

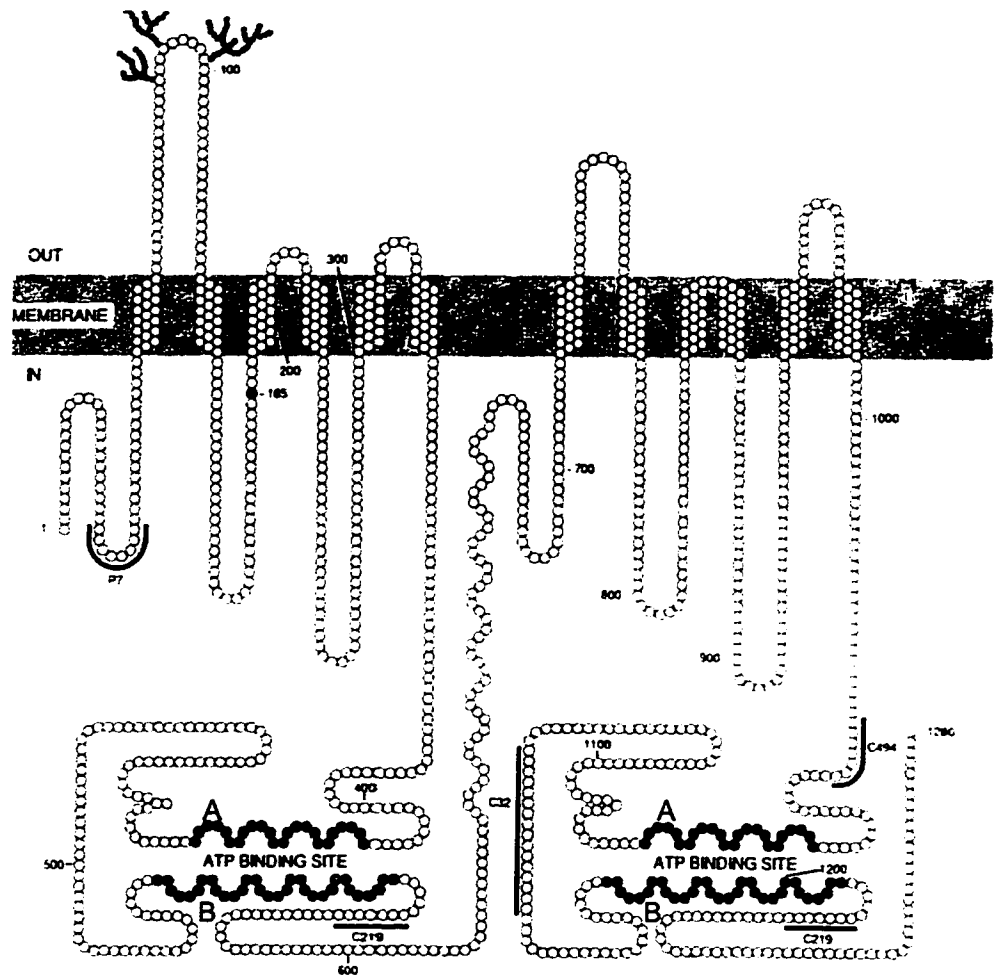


Figure 1.2: Predicted topology of P-glycoprotein in the cell membrane (Juranka *et al.*, 1989). The Walker A and B regions of the ATP-binding sites are indicated. Epitopes for four antibodies are indicated by thick black lines; also indicated are the predicted sites of glycosylation.

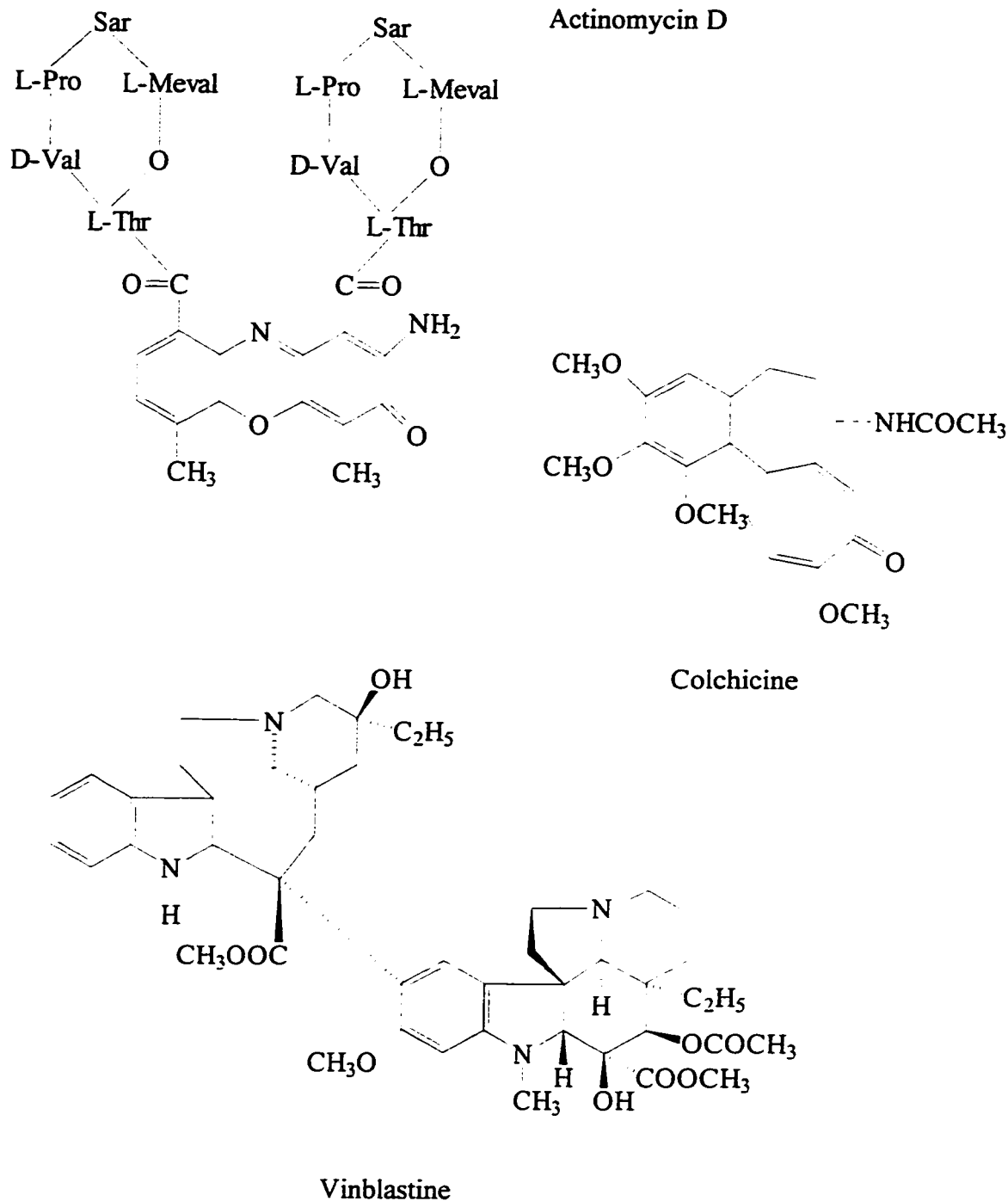
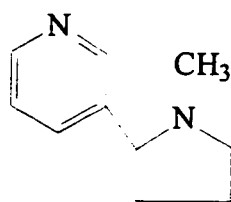
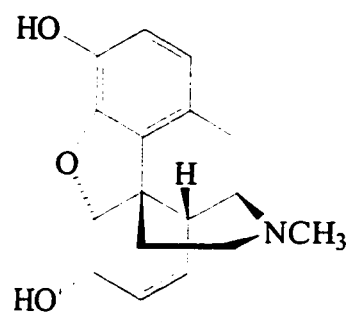


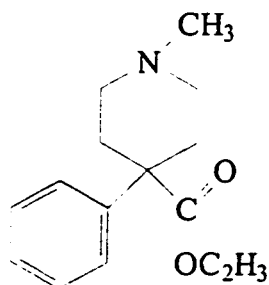
Figure 1.3: Structures of Pgp substrates vinblastine, colchicine and actinomycin D. Note the presence of basic nitrogen atoms and aromatic rings. These tend to be the only characteristics that Pgp substrates have in common. Sar = sarcosine (methylglycine), Meval = methylvaline, Pro = proline, Val = valine, Thr = threonine.



Nicotine



Morphine



Meperidine

Figure 1.4: Structure of nicotine, morphine and the synthetic opiate meperidine - three putative Pgp substrates. Note that meperidine, like nicotine, has a single aromatic ring and basic nitrogen atom.

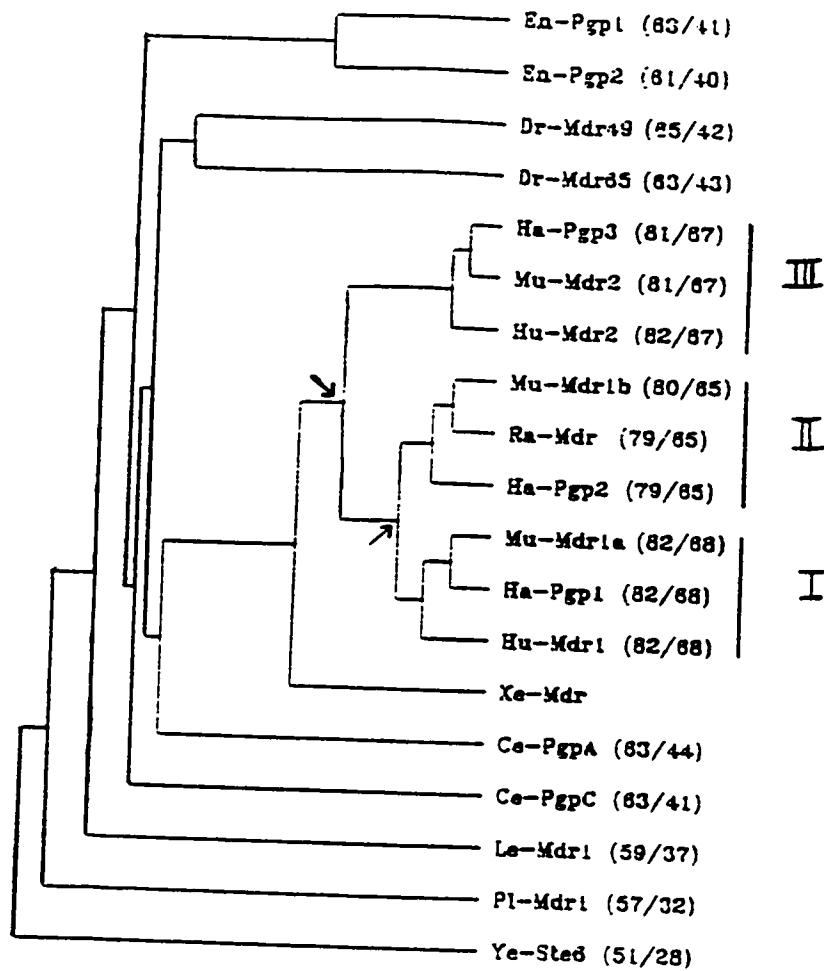


Figure 1.5: A tree diagram (dendrogram) of the evolutionary relationships of members of the P-glycoprotein family, as deduced by a clustering analysis of the amino acid sequences (see Castillo *et al.*, 1995). Arrows indicate putative divergence of the mammalian class III and, subsequently, class I and II genes. The genes used in the analysis are *Entamoeba histolytica* *pgp1* and *pgp2* (En-Pgp1, En-Pgp2), *Drosophila melanogaster* *mdr49* and *mdr65* (Dr-Mdr49, Dr-Mdr65), hamster class I, II and III (Ha-Pgp1, Ha-Pgp2, Ha-Pgp3), mouse class I, II and III (Mu-Mdr1a, Mu-Mdr1b, Mu-Mdr2), rat class II (Ra-Mdr), human class I and III (Hu-Mdr1, Hu-Mdr2), *Xenopus laevis* *mdr* (Xe-mdr), *Caenorhabditis elegans* *pgp1* and *pgp3* (Ce-PgpA, Ce-PgpC), *Leishmania* *ldmdr1* (Le-Mdr1), *Plasmodium* *mdr1* (Pl-Mdr1) and yeast *ste6* (Ye-ste6). The numbers in parentheses represent percent homology/identity to the *Xenopus* gene. This figure is modified from Castillo *et al.* (1995).

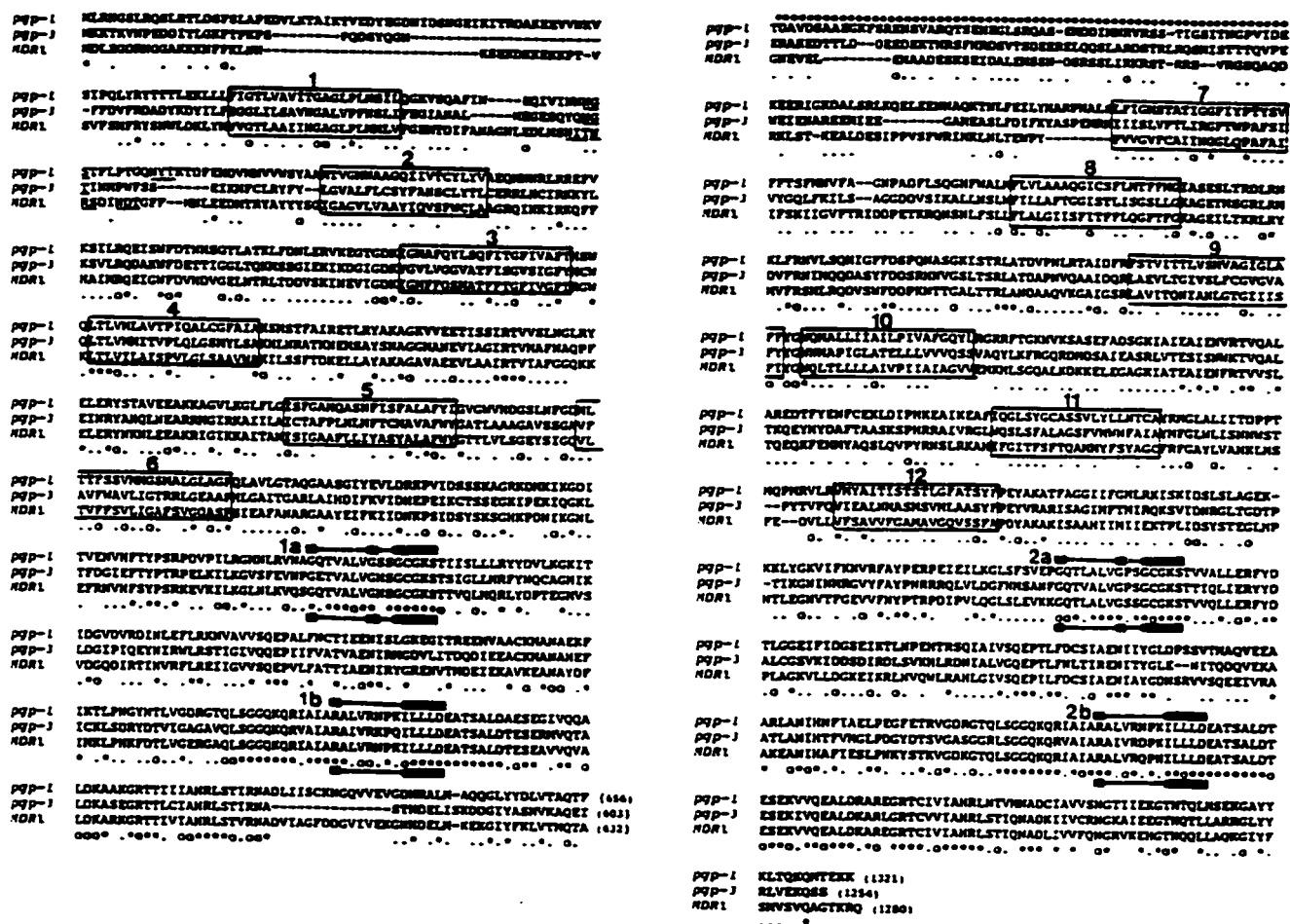


Figure 1.6: Conservation of sequence across species and between gene family members in P-glycoprotein. The amino acid sequences of *pgp1* and *pgp3* from *C. elegans* and *MDR1* from humans are shown. Functionally similar amino acid residues are indicated by periods, identical residues are indicated by open circles or stars. Stars indicate residues that are identical in all human, mouse and hamster genes and *Drosophila melanogaster* genes *mdr49* and *mdr65*. The twelve transmembrane regions are indicated by boxes, and the nucleotide binding sites are indicated by horizontal lines (1a/b, 2a/b) with thicker lines indicating consensus residues. This figure is taken from Lincke *et al.* (1992).

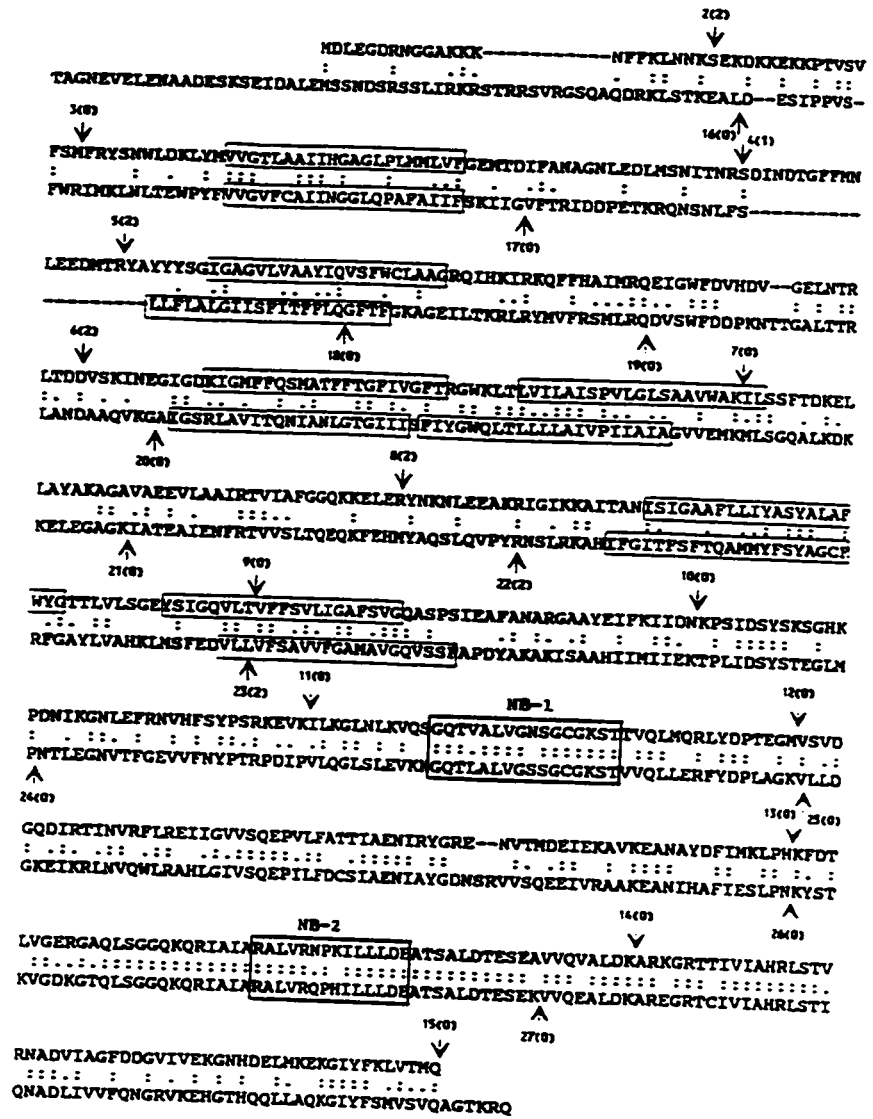
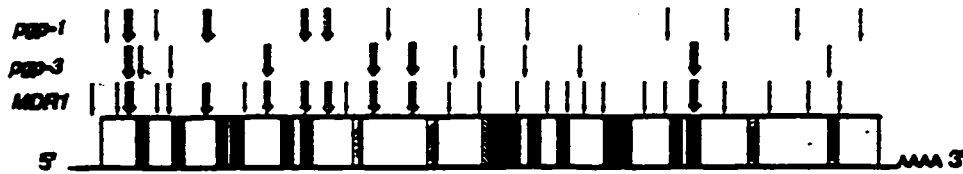


Figure 1.7: Conservation of sequence and intron position in the two homologous halves of the human *MDR* gene. The amino acid sequence of the N-terminal half is positioned above the sequence of the C-terminal half. Identical amino acid residues are indicated by *colons*, functionally similar residues are indicated by *dots*. Transmembrane regions are enclosed in thin boxes and the nucleotide binding consensus regions (NB-1 and NB-2) are indicated by thick boxes. The positions of the 27 introns are indicated by arrows, accompanied by the intron number and, in parentheses, the intron type. Type 0, 1 and 2 introns interrupt the sequence between codons (0) and after the first and second bases of the codon. Note that the only introns exactly conserved in position and type are introns 13 and 26. This figure is taken from Chen *et al.* (1990).

A



B

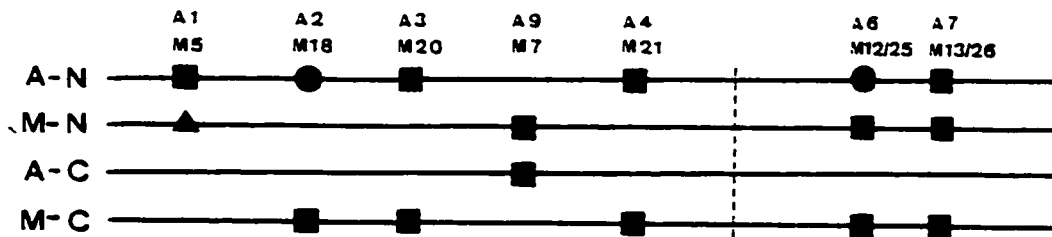


Figure 1.8: A) Comparison of the position of introns relative to the mRNA sequence for *pgp1* and *pgp3* from *C. elegans* and *MDR1* from humans. Arrows represent the positions of the introns; hatched arrows indicate introns that are exactly conserved across at least two of the three sequences. Within the clear box which represents the coding region of the mRNA, black vertical bars represent the twelve transmembrane regions, gray vertical bars represent the consensus sites for the ATP-binding domains, and the hatched box indicates the linker region between the two homologous halves of the molecule. Note that only one intron position is conserved between the two *C. elegans* genes, while several are conserved between an individual *C. elegans* gene and the human gene.

B) Comparison of the position of introns in the two homologous halves of the genes from *Arabidopsis thaliana* and mammals. The lines represent the N- and C-terminal halves of the *Arabidopsis* (A-N, A-C) and mammalian (M-N, M-C) genes. The positions of approximately conserved introns (within 5 codons) are indicated by symbols, and the intron number is also indicated above the lines. Unconserved introns are not indicated. The shape of the symbol indicates the type of intron. Squares, circles and triangles respectively indicate introns that interrupt between codons, after the first base, and after the second base of the codon (known as type 0, 1 and 2 introns). The stippled vertical line indicates the border between the transmembrane domains (*left*) and the ATP-binding domains (*right*).

This figure was adapted from Lincke *et al.* (1992) and Dudler and Hertig (1992).

Chapter 2: Construction and screening of a cDNA library of *Manduca* Malpighian tubules

Introduction

A) Overview of the cloning procedure

The cloning method used in this project involved screening a λ phage cDNA library of the tissue of interest: *Manduca sexta* Malpighian tubules (see Fig 2.1 for flow chart of the procedure). Messenger RNA was isolated from dissected proximal Malpighian tubules, and used to construct a λ ZAPII phage cDNA library according to the protocols provided with the Stratagene library kit (Stratagene, 1993; related in detail below). The cDNA library was plated as phage plaques growing on a bacterial lawn on nutrient-enriched agar. The agar plates were overlaid with nylon transfer membranes, which bound the phage particles from the plaques. The phage particles were lysed and the DNA denatured, then irreversibly cross-linked to the nylon membranes by UV irradiation. These membranes were thus imprinted with the arrangement of phage plaques, each containing cDNA from a particular gene transcript, on the agar plates. A radioactive DNA probe was produced from a clone believed to be similar (or homologous) to the cDNA of interest. The membranes were first incubated in pre-hybridization solution, which contains blocking agents that bind to unoccupied binding sites. The membranes were then transferred to hybridization solution, which is generally the same as pre-hybridization solution with the addition of the radioactive DNA probe. The membranes were incubated in a shaking water bath overnight, and then subjected to a series of washings to remove background radioactivity. The temperature, ionic strength

and percentage formamide (a DNA duplex destabilizing agent) in the hybridization solution and washing solutions determined the stringency of the hybridization procedure. A high stringency (high temperature, low ionic strength) was used if the probe was nearly identical in sequence to the cDNA of interest. A low stringency (low temperature, high ionic strength) was used when the sequence similarity was expected to be low. An alternative way to increase the stringency of hybridization solutions was to add formamide. Each percentage of formamide added to the solution was the equivalent of raising the temperature by 0.6°C (*e.g.* 42°C, 50% formamide was equivalent to 72°C, 0% formamide; Sambrook *et al.*, 1989). After washing, the filters were exposed to X-ray film. Darkened spots on this autoradiograph indicated plaques to which the radioactive probe had hybridized. These dark spots (“positives”) were then compared to the original agar plates. The films were lined up with the plates using reference points, and a small plug of agar corresponding to the positive signal was excised.

This agar plug (a “pick”), which contained a number of phage plaques, was used to prepare a phage stock for secondary plating. The above plating, transfer and hybridization procedures were repeated on a smaller scale. Phage plaques were plated at a lower density than in primary plating, so that single plaques could be excised to get a pure clone. Secondary plating also helped to eliminate false positive results. Pure clones were converted to phagemids and sequenced by the dideoxy chain termination method (Sequenase). The sequence of the clone was sent by electronic mail to a computer that compared the sequence to the other sequences in Genbank. The computer returned a message listing the genes in the database that were most similar to the clone.

B) DNA probes

In total, nine different DNA probes were used to screen the cDNA library. What follows is a description of each probe.

i) mssp11

This probe is a 431-bp fragment of a P-glycoprotein homologue in *Manduca sexta* (Figure 2.2). This fragment was isolated from *Manduca* genomic DNA by polymerase chain reaction (PCR) amplification using degenerate oligonucleotide primers targeting the conserved Walker A and B sites (M. Ell and G. Drouin, unpublished work). The sequence of mssp11 contains a 66-bp intron. This intron is conserved in position with introns in mammalian P-glycoprotein sequences, but not with *Drosophila mdr50*, the only insect P-glycoprotein for which intron/exon structure is known. The fragment shares high sequence similarity to other P-glycoprotein genes (e.g. *mdr49*; Figure 2.3), however, it is not known whether it is from the N-terminal or C-terminal portion of the equivalent full-length gene (Figure 2.4). This probe was placed into the plasmid pBluescript at the *SmaI* restriction site. It was usually removed from the plasmid by restriction digestion with *BamHI* and *EcoRI*.

ii) vacuolar H⁺-ATPase

This probe was kindly provided by J. Dow at University of Glasgow for use as a positive control. It is a 1.4-kb cDNA encoding the 16-kDa proteolipid subunit of the vacuolar H⁺-ATPase which Dow *et al.* (1992) had obtained from a λ ZAPII cDNA library of *Manduca* larval midgut tissue. It had been isolated by DNA probing with the homologous V-ATPase clone from *Drosophila melanogaster*. In their plaque

hybridization experiments, positives were obtained at a ratio of 1 for every 1000 plaques. The probe is contained in pBluescript plasmid, flanked by *EcoRI* and *XhoI* restriction sites.

iii) CHP1

Obtained from the lab of V. Ling, this probe is a 600-bp fragment of the *pgp1* gene, a P-glycoprotein homologue from the Chinese hamster (Riordan *et al.*, 1985). This fragment contains both the A and B sites of the highly conserved nucleotide binding fold (also known as the Walker motif, after Walker *et al.*, 1982), from the C-terminus of the gene (Fig. 2.4). This probe had been used successfully in screening for two other members of the P-glycoprotein gene family (*pgp2*, *pgp3*) in Chinese hamster (Ng *et al.*, 1989). This probe is contained in the plasmid pBluescript, and is flanked by *EcoRI* restriction sites. A fragment of this probe, containing only the Walker B site, was used to construct a Pgp-ZAP construct as a positive control (see Chapter 3). This smaller fragment was also used as a probe; it is designated CHP1A. CHP1A is contained in the plasmid pBluescript and is flanked by *XbaI* and *XhoI* restriction sites. CHP1A is 430 bp in length.

iv) mdr 49, mdr 65

These probes are fragments of two Pgp gene homologues isolated from *Drosophila melanogaster*. The mdr49 fragment is 1.8 kb and includes both the A and B sites of the N-terminal Walker motif and a portion of the N-terminal transmembrane domain. The mdr65 fragment is 1.2 kb and includes a larger portion of the N-terminal transmembrane domain, but only the A site of the N-terminal Walker motif (Figure 2.4). These clones were isolated by DNA screening of a *Drosophila* head cDNA library with a

mouse *mdr1* probe (Wu *et al.*, 1991). Both of these probes are contained in the plasmid pSP72 and are flanked by *EcoRI* restriction sites.

Please note: Throughout the following pages, the use of the unitalicized “*mdr49*” and “*mdr65*” denotes the probes described in the preceding paragraph. When italicized, *mdr49* and *mdr65* refer to the entire genes described by Wu *et al.* (1991).

v) DM13, SP01, TU15, TU19

These are fragments of P-glycoprotein homologues from, respectively, *Drosophila melanogaster*, *Spodoptera frugiperda* and *Tetranychus urticae* (two), which were isolated by M. Ell in the same fashion as mssp11 (Figure 2.4). *Spodoptera* is a lepidopteran like *Manduca*; the larval form is commonly known as the army worm. *Tetranychus urticae* is a species of mite (an arachnid). DM13 is distinct from the three published *Drosophila* Pgp homologues, *mdr49*, *mdr65* and *mdr50* (Gerrard *et al.*, 1993). As of this writing, full sequence data on these probes was not available, but limited sequence data had confirmed the clones to be homologous to Pgp (M. Ell, personal communication). These probes are in the same plasmid as mssp11.

Methods

A) Rearing and dissection of *Manduca sexta*

A *Manduca sexta* colony was established at the animal care facility of the Loeb Medical Research Institute with eggs purchased from Carolina Biological. The larvae were fed an artificial diet that contained no nicotine (a variation of that described in Yamamoto, 1969; see Appendix A). It had previously been established that the presence of nicotine in the diet of *Manduca*, was not required for nicotine resistance or P-glycoprotein immunoreactivity (Murray, 1996). Larvae were housed in a cabinet that

was maintained at room temperature and on a light/dark cycle of 16:8 hours. Eggs were hatched in a petri dish containing damp filter paper, and the larvae were transferred to cubes of diet shortly after hatching. After two days, larvae were transferred to 24-well plates to isolate them from each other (the larvae had occasionally exhibited cannibalistic behaviour when reared *en masse*). When the larvae had grown too big for the wells, they were transferred to pill vials, where they remained until their fifth instar stage. The larvae were checked every day to ensure they were supplied with adequate diet. When new diet was added, old uneaten diet and faeces were discarded to minimize fungal contamination.

Fifth instar larvae were anaesthetized by refrigeration at 4°C for 30 min. The larvae were decapitated using dissecting scissors, and the bodies were pinned to a dissection tray. An incision was made along the dorsal length of the body, and the body wall was pinned back to reveal the innards. The proximal Malpighian tubules were thus exposed; these were dissected out and flash-frozen on liquid nitrogen. The midgut of the larvae was then removed, to reveal the abdominal nerve cord. This tissue was also dissected out (including four or five ganglia, along with some associated tracheal tissue) and flash-frozen. All tissues were stored at -80°C until use (the nervous tissue was not used in this project).

B) Construction of a cDNA library from *Manduca sexta* Malpighian tubule tissue.

i) Tissue homogenization and total RNA extraction

a) Special buffers

CHAOS buffer	4.25 M guanidine thiocyanate
(<i>Homogenization</i>	25 mM sodium citrate, pH 7
<i>buffer</i>)	0.5 % sodium lauryl sarcosinate (sarcosine)

in RNase-free H₂O
immediately prior to use the following were added:
2-mercaptoethanol to 0.1 M (0.36 mL/50 mL)
and 5 drop Antifoam A/50 mL

b) Other preparations

All glassware was baked at 200°C overnight. Other materials were treated with DEPC solution (diethylpyrocarbonate, 0.1% in deionized water) and autoclaved. *RNase-free water* is DEPC solution that has been autoclaved (non-autoclaved DEPC solution reacts rapidly with amines and thus should not be combined with Tris or other amine buffers). Latex gloves were worn at all times and all solutions, including the homogenate, were kept on ice during the following procedure. The Polytron homogenizer tip was pre-soaked for several hours in 0.1 M NaOH. All of these precautions were necessary to avoid degradation of the RNA by RNA-digesting enzymes (RNases).

c) Procedure

Five mL of CHAOS buffer were added to a test tube containing Malpighian tubule tissue from 35 *Manduca sexta* fifth-instar larvae. After rinsing the Polytron tip twice in RNase-free water, the tissue was homogenized in 5-10 second bursts at high speed.

This was followed by the addition of the following solutions in order, with brief mixing by inversion after each addition: 0.5 mL of 2 M sodium acetate, pH 4; 5 mL of water-saturated phenol; 1 mL of CI (24 parts chloroform, 1 part isoamyl alcohol). The solution was vortexed for 30 seconds, and chilled on ice for 15 minutes.

This was followed by centrifugation at 2600g for 20 minutes at 4°C. Three phases were visible in the tube following centrifugation: an aqueous top layer, a phenolic

bottom layer, and an interphase consisting of a white precipitate. The top layer was removed, with extra care so as not to disturb the interphase, and divided into 4 microcentrifuge tubes.

The tubes were spun at 14000g for 15 minutes at 4°C in a microcentrifuge. The supernatants were removed into a single 15 mL conical tube, and an equal volume (~ 4.5 mL) of ice-cold isopropanol was added. The solution was left 2 days at -20°C to allow the RNA to precipitate. This was followed by another spin (30 min., 4°C, 2600g). The supernatant was removed and discarded. Ten mL of 70% ethanol (-20°C) was used to resuspend the pellet, followed by another spin (20 min., 4°C, 2600g). This resuspension/spin step was repeated once. This was followed by a third resuspension using 3 mL of 70% ethanol, splitting the resuspension into 2 microcentrifuge tubes, and a spin in a microcentrifuge (15 min., 4°C, 14000g).

The supernatants were removed, and the pellets were allowed to air dry at 4°C for 1 h, 45 min. After drying, the pellets were resuspended in 200 µL RNase-free water. The concentration of the RNA was determined approximately by UV spectroscopy.

d) Notes

i) Guanidine thiocyanate is a strong protein denaturing agent. Along with mercaptoethanol, which disrupts the disulfide bridges in proteins, it acted to disintegrate cellular structures and dissociate nucleoproteins from nucleic acids. This is a chaotropic effect, hence the name CHAOS buffer. These reagents also served to inactivate any endogenous RNases that may have been released by the disrupted cells.

ii) In the acid-phenol-chloroform extraction, the RNA was dissolved in the aqueous top layer, while DNA and proteins were present in the interphase. Therefore, care was taken not to disturb the interphase when removing the top layer, as this could have contaminated the RNA sample with DNA.

iii) An interesting observation was that, in the acid-phenol-chloroform extraction, the top layer retained the slight yellow colour of the original tissue. This effect was even more evident in a previous experiment in which purple-coloured *Aplysia californica* body wall tissue was used to test this extraction protocol. A strong purple pigment co-extracted with the RNA, resulting in a purple total RNA sample. However, the pigment did not carry through to the mRNA stage.

iv) This protocol was adapted from the method of Chomzynski and Sacchi (1987).

ii) mRNA extraction

a) Special buffers

2x oligo-dT column	40 mM Tris, pH 7.5
loading buffer	2 mM EDTA
	0.5 M LiCl
	0.1 % sodium lauryl sarcosinate
	in RNase-free H ₂ O

The Tris/EDTA/LiCl solution was autoclaved prior to the addition of sarcosine stock at 65°C.

oligo-dT column	10 mM Tris, pH 7.5
elution buffer	1 mM EDTA
	0.5 % sodium dodecyl sulfate (SDS)
	in RNase-free H ₂ O

As above, the solution was autoclaved, then equilibrated to 65°C. The SDS stock solution (10%; also equilibrated to 65°C) was then added.

b) Other preparations

Oligo-dT column preparation: A small plug of DEPC-treated glass wool was inserted into a baked glass Pasteur pipet. Oligo-dT cellulose (0.15 g) was suspended in 0.1 M NaOH, and then poured into the Pasteur pipet. The column was washed with 5 mL of RNase-free water, followed by 3 mL of 1x column-loading buffer.

c) Procedure

The total RNA sample (180 μ L volume) was heated at 65°C for 5 minutes and cooled on ice for 15 min. An equal volume of 2x column-loading buffer was added to the RNA sample, which was then pipetted onto the column. The autopipet (with 1000 μ L tip) was inserted into the top of the column, and used to create positive pressure on the column. The eluant was collected in a fresh tube and reheated, cooled, and reapplied to the column as above. The eluant from this second application was collected as fraction 1.

One mL of 1x column-loading buffer was added to the column: it was also collected as part of fraction 1. Four more additions and collections using 1x column-loading buffer were performed; these were designated fractions 2 through 5.

The mRNA was eluted in 5 subsequent fractions (6 through 10) using 0.3 mL of elution buffer. The concentration of RNA in each fraction was determined by UV spectroscopy. Fractions 8, 9 and 10 were pooled (4.5 mg mRNA). Fifty μ L RNase-free 3 M sodium acetate and 1 mL 100% ethanol were added to the pooled fractions, and the mixture was stored at -20°C overnight to precipitate the RNA.

d) Notes

i) The polyadenylated tail of mRNA bound to the oligo-dT cellulose in the high-salt column-loading buffer. Most of the non-polyadenylated RNAs (ribosomal, transfer, etc.) washed through in the high salt buffer and was collected as "poly A-". The mRNA was then eluted from the column in the low-salt elution buffer and collected as "poly A+".

iii) Sodium lauryl sarcosinate was used as the detergent because SDS is insoluble in concentrated salt solutions. Sarcosine is more soluble in lithium chloride than in sodium chloride.

iv) This method was adapted from Sambrook *et al.* (1989).

iii) cDNA synthesis and insertion into λ ZAP II vector

The cDNA library was prepared using Stratagene's ZAP-cDNA Synthesis Kit, and thus the following method is taken from the manual that accompanied the kit (Stratagene, 1993) with any variations noted. Figure 2.5 (Stratagene, 1993) summarizes the cDNA synthesis procedure.

The RNA sample from above was spun to pellet the RNA (4°C, 15 min., 14000g). The supernatant was discarded and the pellet was washed with 70% ethanol and left standing on ice for 15 minutes. The tube was then spun again as above, the supernatant was removed and discarded, and the pellet dried for 2 hours, on ice. The pellet was then resuspended in 36.5 μ L RNase-free H₂O.

a) First strand cDNA synthesis

First strand synthesis of cDNA was performed in a reaction volume of 50 μ L.

The following reaction mixture was prepared using stock solutions provided in the kit:

1x first strand reaction buffer (50 mM Tris, pH 8.3; 75 mM KCl, 30 mM MgCl₂)
first strand nucleotide mixture (0.6 mM each of dATP, dTTP, dGTP, 0.3 mM
5-methyl-dCTP)
linker-primer oligonucleotide (2.8 μ g)
RNase block I (40 units)
RNA sample (4.5 μ g; a control using 5 μ g of Stratagene's test RNA was
performed simultaneously)
Moloney-Murine leukemia virus reverse transcriptase (M-MuLVRT; 50 units)

Before adding the reverse transcriptase, the mixture was incubated for 10 minutes at room temperature, to allow the linker-primer to anneal to the RNA. After addition of the reverse transcriptase, 5 μ L of the reaction mixture was transferred to a separate tube that contained 0.5 μ L of α -³²P-labeled dATP. All the first-strand reactions, including the radioactive aliquot, were then incubated at 37°C for 1 hour. After this incubation period, the nonradioactive first strand reaction was placed on ice, and the radioactive sample was stored at -20°C for gel analysis at a later time.

b) Second strand synthesis

The following reagents were added to the first strand mixture to the final concentrations given at a volume of 400 μ L. The mixture was vortexed briefly just before adding the last two components. All the reagents were kept below 16°C before addition of the DNA polymerase.

1x second strand buffer (which in combination with the first-strand buffer,
resulted in a total of 24.4 mM Tris, pH 8.3; 8.4 mM KCl, 90.6 mM
CaCl₂, 4.9 mM MgCl₂)

second strand nucleotide mixture (0.2 mM dATP, dGTP, dTTP, 0.4 mM dCTP,
also 0.03 mM 5-methyl dCTP remaining from first strand reaction)
sterile distilled water (to 400 μ L)
 α -³²P-labeled dATP (20 μ Ci)
RNase H (4.5 units)
DNA polymerase I (100 units)

The mixture was incubated at 16°C for 2.5 h, and then immediately placed on ice. The mixture was extracted with phenol:chloroform:isoamyl alcohol (PCI) (25:24:1), and chloroform:isoamyl alcohol (CI) (24:1), and then precipitated with sodium acetate/ethanol. The pellet was washed gently with 80% ethanol. The pellet was dried under partial vacuum and then resuspended in 45 μ L sterile water. A 3.0 μ L aliquot was removed for size analysis.

c) Blunting the cDNA termini

The following were added to the remaining 42 μ L of the resuspended cDNA for the final concentrations given in a volume of 53 μ L:

Buffer #3 (50 mM Tris-HCl, pH 7.5, 7 mM MgCl₂)
dNTP mix (0.23 mM each of dATP, dCTP, dGTP, dTTP)
Klenow fragment (5 units)

The mixture was incubated at 37°C for precisely 30 minutes. Forty-seven μ L of distilled water were added to bring the volume up to 100 μ L. The sample was extracted with an equal volume of PCI, followed by an extraction with CI. The supernatant was transferred to a new tube and precipitated on ice following addition of 7.0 μ L 3M sodium acetate and 226 μ L 100% ethanol. After 30 minutes, the tube was spun in a microfuge at 4°C, maximum speed for 60 minutes. The pellet was washed in 300 μ L of 80% ethanol and then allowed to dry *in vacuo*.

d) Ligation of EcoRI adaptors

The dried pellet was resuspended in 7.0 μL of the EcoRI adaptor solution, and the following were added to the final concentrations given in a volume of 10 μL :

Buffer #3 (50 mM Tris-HCl, pH 7.5, 7 mM MgCl_2)
1 mM rATP
T4 DNA ligase (4 Weiss units)

The mixture was incubated at 4°C overnight, after which the ligase was heat-inactivated by incubating at 70°C for 30 minutes.

e) Phosphorylating the EcoRI ends

The heat-inactivated mixture was allowed to cool for 5 minutes, then the following were added to the final concentrations given in a volume of 20 μL :

Buffer #3 (50 mM Tris-HCl, pH 7.5, 7 mM MgCl_2)
1 mM rATP
sterile water (6 μL)
T4 polynucleotide kinase (10 units)

The mixture was incubated at 37°C for 30 minutes, then the kinase was heat-inactivated by incubation for 30 minutes at 70°C. Condensation was spun down in a microfuge, and the mixture was allowed to cool for 5 minutes.

f) XhoI digestion

To this mixture the following were added to the final concentrations given in a volume of 51 μL :

XhoI buffer supplement (which in combination with Buffer #3 added previously gives 102 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10.4 mM MgCl_2)
XhoI (120 units)

This mixture was incubated at 37°C for 1.5 hours, then cooled to room temperature.

g) Size fractionation of cDNA

Five μL of 10x STE was added to the mixture for a final composition of: 182 mM NaCl, 36 mM Tris-HCl, pH 7.5, 9.5 mM MgCl_2 , 9 mM EDTA. The mixture was loaded onto a prepared Sephacryl S-400 spin column (see Stratagene, 1993, p 30 for column preparation). The column was spun on a table top centrifuge at $400 \times g$ for 2 minutes. The resulting eluant was designated fraction 1. Sixty μL of 1x STE were added to the column, which was spun as above to collect fraction 2. A third fraction was collected in a similar manner. Five- μL aliquots of each sample were analyzed by agarose gel electrophoresis (not shown). The fractions were extracted once with an equal volume of PCI, and once with an equal volume of CI. The fractions were then precipitated by addition of 2 volumes of 100% ethanol (the STE buffer contains adequate salt for precipitation) and incubation at -20°C overnight. The fractions were pelleted in a microfuge (4°C , 60 min, max. speed), and washed carefully with 80% ethanol. The pellets were dried *in vacuo* and then resuspended in 3.0 μL sterile water. The amount of DNA in each fraction was quantitated by spotting 0.5 μL of the resuspended DNA on an ethidium bromide agarose plate, and comparing to a DNA standard. Fractions 1 and 2 were pooled to give a total of 120 ng of cDNA for the ligation step.

h) Ligation of cDNA into Uni-ZAP vector arms

The following were added to 120 ng of cDNA in 4.5 μL volume, to give the concentrations given in a volume of 7 μL :

Buffer #3 (35 mM Tris-HCl, pH 7.5, 4.9 mM MgCl_2)

0.7 mM rATP

1 μg Uni-ZAP XR vector arms

2 Weiss units T4 DNA ligase

A test insert provided by Stratagene was ligated at the same time. The reaction mixtures were incubated at 40°C for 2 days. *Note:* the concentrations of Buffer #3 and rATP were more dilute than indicated in the Stratagene protocol, because the cDNA had been resuspended in a larger volume (4.5 µL instead of 2.5 µL) than recommended.

iv) Packaging of Uni-ZAP vector into phage particles, and plating/titering

Upon completion of the ligation step, the naked phage DNA was present as a series of concatenated linear λ genomes – the same form the λ DNA takes following “rolling circle” replication in the lytic pathway of the λ phage life cycle. The DNA was then packaged into phage particles using Gigapack II Gold packaging extract (Stratagene, La Jolla, CA). The packaging steps cleave the concatenated DNA and package it with the proteins necessary to produce infectious phage particles. The two packaging extracts are called the “Sonic” extract, which contains the primary phage head proteins, and the “Freeze-Thaw” extract which contains the rest of the head proteins and the tail proteins.

a) Media and Buffers

LB medium - for 1 L medium:

- 10 g NaCl
- 10 g Bactotryptone
- 5 g Yeast extract
- [15 g agar - if making plates]
- autoclaved
- [antibiotics (sterile) - if using]

SM buffer - for 1 L of buffer:

- 5.8 g NaCl
- 2.0 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$
- 50.0 mL 1 M Tris-HCl, pH 7.5

5.0 mL 2% gelatin
autoclaved

NZY plates - for 1 L of medium:

5 g NaCl
2 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$
5 g Yeast extract
10 g NZ Amine (casein hydrolysate)
distilled water up to 800 mL
adjusted to pH 7.5 with NaOH
15 g agar
distilled water up to 1 L
autoclaved, equilibrated to 50°C, poured plates

NZY top agar - as above, but substituted 0.7% agarose for the 15 g agar.

b) Host bacteria

The host cells used for plating and titering are an *E. coli* strain designated XL1-Blue MRF'. This strain is mcrA^- and mcrB^- , which means that, unlike most strains of *E. coli*, this strain will not digest DNA containing 5'-methyl C (which was used in the cDNA synthesis procedure; Stratagene, 1993).

The glycerol stock of XL1-Blue MRF' was streaked on an LB-tet plate (12.5 $\mu\text{g/mL}$ tetracycline) and incubated overnight at 37°C. A single colony was used to inoculate 50 mL of LB medium in an Erlenmeyer flask, supplemented with 0.2% maltose and 10 mM MgSO_4 . The flask was incubated, with shaking (~200 rpm), at 37°C for 5 hours. The optical density (600 nm) of the culture was measured (it should not be allowed to grow past 1.0 OD_{600}). The cells were pelleted at 2000 rpm for 10 min, and then resuspended in sterile 10 mM MgSO_4 to an OD_{600} of 0.5.

c) Packaging procedure

A yellow tube ("Sonic" extract) from the Gigapack II Gold kit was thawed. A red tube ("Freeze-Thaw" extract) was warmed with fingers until just beginning to thaw. One μL of the DNA from the ligation reaction was immediately added to the Freeze-Thaw extract; the tube was then placed on ice. Fifteen μL of Sonic extract were then added to the Freeze-Thaw extract. The mixture was stirred with care so as not to introduce air bubbles, and then incubated at room temperature for 1.5 hours. SM buffer (500 μL) was then added, along with 20 μL of chloroform. The tube was spun in a microcentrifuge briefly to pellet the debris, and then stored at 4°C. After plating and titering (of this packaging reaction *and* of a test packaging reaction provided by Stratagene) showed the expected results, the remainder of the ligated ZAP-cDNA was packaged as above.

d) Plating and titering

One μL of the packaged reaction, and 1 μL of a 10^{-1} dilution, were added to separate 10 mL test tubes, along with 200 μL of the XL1-Blue resuspended cells. The tubes were incubated, with shaking, for 15 minutes at 37°C. NZY top agar was equilibrated to 50°C, and NZY plates were warmed to 37°C. The tubes were removed from the incubator; and 6 mL of top agar was added, along with 1.8 mg IPTG and 7.5 mg X-gal. Each tube was capped, inverted to mix, and then poured onto an NZY plate. The plates were allowed to stand for 10 minutes for the top agar to solidify, then they were inverted and incubated overnight at 37°C. From the number of plaques seen on the plates the following day the titer of the primary library was determined. Also, the number of blue plaques versus white plaques indicated the frequency of non-

recombination (in this case, no blue plaques were seen). The total titer for the primary library was determined to be 547,500 plaque-forming units (pfu). This was a sufficiently high titer to proceed to the amplification step.

v) Amplification of the primary library

The packaging mixtures were pooled and divided into ten fractions (9 fractions of 50,000 pfu, 1 fraction of 25,000 pfu). To these fractions 600 μ L of XL1 Blue cells ($OD_{600} = 0.5$) were added, and the mixtures were incubated at 37°C with shaking for 15 min. The phage/cell mixtures were then plated on NZY plates, as above, and incubated at 37°C for 8 hours. The plates were then overlaid with SM buffer (enough to cover the plates) and they were incubated at 4°C with slow rotation for 2 days. More SM buffer was added, as much of the original buffer had been absorbed by the agar. The following day the buffer was collected off the plates. The amplified fractions were kept in separate tubes; the volume of buffer in each fraction was 10-15 mL. The titer of the amplified library was $\sim 3 \times 10^9$ pfu/mL.

C) DNA Screening of cDNA library, Round 1

i) Phage plating and nytran lifts

For the first round of DNA screening, the cDNA library was plated on ten plates at a plaque density of 50,000 plaques per 150 mm plate, as suggested by the cDNA library kit manual (Stratagene, 1993), for a total of 500,000 pfu. One μ L from each of the ten fractions of the amplified library was diluted in 600 μ L of SM buffer. Ten μ L of this dilution were combined with 600 μ L of XL1-Blue cells ($OD_{600} = 0.5$, resuspended in 10 mM $MgSO_4$) in a test tube, and incubated at 37°C, with shaking, for 15 minutes.

Seven mL of NZY top agar were added, and the mixture spread over a large NZY agar plate. The top agar was allowed to gel (10 minutes) and then the plates were inverted and incubated at 37°C overnight. The next day the plates were cooled at 4°C for 30 min - 1 hour, to harden the top agarose, so it would not stick to the nytran. Nytran membranes were labeled with a pencil, two for each plate (e.g. 1A, 1B). The dry filters were then placed, label side down, in contact with the NZY plates. An 18-gauge needle was used to create asymmetric orientation holes, poking through the nytran membrane, into the agar plate (*Note*: in later screenings, the needle was first dabbed in India ink, to make the holes more visible). The filters were removed after 4 minutes, and a duplicate filter was “lifted” from the same plate in the same way. To denature the DNA, the filters were transferred consecutively (for 4 minutes each) to Whatman blotting paper soaked in the following solutions:

- 1) denaturing solution (0.5 M NaOH, 1.5 M NaCl)
- 2) neutralizing solution (0.5 M Tris pH 8, 1.5 M NaCl)
- 3) 2x SSC (300 mM NaCl, 30 mM trisodium citrate).

The filters were wet in 2x SSC and transferred to a cellophane-covered support. The support was placed inside the “UV Stratalinker 1800” (Stratagene, La Jolla, CA), which was set to “autocrosslink” mode. This exposed the blots to 1200 (x100) µJ of UV radiation. The blots were then transferred to 2x SSC again in preparation for pre-hybridization.

ii) Probe labeling procedure

Radioactively labeled probes were created using reagents borrowed from J. Ngsee. A culture of *E. coli* cells harbouring the probe plasmid was grown overnight in LB-ampicillin. The plasmid DNA was isolated using the Qiagen Q-midi plasmid prep kit (Qiagen, Inc., Chatsworth, CA). The probe fragment was removed from the plasmid by digestion with the appropriate restriction enzyme(s). The fragment was purified by agarose gel electrophoresis (Sambrook *et al.*, 1989) followed by the Geneclean glass milk DNA purification system (Bio 101). The mass of DNA in the purified fragment was determined approximately by comparing to a known standard on an agarose gel.

Purified template DNA (25-200 ng) was combined with 5 μ L Random Priming buffer (200 mM Tris-HCl pH 7.5, 40 mM $MgCl_2$, 15 ng/ μ L random hexamers), and distilled water added to 10 μ L. This mixture was boiled for 5 minutes, then cooled on ice. Then 4 μ L of a nucleotide mix containing 2.5 mg/mL acetylated BSA and 1.25 mM each of dGTP, dATP and dTTP were added, followed by 5 μ L of ^{32}P -dCTP (10 μ Ci/ μ L; 3000 or 6000 Ci/mmol) and 4 units of Klenow fragment. This mixture was incubated at 37°C for 1 hour. A 0.5 μ L aliquot of this reaction was removed and added to 5 mL of scintillation fluid for later counting.

The labeled DNA probe was purified on a Stratagene Nuc-Push column, to remove unincorporated nucleotides. A disposable Sephadex size-exclusion column was prewet with 70 μ L STE buffer (100 mM NaCl, 20 mM Tris, pH 7.5, 10 mM EDTA). Fifty μ L of STE were added to the 20 μ L volume of the random labeling reaction, and the total volume was loaded onto the column. This volume was forced through the

column using the shielded Nuc-Push device, and collected in a tube at the bottom. Another 70 μL of STE buffer was loaded onto the column and collected into the same tube as combined fraction “1-2”. Fractions three through five were subsequently collected from the column in a similar manner. The volume of the fractions was measured, and from each a 0.5 μL aliquot was removed and added to 5 mL of scintillation fluid. These fractions were counted with a Beckman scintillation counter. In most cases, only the combined fraction “1-2” was subsequently used.

Scintillation analysis was done on aliquots of the labeling mixture and the purified probe to determine probe characteristics. The percentage of radioactivity incorporated into the DNA probe was determined by calculating the total counts present in the purified probe fraction, and dividing by the total counts in the labeling mixture. The amount of newly synthesized DNA produced in the reaction labeling mixture was calculated according to the following equation (from the manual provided by Gibco BRL with the random priming kit):

$$\text{new DNA} = \frac{\mu\text{Ci dCTP} \times 13.2 \times \% \text{ incorporation}}{\text{specific activity dCTP (Ci / mmol)}} \quad (1)$$

The purified probe was boiled for 5 minutes, then cooled on ice. It was then added to the hybridization solution.

iii) Probing with CHP1

The nytran filters were first probed with the hamster homologue CHP1. The probe was prepared by random priming. The filters were prehybridized in a 50% formamide prehybridization solution

50% formamide
6x SSC (900 mM NaCl, 90 mM trisodium citrate)
5x Denhardt's solution (10 mg/mL each of polyvinylpyrrolidone, Ficoll and BSA)
0.5% sodium dodecylsulfate (SDS)
100 µg/mL sheared, denatured (boil 5 minutes, cool on ice) salmon sperm DNA

The filters were prehybridized for 4 hours at 42°C and hybridized overnight at 42°C. The following day, the filters were rinsed twice, then washed for 30 minutes at 42°C in 5x SSC, 0.2% SDS. The filters were then exposed to film for two days. After the exposure, the orientation holes were marked on the autoradiograph, and the films were aligned. The filters were stripped of the radioactive probe by washing twice for 15 minutes with boiling 0.04x SSC (6 mM NaCl, 0.6 mM trisodium citrate), 0.2% SDS.

iv) Probing with mssp11

The stripped filters were prehybridized again, this time in a completely aqueous solution (*i.e.* no formamide) at 55°C for 1.5 hours. Hybridization took place overnight at 55-58°C. The following day the filters were rinsed twice, then washed with 5x SSC, 0.2% SDS for 30 minutes at 40°C, then an additional 30 minutes at 42°C. The filters were wrapped in cellophane and exposed to film. Thirteen putative positives were identified from the duplicate autoradiographs. These 13 were “picked” by coring the plaque from the agar plate with the wide end of a sterile Pasteur pipette. The cores were placed into 200 µL SM buffer (with 1 drop chloroform), and left overnight at 4°C for the phage to diffuse into the SM medium. These phage stocks were titered, then used for secondary screening. The phage was plated out in essentially the same way as for primary screening, except that smaller NZY agar plates were used (80 mm), and the plaque density was very low (50-200 pfu per plate). Nytran lifts were prepared as per the

primary screening protocol, using smaller circles of nytran. The probing was carried out as per the primary screening protocol. The primary library filters were stripped as described above.

It was determined that plating with XL1-Blue cells at $OD_{600} = 0.5$ might have produced a bacterial lawn that was too thin; consequently, there might not have been enough DNA in the plaques to produce a positive signal (P. Juranka, personal communication). New plates were made using cells resuspended at $OD_{600} = 5.0$, and primary screening was carried out again, exactly as outlined in this section.

iii) Probing with V-ATPase

Two of the stripped filters were probed with V-ATPase, a gene known to be present in *Manduca* midgut (Dow *et al.*, 1992). The filters were pre-hybridized for 1h, 15 min at 55°C in aqueous pre-hybridization solution. The filters were hybridized overnight at 55-59°C with shaking. The following day, the filters were rinsed twice with 5x SSC, 0.2% SDS, then washed in same for 30 minutes at 49°C. This was followed by a 1h, 20 min wash in 5x SSC, 0.2% SDS at 55°C, and again by an hour-long wash in 2.5x SSC, 0.1% SDS at 55°C. The filters were exposed overnight, but demonstrated very high background, so the filters were rewashed at high stringency (30 min., 0.1x SSC, 65°C) and re-exposed. Following this exposure the filters were stripped as outlined above.

iv) Probing with *mdr49*, *mdr65*

The stripped filters were pre-hybridized in aqueous pre-hybridization solution for 3h20 at 50°C. The probes were prepared separately by random priming (as above), then pooled and added to the pre-hybridization solution. The filters were hybridized overnight

at 50°C. The following day, the filters were rinsed and then washed twice (50 min., 50 min.) in 5x SSC, 0.2% SDS at 42°C. The filters were exposed to film. Nine putative positives were identified, and secondary plating was carried out as above.

D) DNA screening of cDNA library, Round 2

The cDNA library was screened again, after the results from a plaque density experiment indicated that the plaque density in the previous screening had been too high (see Chapter 3). The library was plated on 20 plates at a density of ~15 000 pfu per large 150-mm plate, for a total of 300,000 pfu. Two platings were done for each of the ten fractions of the library, and duplicate filters were lifted in the same way as for the first screening. The DNA was denatured and cross-linked to the membranes in the same way as for the first screening.

i) Probing with a DNA probe cocktail: mssp11, DM13, SP01, TU15, TU19

The filters were first probed with a cocktail of 5 probes: mssp11, DM13, TU19, TU15 and SP01. The filters were pre-hybridized in 30% formamide pre-hybridization solution at 37°C for >1 hour. The probes were prepared individually by random priming, and added to the hybridization solution as a cocktail. The filters were hybridized overnight at 37°C with shaking. The following day the filters were washed for 15 minutes at 37°C with 5x SSC, 0.2% SDS, 15 minutes at 42°C with 2.5x SSC, 0.1% SDS, and 15 minutes at 42°C with 2x SSC, 0.2% SDS. The filters were wrapped in cellophane and exposed to film. Following exposure, the filters were stripped as described above.

ii) Probing with a second cocktail: CHP1A, mdr49, mdr65, DM13, SP01

Then the filters were probed with a second cocktail of probes: CHP1A, mdr65, mdr49, and again, DM13 and SP01. The filters were prehybridized in 30% formamide

pre-hybridization solution at 37°C for 3 days, with shaking. The probes were prepared as usual, and added to the hybridization solution as a cocktail. The filters were hybridized at 37°C for 1.5 days. The filters were then washed in 5x SSC, 0.2% SDS, 37°C for 40 minutes. The filters were wrapped in cellophane and exposed to film.

Positive signals were detected on the films at a ratio of 1 for every 1500 plaques. Seventeen of the positives were picked into SM buffer. Secondary plating of these picks was done as outlined in the first screening procedure. The secondary filters were pre-hybridized as for primary plating. The same five probes were added to the hybridization solution in a cocktail. The filters were hybridized at 37°C, with shaking, for 1.5 days, then washed at 37°C for 30 minutes with 5x SSC, 0.2% SDS. The filters were wrapped in cellophane and exposed to film.

Six plaques each that demonstrated positive signals from secondary plates 1 and 6 were picked into 0.5 mL SM buffer. These phage were converted to pBluescript plasmids by *in vivo* excision, as outlined in the Stratagene manual (Stratagene, 1993). Plasmid mini-preps of these were prepared; the plasmids were cut with *EcoRI* and *XhoI* and run on an agarose gel to determine the sizes of their inserts. Plasmid midi-preps of clones 6-1 and 6-5 were prepared and sequencing of the inserts was done using the Sequenase dideoxy sequencing kit (Sequenase). The sequence data were transmitted to the BLAST Genbank searching program at NIH by electronic mail (Gish and States, 1993; Altschul *et al.*, 1990).

Results

A) Construction of a λ ZAP cDNA library from *Manduca* Malpighian tubule tissue

A cDNA library was constructed using the Malpighian tubules of 35 fifth-instar *Manduca sexta* as the RNA source. The total RNA was extracted using a variation of the guanidinium thiocyanate/acid/phenol/chloroform method of Chomzynski and Sacchi (1987). Messenger RNA was purified from the total RNA by oligo-dT chromatography (Sambrook *et al.*, 1989). The library was constructed using Stratagene's ZAP-cDNA synthesis kit and packaged with Gigapack II Gold packaging extract (Stratagene, La Jolla, CA). Size analysis of the cDNA during the procedure showed that cDNAs of adequate size were isolated (Figure 2.6). The titer of the primary library was 550,000 plaque-forming units (pfu). This primary library was divided into 10 equal fractions which were separately amplified. After amplification the titer was 3×10^9 pfu/mL.

The cDNA inserts in the λ ZAP vector were shown to be of adequate size for the purposes of the project. Over the course of this project, the inserts of 35 distinct clones were excised and sized against a molecular weight marker in agarose gel electrophoresis. The size distribution of these inserts is shown in Figure 2.7. The mean size of the inserts is 1.44 ± 0.61 kilobasepairs (kbp). The range of insert size is 0.28 to 4.6 kbp. The total size of the open reading frame of P-glycoprotein is 3.8 kbp, and the highly conserved Walker motif of the nucleotide binding fold is within the last 800 base pairs of the open reading frame (see Figure 2.4). Since the cDNA synthesis proceeds from the 3' end of the message toward the 5' end, even truncated cDNAs should be detectable by the probes.

which are mostly derived from the Walker motifs. Even if the first cDNAs to be identified were truncated, they could be used to subsequently probe for the whole gene.

B) cDNA library screening, Round 1

i) Probing with CHP1

Table 2.1 summarizes information about the radioactive labeling of the CHP1 probe (tables placed at end of Chapter 2). No positive signals were identified from the autoradiographs of the filters screened with this probe.

ii) Probing with mssp11

Table 2.2 summarizes information about the radioactive labeling of the mssp11 probe. None of the 13 picks from primary screening was confirmed on secondary screening.

Upon repeating the experiment using a higher concentration of *E. coli* in the platings, less background signal was seen on the autoradiographs, but no positives were identified.

iii) Probing with V-ATPase

Table 2.3 summarizes information about the radioactive labeling of the V-ATPase probe. This probing served as a positive control for the screening procedure, as this gene is known to be expressed in Malpighian tubules (Dow *et al.*, 1992). The probing resulted in a large number of strong positive signals (Figure 2.8). The positive signals are round or oval, with characteristic smears or “rocket tails” which are the result of the slight smudging of plaque DNA which occurs as the nytran membrane is peeled off the agar plate. The gene constitutes a high proportion of the tubule library, approximately 1 in 500 cDNAs.

iv) Probing with *mdr49* and *mdr65*

Table 2.4 summarizes information about the radioactive labeling of the *mdr49* and *mdr65* probes. Putative positive signals were seen in the primary screening, however, not one of these signals was confirmed upon secondary screening.

C) cDNA library screening, Round 2

i) Probing with DNA probe cocktail: *mssp11*, *DM13*, *SP01*, *TU15*, *TU19*

The DNA probes were individually labeled by the random primer method and then combined into a probe cocktail. Table 2.5 summarizes information about the radioactive labeling of the probes. No positive signals were seen on the autoradiographs for this experiment.

ii) Probing with a second probe cocktail: *CHP1A*, *mdr49*, *mdr65*, *SP01*, *DM13*

Table 2.6 summarizes information about the radioactive labeling of the probes. This probing yielded a large number of positives: approximately 100 positives per plate. for a rate of 1 in 150 plaques (Fig. 2.9). Sixteen of the strongest positives from eight different plates were picked for secondary plating, and two “picks” were also removed from areas that were clearly negative.

Table 2.7 summarizes information about the radioactive labeling of the probes prepared for the secondary screening. On the autoradiographs of the secondary screening the negative picks were clearly shown to be negative in the secondary screening, but only some, not all, of the lifts from “positive” picks resulted in clearly positive signals on the secondaries. Six pure clones each were picked from two of the secondary plates designated 1 and 6. These clones were converted to phagemids (plasmids) and the inserts were sized by agarose gel electrophoresis (Fig 2.10). The six clones from secondary

plate 1 (1-1...1-6) had inserts of completely different sizes, indicating that false positives did account for some of the signals seen. Of the six clones from secondary plate 6, four (6-1, 6-2, 6-3, 6-5) had inserts that were the same, while the other two were different. This indicated that there was a mix of true and false signals generated by this probing. The clones 6-1 and 6-2 were partially sequenced, and found to be identical. However, when the sequence was sent to the BLAST server on the Internet for comparison to known sequences in Genbank, it was identified as a chymotrypsinogen transcript (i.e. the inactive precursor of the digestive protease chymotrypsin).

Discussion

A *Manduca* Malpighian tubule cDNA library was constructed, and it possesses many of the qualities of a good library: It is of high titre (5.5×10^5 pfu in the primary library), which means that unless the Pgp homologue is expressed only in very low quantities, it is likely to be represented in the library (see further discussion of this point below).

The cDNA inserts appear to be of adequate size. There is a good distribution of sizes; a majority of the inserts are 1 to 2 kilobases in size, while there are also many that are larger (the largest insert that was excised from λ ZAP was 4.6 kb; cDNAs as large as 6 kb were seen in the autoradiograph of the second strand reaction, Fig. 2.3). The size of the inserts is important because reverse transcriptase will not always convert the entire mRNA transcript to a cDNA of equivalent length; occasionally the cDNAs are truncated. The reverse transcriptase works from the poly-A tail of the transcript towards the 5' end, thus any truncated cDNAs will represent the 3' end of the molecule. In P-glycoprotein,

the highly conserved Walker sequence is within the last 800 bases of the transcript (Figure 2.4). Hence, even if many of the Pgp cDNAs are truncated, they should still be detectable by the DNA probes which are derived from this conserved region of the molecule. Given that the full sequence of Pgp is approximately 3.8 kbp, it is possible that the transcript is present in its entirety.

Another indication that the library is of good quality is that I was able to identify and isolate *Manduca* genes. A DNA probe of the previously cloned (Dow *et al.*, 1992) *Manduca* vacuolar ATPase identified a large number of strongly positive plaques. (Figure 2.8). As well, several other *Manduca* genes were identified as the result of falsely positive signals appearing during the cloning process. These clones were brought through to the sequencing stage of the cloning process, and the limited sequence was enough to identify them with a BLAST search. Two such genes had been previously cloned from *Manduca*, cytochrome c oxidase (subunit 1), and chymotrypsinogen, but two others had not been cloned from *Manduca* before. One was pyruvate carboxylase, identified by its homology to mosquito (65%) and human (58%) versions of the gene. The other was ADP/ATP translocase, which was homologous to the *Drosophila* (72%) and bovine (59%) equivalents listed in Genbank. This latter clone was sequenced in its entirety and used as a probe in Southern and Northern blot analyses (see Chapter 4).

Despite having a seemingly high quality cDNA library, clones of a *Manduca* Pgp homologue were not obtained. The next chapter describes experiments which were performed to try to determine reasons for this result.

Table 2.1: Probe labeling and radioactivity data for the hamster Pgp-homologous probe CHP1.

Probe characteristic	Probe
	CHP1
probe template size (bp)	600
template DNA (ng)	180
newly synth. DNA (ng)	n/a
percentage incorporation	n/a
specific activity (cpm/ μ g)	1.8×10^8
activity in hybrid. solution (cpm/mL)	3.2×10^5
[DNA] in hybrid. solution (ng/mL)	1.8

Table 2.2: Probe labeling and radioactivity data for the *Manduca* genomic fragment mssp11.

Probe characteristic	Probe
	mssp11
probe template size (bp)	450
template DNA (ng)	80
newly synth. DNA (ng)	4.0
percentage incorporation	18%
specific activity (cpm/ μ g)	1.1×10^8
activity in hybrid. solution (cpm/mL)	9.3×10^4
[DNA] in hybrid. solution (ng/mL)	0.84

Table 2.3: Probe labeling and radioactivity data for the *Manduca* V-ATPase probe.

Probe characteristic	Probe
	V-ATPase
probe template size (bp)	1400
template DNA (ng)	300
newly synth. DNA (ng)	3.1
percentage incorporation	14%
specific activity (cpm/ μ g)	3.6×10^7
activity in hybrid. solution (cpm/mL)	7.6×10^4
[DNA] in hybrid. solution (ng/mL)	3.0

Table 2.4: Probe labeling and radioactivity data for two Pgp-homologous probes from *Drosophila melanogaster*, mdr49 and mdr65.

Probe characteristics	Probe	
	mdr49	mdr65
probe template size (bp)	1800	1200
template DNA (ng)	300	240
newly synth. DNA (ng)	2.6	4.6
percentage incorporation	12%	21%
specific activity (cpm/ μ g)	4.5×10^7	1.1×10^7
activity in hybrid. solution (cpm/mL)	1.36×10^5	2.6×10^5
[DNA] in hybrid. solution (ng/mL)	3.0	2.4

Table 2.5: Probe labeling and radioactivity data for five Pgp-homologous probes, the *Manduca* genomic fragment mssp11, DM13 from *Drosophila melanogaster*, SP01 from *Spodoptera frugiperda*, TU19 and TU15 from *Tetranicus urticae*.

Probe characteristics	Probe				
	mssp11	DM13	SP01	TU19	TU15
probe template size (bp)	450	490	485	490	750
template DNA (ng)	25	25	25	25	25
newly synth. DNA (ng)	1.6	1.7	1.6	1.7	5.1
percentage incorporation	7.4%	7.9%	7.1%	7.8%	23%
specific activity (cpm/ μ g)	7.8×10^8	7.0×10^8	8.0×10^8	7.3×10^8	2.4×10^9
activity in hybrid. solution (cpm/mL)	1.0×10^5	9.3×10^4	1.0×10^5	9.7×10^4	3.6×10^5
[DNA] in hybrid. solution (ng/mL)	0.13	0.13	0.13	0.13	0.15

Table 2.6: Probe labeling and radioactivity data for five Pgp-homologous probes, CHP1A from Chinese hamster, mdr49, mdr65 and DM13 from *Drosophila melanogaster*, SP01 from *Spodoptera frugiperda*.

Probe characteristics	Probe				
	CHP1A	mdr49	mdr65	DM13	SP01
probe template size (bp)	430	1800	1200	490	485
template DNA (ng)	25	25	25	25	25
newly synth. DNA (ng)	2.9	1.1	1.2	0.88	5.6
percentage incorporation	13%	5.1%	5.5%	4.0%	24%
specific activity (cpm/ μ g)	8.8×10^8	2.8×10^8	4.0×10^8	2.4×10^8	7.8×10^8
activity in hybrid. Solution (cpm/mL)	1.2×10^5	3.6×10^4	5.3×10^4	3.1×10^4	1.2×10^5
[DNA] in hybrid. solution (ng/mL)	0.14	0.12	0.12	0.12	0.14

Table 2.7: Probe labeling and radioactivity data for five Pgp-homologous probes. CHPIA from Chinese hamster, mdr49, mdr65 and DM13 from *Drosophila melanogaster*. SP01 from *Spodoptera frugiperda* (secondary screening).

Probe characteristics	Probe				
	CHPIA	mdr49	mdr65	DM13	SP01
probe template size (bp)	430	1800	1200	490	485
template DNA (ng)	25	25	25	25	25
newly synth. DNA (ng)	1.3	n/a	1.0	1.8	2.6
percentage incorporation	7.4%	n/a	6.0%	10%	15%
specific activity (cpm/ μ g)	2.1×10^8	8.0×10^8	3.5×10^8	2.5×10^8	4.0×10^8
activity in hybrid. Solution (cpm/mL)	5.6×10^4	2.0×10^5	9.0×10^4	6.6×10^4	1.1×10^5
[DNA] in hybrid. solution (ng/mL)	0.26	0.25	0.26	0.27	0.28

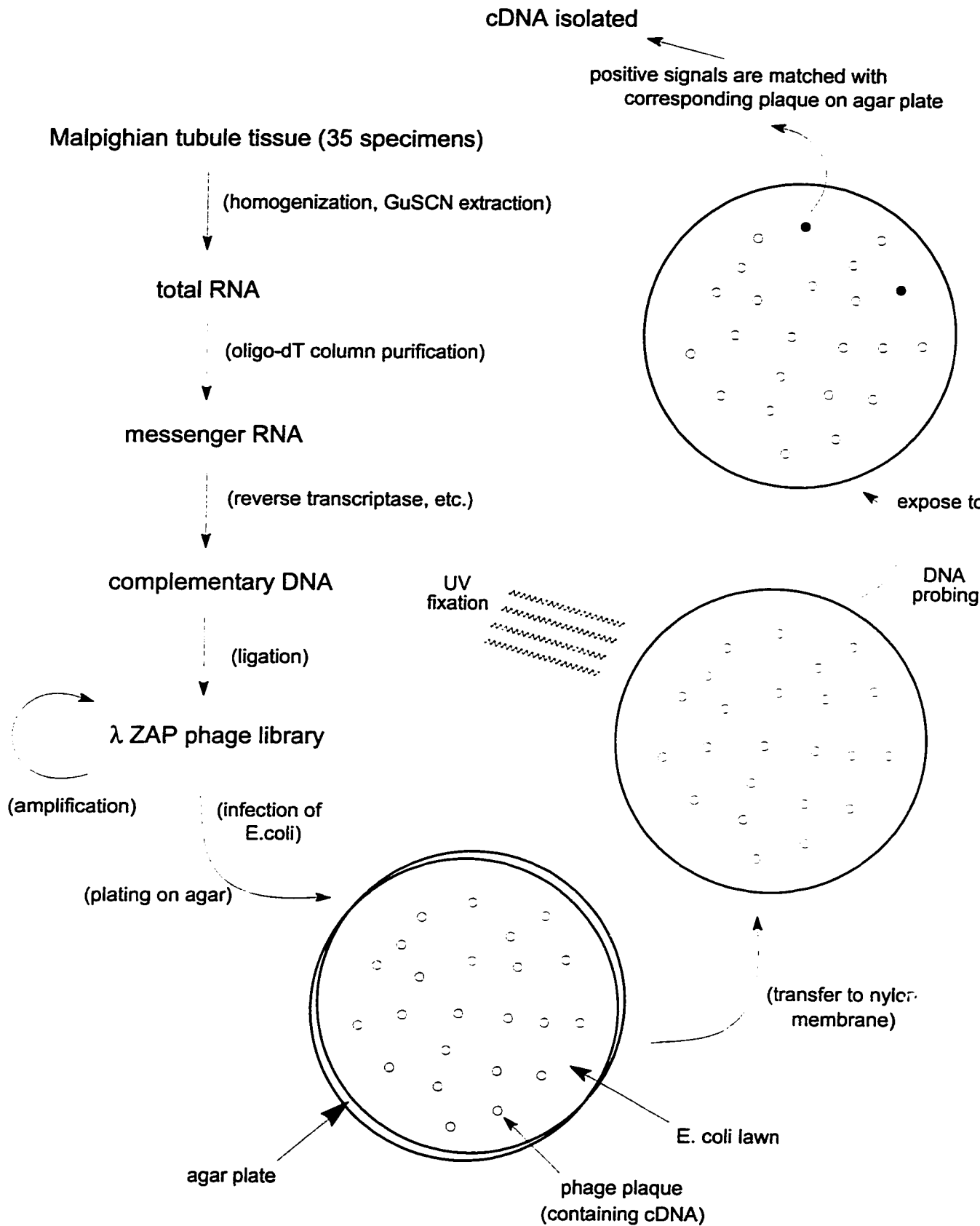


Figure 2.1: Flow chart illustrating cDNA library construction and screening procedure. Please see text for details.

```

      10      20      30      40      50      60
5' GGCAAACGGTGGCGGTGGTGGGGGAAAGTGGCTGCGGTAAAAGTACATTAATACAAATTT
      70      80      90     100     110     120
TAATGCGTTTCTATGATCCTGATTCTGGTTTAATTGTAAGTATATCATATTCTAGAGCAA
                                     XbaI
      130     140     150     160     170     180
CAAAGTTTAATAAAAAATATATTTTGATTTAATTCTTTAGGAGGTTGATGGTATTGATAT
      190     200     210     220     230     240
AAGAGATATCAATATAACTCATTTACGAAGTCACATTGGTTTTGTTGGACAAGAACCGGT
      ^
      EcoRV
      250     260     270     280     290     300
ACTTTTTTAGCGGGACAATAGCGTATAATATAACTTCTGGTCTAGAAAGTCATATTGAGCA
                                     ^
                                     XbaI
      310     320     330     340     350     360
AAGCGAAATTGAAGCTGCTGCCAAGAAGGCCAAAATTCATGATTTTCATTAAATCTCTTCC
      ^
      BglI
      370     380     390     400     410     420
CTTGAAATATGAAACAATGGTTGGCGAAAGAGGAATGCTACTAAGTGGAGGCCAAAAGCA
      ^
      DdeI
      430
ACGCATCGCCA 3'

```

Figure 2.2: Complete sequence of *msspl1*, a 431 bp fragment of a P-glycoprotein gene, isolated by PCR of *Manduca sexta* genomic DNA with degenerate primers to the Walker A and B sites of the ATP-binding domains (Ell and Drouin, unpublished). The sequence contains one 66 bp intron indicated by outline. Sites for commonly-used restriction enzymes are indicated. The fragment is relatively GC-poor at 36%. The fragment is ligated into a pBluescript KS plasmid in the *SmaI* site.

pir||A41249 multidrug resistance protein homolog Mdr49 - fruit fly
(*Drosophila melanogaster*) (M59076) P glycoprotein [*Drosophila melanogaster*] Length = 1302

Plus Strand HSPs:

Score = 114 (52.4 bits), Expect = 4.4e-30, Sum P(3) = 4.4e-30
Identities = 26/53 (49%), Positives = 34/53 (64%), Frame = +3

mssp11 → Query: 138 EVDGIDIRDINITHLRSHIGFVGQEPVLFSGTIAYNITSGLESHIEQSEIEAA 296
++DG D+R +N+ LRS IG VGQEPVLF+ TI NI G S + +AA
mdr49 → Sbjct: 462 KLDGRDLRTLNVGWLRSQIGVVGQEPVLFATTIGENIRYGRPSATQADIEKAA 514

Score = 109 (50.1 bits), Expect = 4.4e-30, Sum P(3) = 4.4e-30
Identities = 21/38 (55%), Positives = 27/38 (71%), Frame = +3

Query: 276 QSEIEAAAKKAKIHDFIKSLPLKYETMVGERGMLLSGG 389
Q++IE AA+ A HDFI LP Y+T VGE+G +SGG
Sbjct: 507 QADIEKAARAANCHDFITRLPKGYDTQVGEKGAQISGG 544

Score = 97 (44.6 bits), Expect = 4.4e-30, Sum P(3) = 4.4e-30
Identities = 16/23 (69%), Positives = 21/23 (91%), Frame = +1

Query: 4 SGCGKSTLIQILMRFYDPDSGLI 72
SGCGKSTLIQ++ RFYDP++G +
Sbjct: 439 SGCGKSTLIQLMQRFYDPEAGSV 461

Score = 86 (39.6 bits), Expect = 5.9e-27, Sum P(3) = 5.9e-27
Identities = 17/39 (43%), Positives = 24/39 (61%), Frame = +3

Query: 270 IEQSEIEAAAKKAKIHDFIKSLPLKYETMVGERGMLLSG 386
+ EI AAKK+ IH+FI +LP Y+T +G+ L G
Sbjct: 1165 VSMQEIIEAAKKSNIHNFISALPQGYDTRLGKTSQLSGG 1203

Score = 83 (38.2 bits), Expect = 9.4e-23, Sum P(3) = 9.4e-23
Identities = 18/39 (46%), Positives = 23/39 (58%), Frame = +3

Query: 141 VDGIDIRDINITHLRSHIGFVGQEPVLFSGTIAYNITSG 257
+ G+ + + LRS +G V QEPVLF TIA NI G
Sbjct: 1120 LSGVPSTEFPLDTLRSLGLVSGEPVLFDRITIAENIAYG 1158

Score = 80 (36.8 bits), Expect = 1.9e-20, Sum P(3) = 1.9e-20
Identities = 14/23 (60%), Positives = 19/23 (82%), Frame = +1

Query: 4 SGCGKSTLIQILMRFYDPDSGLI 72
SG GKST +Q+L+R+YDP SG +
Sbjct: 1096 SGSGKSTCVQLLLRYDPSGVS 1118

N-terminal
half

C-terminal
half

Figure 2.3: Comparison of the predicted amino acid sequence of mssp11 with that of the *Drosophila melanogaster* P-glycoprotein gene *mdr49*, using the BLAST search algorithm (Gish and States, 1993; Altschul *et al.*, 1990). The BLAST program finds High-scoring Segment Pairs (HSPs): regions of the subject sequence (*mdr49*) that have a high similarity to the query sequence (mssp11). The two sequences are shown and identical amino acids are indicated; functionally similar amino acids (“positives”) are indicated by “+”. Here, the program has found three regions in mssp11 with high scores, and is comparing each region to the N-terminal and C-terminal ATP-binding sites of *mdr49*. The percentage of identical and positive amino acids are indicated for each HSP. Note that in this case, the mssp11 sequence shares greater similarity with the N-terminal half of the gene than the C-terminal half; however, this does not hold true in other comparisons.

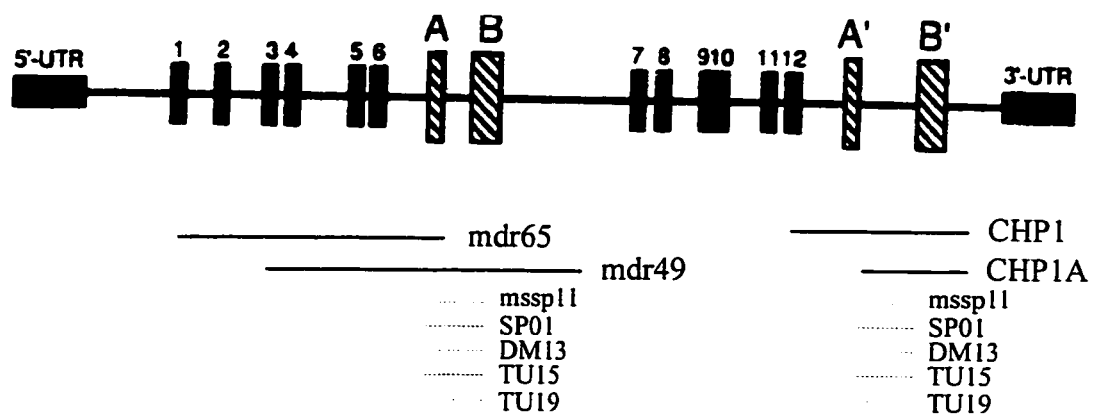


Figure 2.4: Cartoon depicting the positions of the DNA probes relative to a generic P-glycoprotein cDNA sequence. The transmembrane domains (1-12) are indicated, as are the Walker A and B sites of the ATP-binding domains. The exact positions of the genomic clones mssp11, SP01, DM13, TU15 and TU19 are not known. As well, the introns are not included in the representations of these five probes. Adapted from Endicott *et al.* (1991).

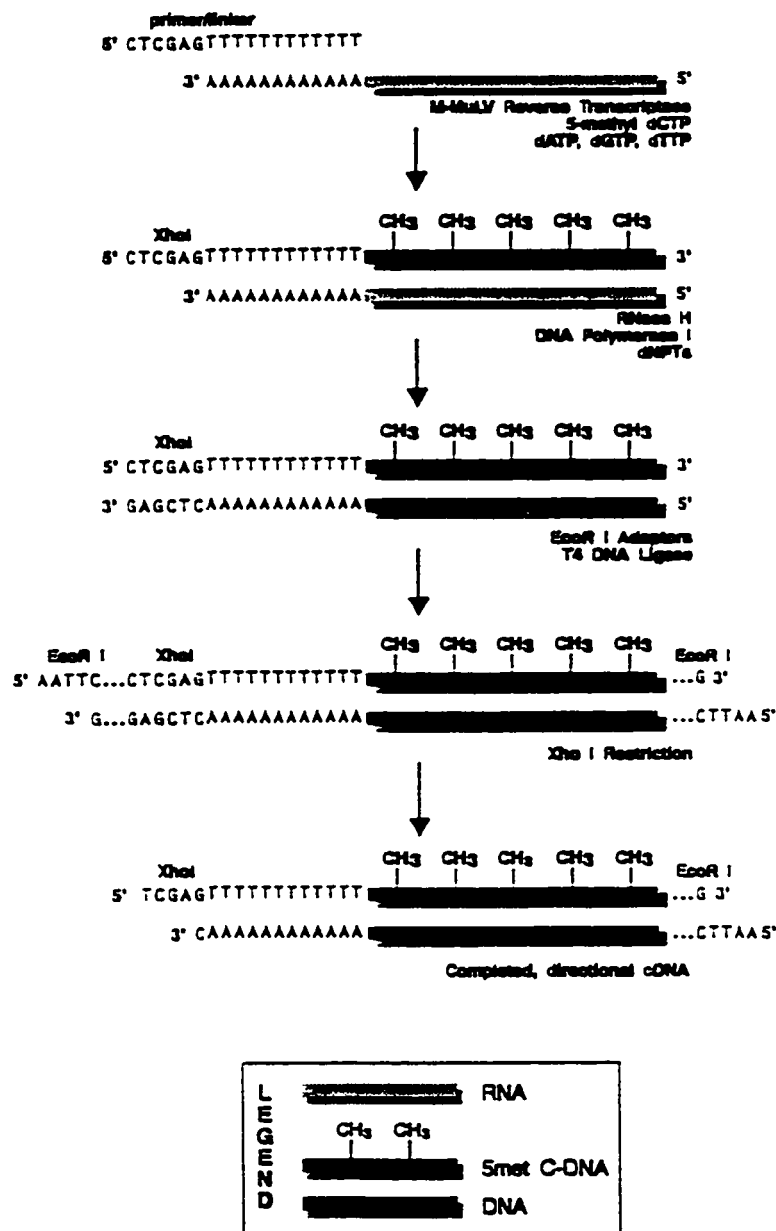


Figure 2.5: Flow chart of the procedure for producing directional *XhoI/EcoRI*-linked cDNA from mRNA (Stratagene, 1993). Messenger RNA is reverse-transcribed with methyl-dCTP so that the cDNA is protected from the subsequent *XhoI* digestion required to make *XhoI/EcoRI* “sticky ends”.

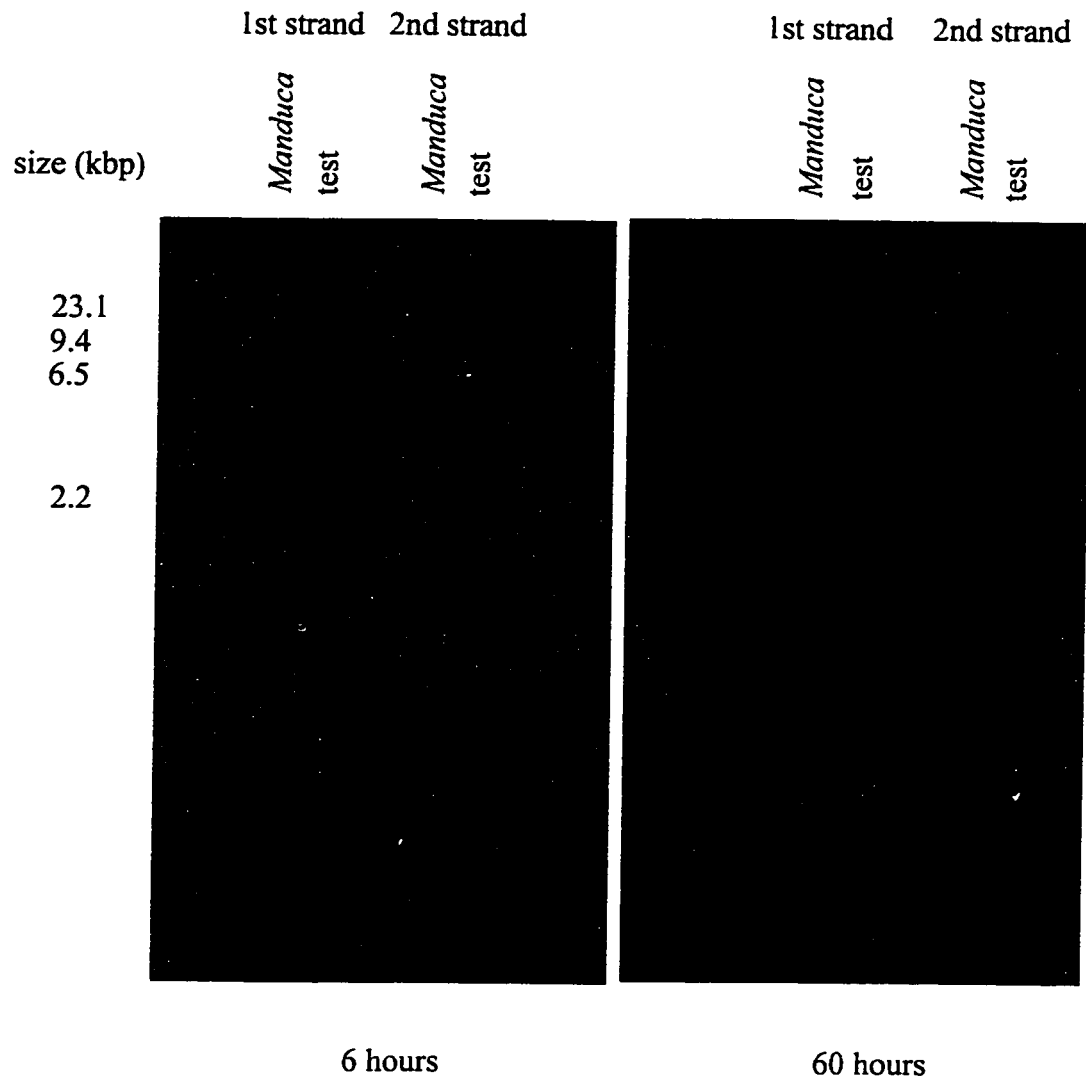


Figure 2.6: Size analysis of first and second strand cDNA reactions. Shown are autoradiographs after 6 and 60 hours of exposure. The left lane is cDNA prepared from *Manduca* mRNA, the right lane is the test cDNA provided with the cDNA synthesis kit. The position of the molecular weight markers is shown. Note that the maximum size of the 2nd strand cDNAs is approximately 6500 bp.

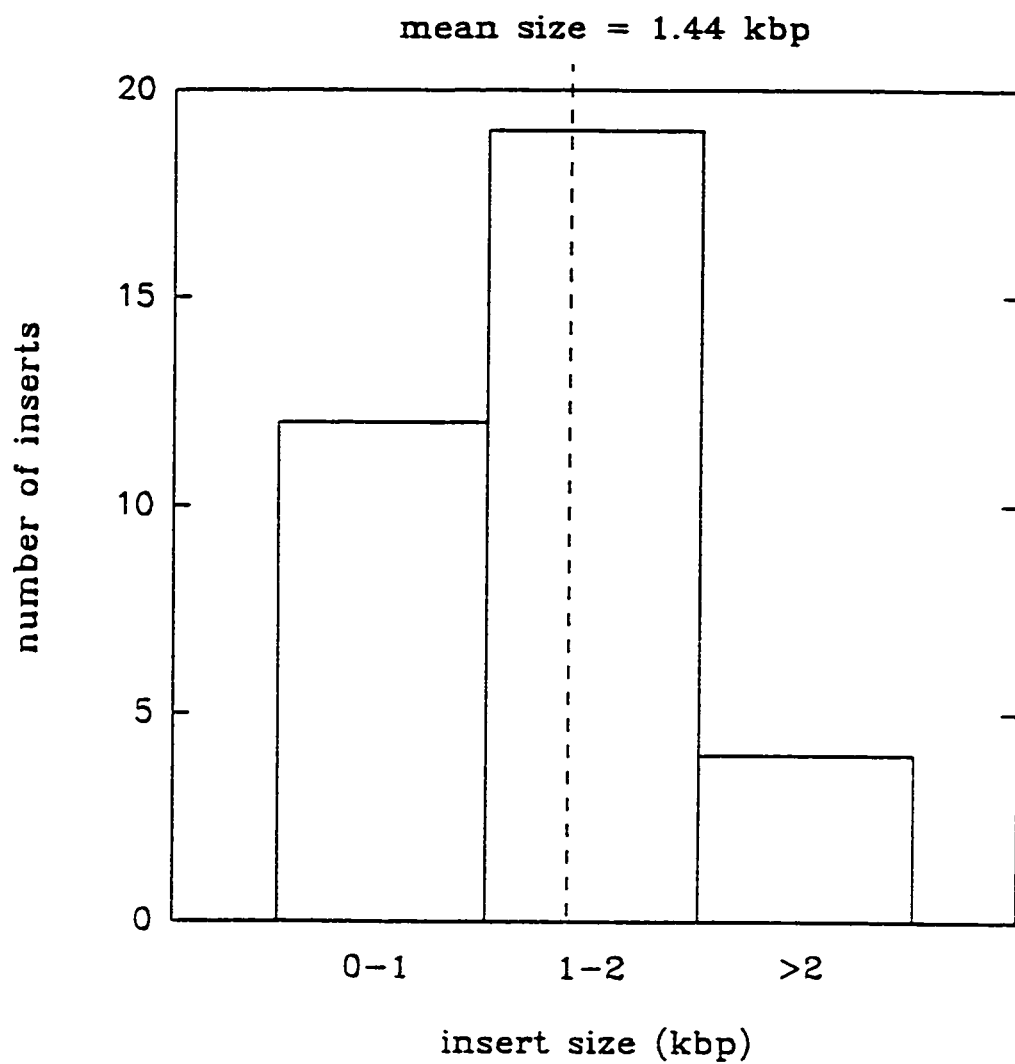


Figure 2.7: Size distribution of 35 clones isolated from the *Manduca* Malpighian tubule library. The range of insert sizes is 0.28 to 4.6 kbp.

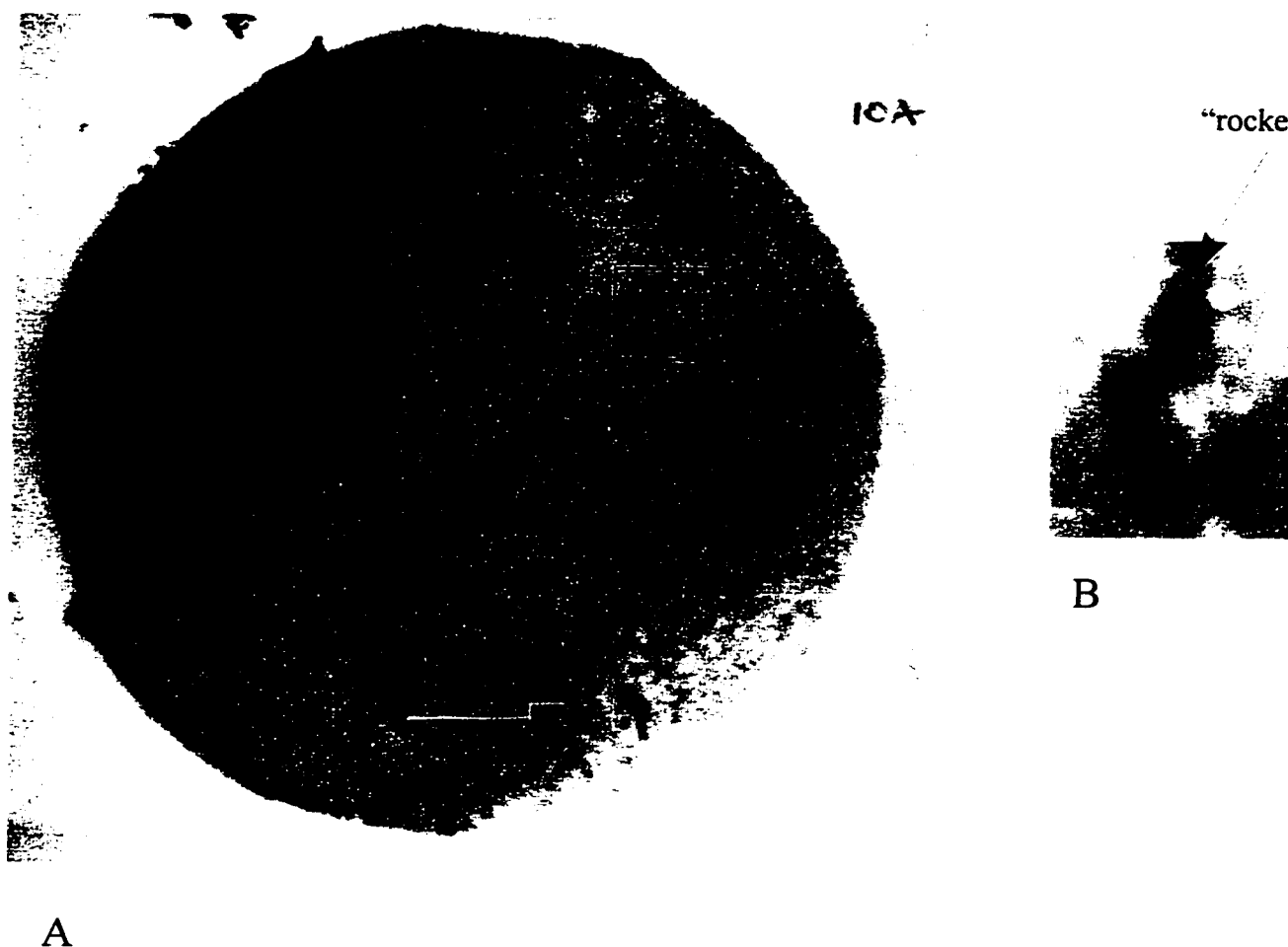


Figure 2.8: A) Autoradiograph of a nytran lift of a *Manduca* Malpighian tubule cDNA library, probed with a *Manduca* V-ATPase DNA probe. The lift was obtained from an agar plate of 50 000 plaque-forming units (pfu) of fraction #10 of the cDNA library. The density of plaques is 320 pfu/cm². Approximately 100 positive signals can be seen. The rectangle indicates the area enlarged in B. B) Enlarged and contrast-enhanced image of a small grouping of positive signals. The arrow indicates a characteristic "rocket tail" formation caused by smearing of the plaque DNA as the nytran filter is lifted from the agar plate.

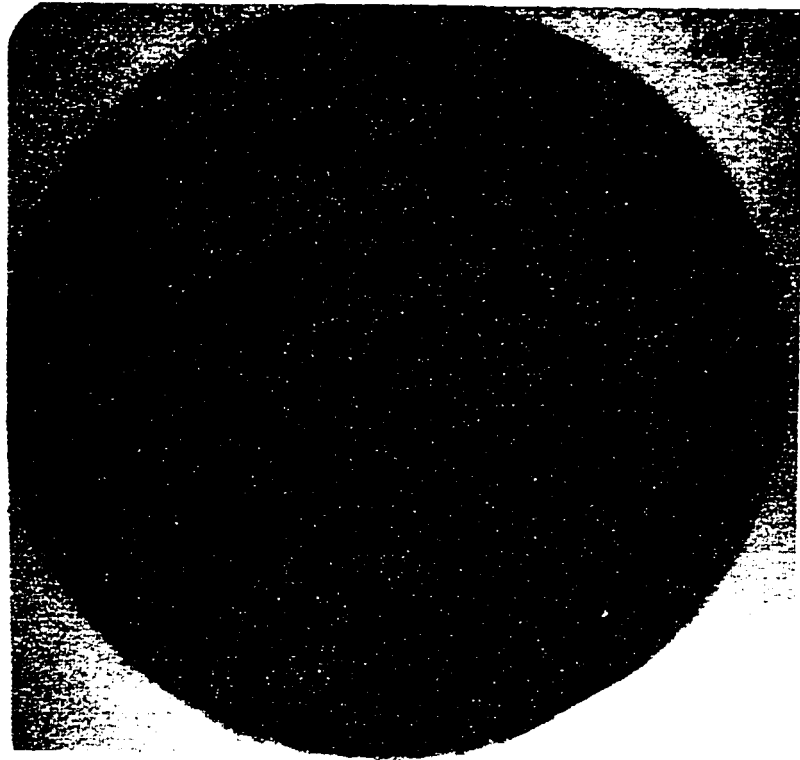


Figure 2.9: Autoradiograph of a nytran lift from the second round of *Manduca* Malpighian tubule cDNA library screening, probing with a cocktail containing CHP1A, mdr49, mdr65, DM13 and SP01. The lift is representative of 15 000 plaque-forming units (pfu), plated at a density of 96 pfu/cm². Approximately 100 putative positives can be seen on the plate. These signals lined up with similar signals on a duplicate filter.

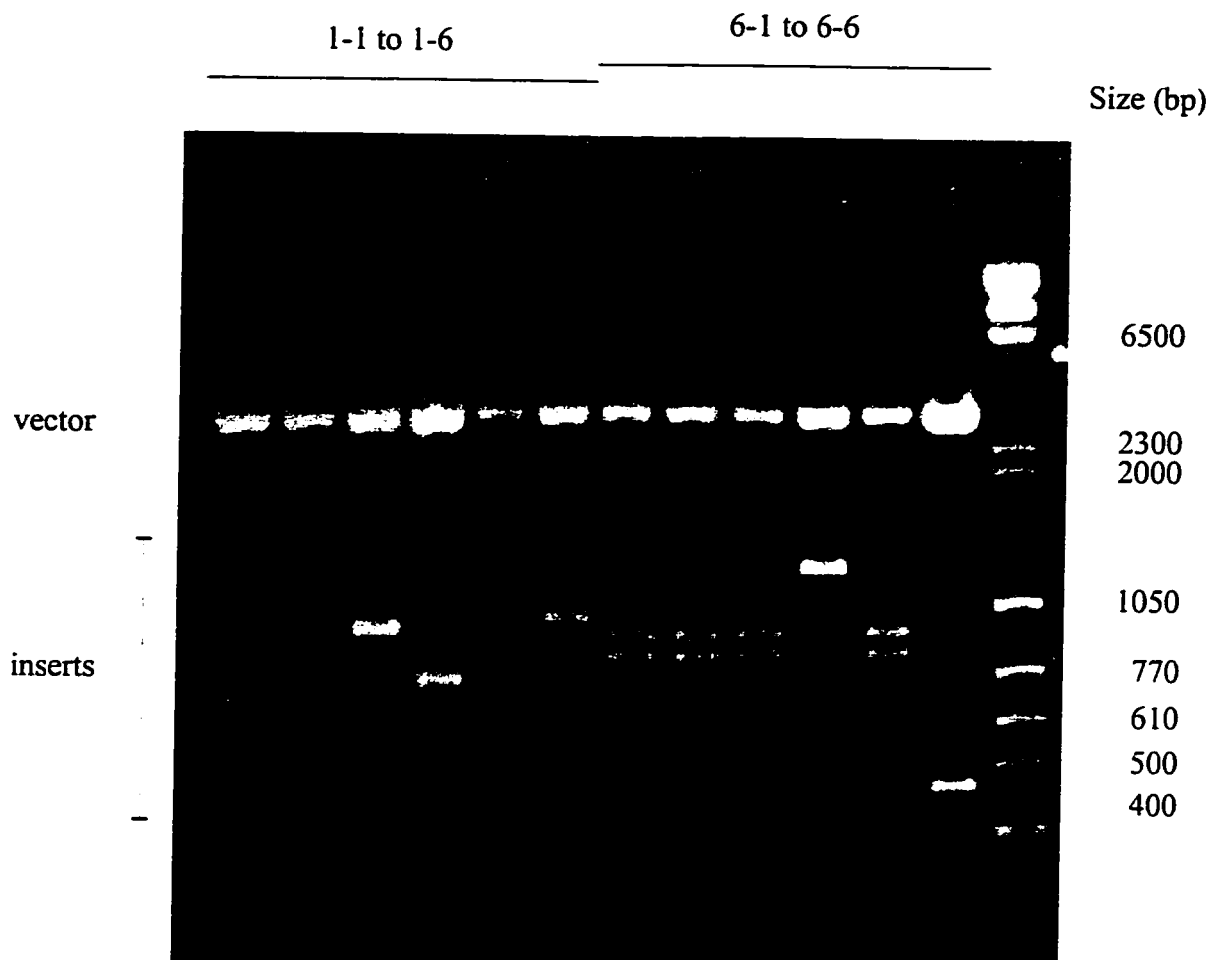


Figure 2.10: Gel analysis of insert sizes in 12 clones, 1-1 to 1-6, and 6-1 to 6-6, which were picked as single positive plaques from plates 1 and 6 during secondary screening with the probe cocktail CHP1A, mdr49, mdr65, SP01 and DM13. The molecular weight marker (λ HindIII+ ϕ XHincIII) is shown at right. Note that the clones picked from plate 1 all have inserts of different sizes, indicating that the positive signals on plate 1 were not indicating siblings of one clone. Four out of six clones from plate 6 have the same insert size (two bands approximately 700 and 800 bp in size) and are most likely siblings of the same clone. Clones 6-1 and 6-5 were subsequently sequenced (see text).

Chapter 3: Troubleshooting the cDNA cloning process

Introduction

The experiments in this chapter were performed in response to the negative results from the cDNA library screenings detailed in the previous chapter. These experiments attempt to answer the following questions:

A) Is cross-species DNA hybridization feasible?

All of the DNA probes used in the cDNA library screening, with the exception of the *Manduca* genomic clone mssp11, were derived from P-glycoprotein genes from other species (mostly other arthropods, but including one mammal). While numerous reports have demonstrated the feasibility of cross-species cloning for Pgp (*e.g.*, Wu *et al.*, 1991, Castillo *et al.*, 1995), it was deemed worthwhile to test the concept first-hand. This was done by doing probe-on-probe hybridizations or “dot blots”. Serial dilutions of the DNA fragments were applied to nylon membranes, which were then probed with individual probes to assess the level of cross-hybridization between species (and between gene family members).

B) What is the optimal plaque density for cDNA library screening?

A control for a Pgp-positive plaque was prepared by ligating a fragment of CHP1 (termed CHP1A) into the same phage vector used for the cDNA library, λ ZAPII. A mock cDNA library screening was then performed, in which a number of different phage plaque agar plates were prepared with a constant number of CHP1A “positives”, and a varying density of plaques of the *Manduca* cDNA library. Nytran lifts of these plates

were prepared and probed with radiolabeled CHP1A in order to obtain information on the effect of plaque crowding on positive signals from individual plaques.

C) Can the probes detect Pgp genes on Southern blots of genomic DNA?

A test of probe sensitivity is to use it in a hybridization of a Southern blot of a restriction digest of genomic DNA from the same organism. In a sense, this experiment is a positive control much like the previous experiment, in that it is a 100% match of the probe to the target. The difference is that in a Southern blot, the quantity of target is much smaller, as it is a single gene in a population of restriction fragments representing an entire genome. This approach was used to test the sensitivity of two probes, mssp11 and CHP1.

D) Can the probes detect Pgp transcripts on Northern blots of *Manduca* RNA?

One of the major issues in a cDNA cloning project is the presence of the target transcript in the tissue of interest. This question was addressed by two Northern blot experiments, one of total RNA from Malpighian tubules and one of mRNA from midgut and Malpighian tubules, probed with mssp11, CHP1, mdr49 and mdr65. Unfortunately, this question is complicated by the issue of probe sensitivity.

E) Is polymerase chain reaction (PCR) feasible as an alternative method of cloning?

Several studies have used PCR in combination with library screening to obtain P-glycoprotein genes (*e.g.*, Gerrard *et al.*, 1993, Castillo *et al.*, 1995). RT-PCR would also be an alternative to Northern blots as a way to study expression of Pgp in *Manduca*. As a first step toward investigating these possibilities, PCR primers directed against the genomic fragment mssp11 were used to try to isolate a Pgp gene fragment from *Manduca* genomic DNA.

Methods

A) Dot blot cross-hybridization experiments

To investigate the feasibility of performing cross-species hybridization with DNA probes, serial dilutions of the Pgp homologue fragments from different species were blotted onto nylon membrane and then probed.

For the first set of dot blots, the fragments were not excised from the vector. Plasmid midi-preps of CHP1A, mssp11, mdr49, mdr65, pBluescript and V-ATPase were dotted onto nylon membrane (Schlesser & Scheuller). Mssp11 was dotted at 300 ng and in seven threefold dilutions down to 0.4 ng. The other fragments were dotted at equimolar concentrations, adjusted for the sizes of the fragments compared to mssp11. For the second set of blots, DNA from plasmid midi-preps of TU19, TU15, DM13, SP01, mssp11, CHP1A, mdr49 and mdr65 was digested with the appropriate restriction enzymes, and the inserts were purified by agarose gel electrophoresis and the GeneClean glassmilk purification system. The mssp11 DNA was dotted onto nylon membrane starting with 2.5 ng in a 0.4 μ L volume, followed by ten successive twofold dilutions down to 2.5 pg. The other fragments were diluted to be equimolar to mssp11.

The DNA on the blots was denatured and cross-linked exactly as for nytran lifts of the cDNA library plates. Three separate hybridization experiments were done:

i) Probing with CHP1, mssp11, mdr49, mdr65

The first set of dot blots were incubated in aqueous pre-hybridization solution for 4 hours at 55°C with shaking. The blots were hybridized overnight at 55°C with shaking. The blots were rinsed briefly, then washed for 30 minutes at 42°C in 5x SSC, 0.2% SDS.

The blots were wrapped in cellophane and exposed to a phosphoimaging screen overnight.

ii) Probing with *mdr49*, *mdr 65*, *DM13* and *SP01*

Four dot blots were pre-hybridized at 37°C in a shaking water bath over 3 days in 200 mL of 30% formamide pre-hybridization solution. DNA probes were labeled with ³²P- α -dCTP by the random labeling technique (see Chapter 2). Each blot was placed into a heat-sealed hybridization bag along with 3 mL of new pre-hybridization solution. The blots were hybridized for 1.5 days at 37°C, with shaking. The blots were rinsed briefly and then washed (separately) in 5x SSC, 0.2% SDS, for 40 minutes in a shaking water bath at 37°C. The blots were wrapped in cellophane and exposed to X-ray film for 17 hours.

ii) Probing with *mssp11*

A dot blot was probed at the same time as an *mssp11* probing of the *CHP1A-ZAP* plaque density experiment. The blot was pre-hybridized in solution containing 50% formamide (but otherwise identical to that outlined above) for 5 hours at 37°C with shaking. The blot was hybridized for 1 day at 37°C with shaking. The blot was rinsed briefly, then washed for 30 minutes at 37°C in 5x SSC, 0.2% SDS. The blot was wrapped in cellophane and exposed to X-ray film for 40 hours.

iv) Stripping

All the blots were stripped of the radioactive probe by washing twice for 15 minutes with boiling 0.04x SSC (6 mM NaCl, 0.6 mM trisodium citrate), 0.2% SDS.

B) Optimizing plaque density using a positive construct, CHP1A-ZAP

A positive control construct was created for DNA screening. By plating a constant number of positive plaques with varying densities of plaques from the cDNA library, an estimate of the optimum plaque density for detecting a positive signal was determined. A λ ZAPII construct of a fragment of the hamster Pgp homologue containing the C-terminal Walker B site was created as a positive control.

i) Preparation of the λ ZAP-CHP1A construct

CHP1A was inserted into λ ZAP in frame with the β -galactosidase reporter gene so that it would also be useful as a positive control for antibody expression cloning. This was accomplished by:

- 1) cutting the CHP1 plasmid with the restriction endonucleases *XbaI* and *XhoI*, gel purifying the 430-bp fragment,
- 2) cutting the λ ZAP arms (from the cDNA library kit) with *XbaI*, removing a 30-bp piece between the *EcoRI* and *XbaI*, and
- 3) ligating the CHP1 fragment (~400 ng) (*XbaI*-CHP1A-*XhoI*) into the modified λ ZAP vector (~250 ng arms) after heat inactivating the *XbaI* restriction enzyme.

This process is illustrated in Figure 3.1.

ii) Plating and lifts

This construct was packaged and amplified in the same manner as the cDNA library (see Chapter 2). The titre of the amplified construct was 6.6×10^6 pfu/ μ L. The construct was plated at a density of 100 plaques per 150 mm plate (first experiment) or 20 pfu per 80 mm plate (second experiment). The positive control was mixed with the

cDNA library at plaque densities of 5.2, 52 and 520 pfu/cm² in the first experiment, and 52, 103, 162, 215, 320, 420 and 520 in the second experiment.. The plates were incubated overnight. Nytran lifts of the plates were performed as for the cDNA library screenings.

iii) Probing

a) Trial 1

The nytran lifts were probed with CHPlA. In the first experiment, the lifts were pre-hybridized at 55-65°C in aqueous pre-hybridization solution for 4 hours. The filters were hybridized overnight at 60°C, with shaking. The filters were rinsed twice and washed for 10 minutes with 5x SSC, 0.2% SDS at 55°C, then exposed to a phosphoimaging screen for two days.

b) Trial 2

In the second experiment, the lifts were pre-hybridized at 55°C for >1 hour in aqueous pre-hybridization solution, then hybridized overnight at 55°C, with shaking. The filters were washed for 1 hour (twice) in 5 x SSC, 0.2% SDS at 42°C, then washed for two hours in 2.5 x SSC, 0.2% SDS at 45°C. The filters were wrapped in cellophane and exposed to X-ray film for 1 day.

c) mssp11

The filters were also probed with mssp11. The filters were pre-hybridized at 37°C in 50% formamide pre-hybridization solution then hybridized overnight at 37°C. The filters were washed once in 5 x SSC, 0.2% SDS at 37°C, then exposed to film.

C) Southern Blot analyses

A Southern blot of genomic DNA from *Manduca* (supplied by M. Ell) was prepared to test for hybridization of mssp11 and CHP1.

i) Preparation of Southern Blot

Four digests of 20 µg *Manduca* genomic DNA with the restriction enzymes *EcoRI*, *HindIII*, *PstI* and *BamHI* (200 Units) were prepared in a volume of 400 µL. As a control for the CHP1 (hamster) probe, an equivalent amount of rodent genomic DNA from B78, a mouse melanoma cell line, was used in a single digest with *EcoRI* (200 Units). The digests proceeded for 2 hours at 37°C; the tubes were tapped with fingers after 1 hour for gentle mixing. During the digestion, two 0.8% agarose gels were prepared in 1x TAE buffer. The digested DNA was precipitated with ethanol and half was loaded onto each gel, so that each lane on the gel should represent 10 µg genomic DNA. Also loaded onto the gel were plasmid controls for the probes to be used. Whole (i.e. undigested) plasmid was loaded in 1, 10 and 100 pg quantities. For gel #1, to be probed with mssp11, the plasmid was pBluescript with mssp11 insert. For gel #2, to be probed with CHP1, the plasmid was pUC with CHP1 insert. The 11 x 13 cm gels were subjected to a voltage of 50 V for 5 min, then the voltage was reduced to 28 V for 14 hours.

The gels were stained with ethidium bromide and photographed over a UV transilluminator. The gels were depurinated in 0.25 M HCl, denatured in 1.5 M NaCl, 0.5 M NaOH and neutralized in 1.5 M NaCl, 1.0 Tris-HCl - pH 8.0. The gels were assembled into a standard upward capillary transfer configuration (Sambrook *et al.*,

1989). The membrane used was Hybond-N fp, an uncharged nylon membrane with 0.45 μm pore size, which was prewet in 20x SSC. The transfer took place overnight with 20x SSC. Following transfer the DNA was cross-linked to the nylon by UV irradiation.

ii) Preparation of radiolabeled DNA probes

As in previous experiments, the DNA probes were prepared using the random primer labeling method, although a new, more convenient method of purification was used. The probes were purified on NICK size-exclusion columns (Pharmacia) which contain a Sephadex G-50 gel bed with dimensions 0.9 x 2.0 cm. The columns were equilibrated with 3 mL of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA). Aliquots of the labeling mixture were removed for later scintillation analysis, and the remainder was added to the column and allowed to sink into the gel bed. Then, 300 μL of TE was added to the column, and allowed to sink into the gel bed, with collection into a waste beaker. This was followed by the addition of 500 μL TE. The eluate collected contained the radiolabeled DNA. An aliquot of the purified probe was removed for scintillation analysis. The purified DNA probe was boiled for 5 minutes and cooled on ice, prior to addition to the hybridization solution.

The purified probe was added to the hybridization solution to a concentration of 1×10^6 cpm/mL.

iii) Probing with mssp11 and CHP1

a) Standard procedure

Standard Denhardt's-based hybridization solution was used, as in previous experiments. The blots were pre-hybridized for greater than 1 hour at 45°C. The

Southern Blots were subjected to overnight hybridization at 45°C in a hybridization oven. Following hybridization, the blots were subjected to several washes of increasing stringency (all wash solutions contained 0.1% SDS):

- 5 x SSC, 30°C, 30 min.
- 2 x SSC, 32°C, 35 min.
- 2 x SSC, 42°C, 45 min.
- 2 x SSC, 57°C, 15 min, 45 min.
- 2 x SSC, 67°C, 30 min.

The blots were exposed to X-ray film at each step. The blots were stripped with boiling 0.1 x SSC, 0.1% SDS for 5 minutes, wrapped in cellophane and stored.

b) Multiple sandwich procedure

The Southern blots were later probed again with mssp11 and CHP1 using a variation of the standard hybridization protocol, the multiple sandwich hybridization method (Wu *et al.*, 1995). The major differences between this protocol and the standard protocol (Sambrook *et al.*, 1989) were: 1) The blots were stacked between pieces of Whatman 3MM paper; 2) No pre-hybridization of the blots was required, 3) The composition of the hybridization solution was different:

- 50% formamide
- 5x SSC
- 1x Denhardt's solution
- 20 mM sodium phosphate, pH 6.8
- 1% SDS
- 5% dextran sulfate

Note that no salmon sperm DNA was used in the hybridization solution. The authors of this protocol note that purification of the probe from the unincorporated nucleotides is not necessary. However purification on a NICK column was done in this instance for consistency in the assay of the specific activity of the probe. The blots were hybridized

overnight at 42°C. Following hybridization the blots were rinsed twice with room temperature 2x SSPE (300 mM NaCl, 20 mM NaH₂PO₄, 2 mM EDTA, pH 7.4), 0.5% SDS, followed by a 15 minute wash in this same solution at 68°C. This was followed by a 15 minute wash in 0.2x SSPE, 0.1% SDS at 68°C. The blots were exposed to Hyperfilm (Amersham) at -70°C for 5 days.

iv) Probing with V-ATPase

As a control, a blot was also probed with V-ATPase. Hybridization conditions were the same as for the previous experiment (standard hybridization). Following hybridization, the blot was washed in the following series of washes (all washes contained 0.1% SDS):

2 x SSC, 42°C, 30 min., 30 min.
2 x SSC, 57°C, 15 min., 45 min.

The blot was exposed to film after each step.

v) Probing with ADP-ATP translocase (AAT) cDNA clone

A blot was also probed with a cDNA clone previously isolated from my cDNA library, a partial cDNA of the ADP-ATP translocase gene. This was done both as a second control for the previous experiments and for the intrinsic value of this original data. This work is described in Chapter 4.

D) Northern Blot analyses

i) Northern Blot of total RNA from *Manduca* Malpighian tubules, mouse brain and mouse liver

Total RNA was extracted from samples of *Manduca* Malpighian tubule tissue (from 117 individuals) in the same manner as for cDNA library construction (see Chapter

2). Total RNA was also extracted from mouse brain, and mouse liver, for comparison. The following protocol is taken from Fourney *et al.*, 1988.

Approximately 10 µg each of total RNA from *Manduca* Malpighian tubules, mouse liver and mouse brain, were loaded into wells in a 1.5% agarose-formaldehyde gel in a 10 µL volume following heat treatment at 65°C for 15 minutes and addition of 1 µL 1µg/µL ethidium bromide. This was repeated in adjacent wells, so that there were two blots available. The formaldehyde gel was run at 45 volts for 5 hours. A photograph of the gel was taken over a UV transilluminator. The gel was soaked in 10x SSC for 20 minutes, twice. The nylon membrane was soaked in distilled water for 5 minutes, followed by 10x SSC for 5 minutes. The blotting was accomplished by standard upward capillary transfer (Sambrook *et al.*, 1989). The capillary transfer of the RNA was allowed to proceed for 20 hours. The membrane was then removed from the gel and placed (RNA side up) in 2x SSC. The RNA was crosslinked to the membrane as outlined above for the dot blots. The blot was then dried between two pieces of Whatman paper, and cut in two.

a) Probing with mssp11 and V-ATPase

The blots were pre-hybridized in 50 mL aqueous pre-hybridization solution for 3h35 at 55°C. The probes were prepared as outlined above. The blots were hybridized separately at 57°C, with shaking, for 1.5 days. The blots were rinsed twice and then washed for 30 minutes at 42°C in 5x SSC, 0.1% SDS. The blots were wrapped in cellophane and exposed to film. The blots were then stripped as above.

b) Probing with CHP1 and *mdr49/mdr65*

The stripped blots were pre-hybridized as above. The probes were prepared as usual, however, *mdr49* and *mdr 65* were labeled together in one reaction instead of separately. The blots were hybridized separately at 55°C, with shaking, overnight. The blots were washed once with 5x SSC, 0.2% SDS at 45°C for 30 minutes. The blots were then wrapped in cellophane and exposed to film. Following exposure the blots were stripped as above.

c) Probing with ubiquitin

The Northern was also probed with a *Manduca* ubiquitin cDNA obtained from L.M. Schwartz (Bishoff and Schwartz, 1990), by way of M. Ell. A stripped blot was pre-hybridized in aqueous pre-hybridization solution at 55°C for 4 hours. The blot was hybridized overnight at 54°C, with shaking. The blot was rinsed twice then washed for 30 minutes at 44°C in 5x SSC, 0.1% SDS. The blot was wrapped in cellophane and exposed to film.

ii) Northern Blot of mRNA from 4th and 5th instar *Manduca* larvae

A Northern blot was prepared from tissues of 4th and 5th instar *Manduca* larvae. The larvae were anaesthetized by chilling at 4°C for 20 minutes, and sacrificed by decapitation. The larvae were grossly dissected into two tissue fractions: gut (including gut and Malpighian tubules) and body wall (including muscle, epidermis, fat body, trachea and abdominal nerve cord). Total RNA was isolated from these tissue fractions by the guanidinium isothiocyanate method employed in previous experiments (modified from Chomzynski and Sacchi, 1987). Messenger RNA was isolated from these

preparations using Oligo-tex mRNA spin columns (Qiagen). For comparison, the Oligo-tex system was also used to isolate mRNA directly from 100 mg of 4th instar gut tissue.

These five RNA samples (4th instar gut, 4th instar body wall, 5th instar gut, 5th instar body wall RNA isolated in two steps, and 4th instar gut mRNA directly isolated from tissue) were mixed with formamide RNA loading buffer, denatured at 65°C, and loaded onto a 1% agarose, 5% formaldehyde gel prepared in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH = 7.0). The samples were fractionated by electrophoresis at 80 V for 3.5 h. The RNA was then blotted onto Nytran-Plus Maximum Strength nylon charged membrane (S&S), using a standard upward capillary transfer method (Sambrook *et al.*, 1989). After transfer, the RNA was cross-linked to the nylon membrane by exposure to UV radiation.

a) Probing with mssp11

I) Standard hybridization

The Northern blot was probed with radiolabeled mssp11. Hybridization was overnight in standard Denhardt's-based aqueous hybridization solution at 55°C, the temperature was chosen to coincide with that used in the previous Northern Blot of total RNA. Following hybridization the blot was subjected to the following washes (all wash solutions contained 0.1% SDS):

5 x SSC, 42°C, 30 min.
2 x SSC, 47°C, 30 min., 30 min.
2 x SSC, 65°C, 10 min.

The blot was exposed to film after each step. The blot was stripped in 50% formamide, 2 x SSPE, 65°C, 1 hour ("gentle stripping", Sambrook *et al.*, 1989).

II) Multiple sandwich hybridization

The same Northern blot was also probed with mssp11 using the multiple sandwich hybridization technique. Hybridization wash conditions were the same as described in Section C-iii-b above for the Southern blots.

b) Probing with AAT

The same blot was also probed with AAT, both as a control for the quality of the RNA preparation, and for the intrinsic value of this original data. This work is reported in Chapter 4.

E) PCR of *Manduca* genomic DNA with primers to mssp11

PCR primers to the sequence of mssp11 were constructed (Figure 3.2). These primers were used in a PCR of *Manduca* genomic DNA. PCR reactions were prepared with Vent™ DNA Polymerase (New England Biolabs) according to the manufacturer's instructions. Template DNA for 3 reactions was 0.2, 1.0 and 2.0 µg *Manduca* genomic DNA (same stock used for the Southern blots). Positive (mssp11 plasmid) and negative (no template) controls were also included. The reactions were topped with mineral oil and loaded into a thermocycler programmed as follows:

Initial denaturation	2 min	92°C
30 cycles	45 s	55°C
	30 s	72°C
	45 s	92°C

Gel analysis of aliquots of the product showed multiple weak bands of a range of sizes, a strong single band from the plasmid control and no bands in the negative control.

Aliquots of the reactions were used in a second amplification cycle. Each of the original three reactions were used to prepare three new reactions with 0, 2, and 4 mM

added magnesium ion (for a total of nine experimental reactions). Positive and negative controls were done as before. The reaction conditions were slightly altered with an annealing temperature of 60°C instead of 55°C.

Gel analysis of aliquots of the products showed that in all reactions one or two bands of approximately the same size as the positive control were more represented than other bands (see Results). These bands were cut from the gel, purified with glassmilk (Geneclean II, Bio 101, Inc.), and digested with *EcoRV*, a restriction enzyme which cleaves *msspl* I into two smaller pieces.

In order to clone these PCR fragments so they could be sequenced, the remainders of the second-round amplification reactions were pooled and separated by agarose gel electrophoresis. The major band(s) was excised and purified with glassmilk. The fragments were ligated into the *EcoRV* site of pBluescript KS with T4 ligase (Sambrook *et al.*, 1989). Competent XL1-Blue *E. coli* cells were transfected with the purified plasmid by electroporation (Bio-Rad *E. coli* pulser protocol) with a voltage of 2.5 kV. The transfected cells were plated on LB-amp plates treated with 800 µg IPTG and 800 µg X-gal.

A total of 71 white colonies were picked from the original platings of the transfected cells. Forty of these colonies were white when plated a second time on a patch plate. Plasmid DNA preparations were made from these colonies (Wizard mini-preps; Promega), and aliquots of each were digested with *EcoRI* and *HindIII* restriction enzymes and separated by agarose gel electrophoresis. Nine of the plasmids were shown to have inserts. These nine inserts were sequenced by automated sequencing (Li-Cor,

Inc.). The sequences were subjected to the BLAST search program (World Wide Web version) to compare them to sequences in Genbank (Gish and States, 1993; Altschul *et al.*, 1990)

Results

A) Dot blot cross-hybridization experiments

i) First series

Table 3.1 summarizes labeling information about the probes used in the first set of dot blot experiments. The phosphoimager record of dot blots is shown in Figure 3.3. Note that for each of these experiments, comparisons can only be made between rows of dilutions on the same blot. Comparison across blots is not appropriate as there was some variation in the hybridization conditions of each blot probed. Figure 3.3 shows the results of the first series of dot blots, in which threefold serial dilutions of plasmids are probed. The dilutions run from 300 ng down to 0.4 ng DNA for *mssp11*, with the other probes diluted to be equimolar to *mssp11*. All of the four probes hybridize strongly to their corresponding row of dilutions, as expected. *CHP1A* hybridizes to *mssp11* about 1/27 as well as it does to itself. It also binds to *mdr49* 1/243 as well as it does to itself. It does not bind appreciably to *mdr65*. *Mssp11* binds to *CHP1A* about 1/27 as well as it does to itself, but does not appear to bind any of the others appreciably. *Mdr49* and *mdr65* appear to bind only to their corresponding rows of dilutions.

ii) Second series

Table 3.2 summarizes information about the probes used in the second series of dot blots. Figure 3.4 shows the results of the second series of dot blots in which only the purified probe fragments were dotted to the membrane in two fold dilutions from 2.5 ng

down to 2.5 pg. As with the first experiment, mdr65 did not bind to anything but its own corresponding row of dilutions. However, probing with mdr49 resulted in a very faint signal from the first dilution of mdr65 (may not be visible in Figure 3.4, but it was detectable on the autorad). This indicated that mdr49 bound to mdr65 1/500 as well as it did to itself. DM13, a *Drosophila* probe, hybridized to two other arthropod probes SP01 and TU19 equally as well as to itself. It also bound to mssp11 1/125 as well and to CHP1A 1/250 as well. TU15 was bound at about 1/360, and mdr49 and mdr65 were not bound at all. The results were similar for probing with SP01, in that TU19 and DM13 were bound equally as well as the dilutions of SP01 itself. However, SP01 bound the second arachnid homolog, TU15 at 1/32. It also bound to mssp11 at 1/64, to mdr65 at 1/250 and to CHP1A at 1/1000. There was no appreciable binding to mdr49.

Table 3.3 summarizes information about the mssp11 probe used to probe a dot blot. Probing with mssp11 (Figure 3.4) gave the following ratios: DM13 = 1/32, TU19 and TU15 = 1/64, SP01 = 1/250, CHP1A = 1/750, mdr49 and mdr65 = 1/1000. The results of the second series of dot blots are summarized in Table 3.4

B) Optimizing plaque density using a positive construct, CHP1A-ZAP

i) Trial 1

Table 3.5 summarizes information about the CHP1A probe used in the first plaque density experiments. Probing of the ZAP-CHP1A construct yielded dramatic results. In the first experiment the positive control was mixed with the cDNA library at three plaque densities of 5.2, 52 and 520 pfu/cm². The lifts from the plates with the two lower plaque densities showed positive signals when probed with CHP1 (Figure 3.5). However, the highest plaque density of 520 pfu/cm² (which is equivalent to 30 000 pfu

per 85-mm plate, or 80 000 pfu per 140-mm plate) showed a marked attenuation of the signal, such that it was barely discernible above the background (Figure 3.5).

ii) Trial 2

Table 3.6 summarizes information about the CHP1A probe used in the second plaque density experiment. In this second experiment, seven densities between 52 and 520 pfu/cm² were plated and probed. The 52 pfu/cm² lift again resulted in strong positive signals; they were as strong as the signals apparent on the lift of the control construct alone (Figure 3.6). The occasional strong signal could be seen on the 162 pfu/cm² lift, and weak signals were discernible all the way up to 520 pfu/cm². However, the signal from the positive plaques is strongly attenuated at densities higher than 520 pfu/cm².

iii) Probing CHP1A positive control plaques with mssp11

The nytran lifts were also probed with mssp11. Probe labeling information for this experiment is identical to that described in Table 3.3. No positive signals could be discerned in the resulting autoradiograph (not shown).

C) Southern blot analyses

DNA loading on the Southern blot was seen to be somewhat uneven in a photograph of the agarose gel following electrophoresis (Figure 3.7). Loading in the *HindIII* lane was below average and loading in the mouse lane was above average. These differences were reflected in the experiments below.

i) Probing with msspl1 and CHP1

a) Standard hybridization

Table 3.7 summarizes information about msspl1 and CHP1 probe labeling in this experiment. With the standard procedure no bands were detected by either of the probes in the *Manduca* and mouse genomic DNA lanes. The probes did hybridize to the plasmid controls (1, 10, 100 pg of plasmid; not shown).

b) Multiple sandwich hybridization procedure

Table 3.8 summarizes information about the labeling of msspl1 and CHP1 probes in this experiment. With the multiple sandwich procedure, no bands were detected by either probe in the *Manduca* genomic DNA lanes. However, very faint bands were detected by the hamster probe CHP1 in the mouse genomic DNA lane (Figure 3.8). These bands form a pattern that is very similar to that seen in an identical experiment performed by Riordan *et al.* (1985). There is however, a small difference in the sizes of the bands compared to Riordan *et al.* (1985; Figure 3.8). The reason for this discrepancy is unknown. There is also a difference in the stoichiometry of the bands compared to Riordan *et al.* (1985). This likely indicates uneven hybridization of my probe to the three Pgp gene family members in mouse, although the reason why this was not also the case for the Riordan *et al.* (1985) experiment is not clear.

ii) Probing with V-ATPase

The V-ATPase probe was used as a control for the quality of the genomic DNA. Table 3.9 summarizes information about the probe used in this experiment. This probe identifies a band in the *PstI* lane and the *EcoRI* lane of *Manduca* genomic DNA showing that the negative results in these lanes in the previous experiment were not the result of

degraded DNA. Lack of detection in the *HindIII* lane is consistent with the below average DNA loading on the gel seen in Figure 3.7, however, lack of detection in the *BamHI* lane is not consistent with the DNA loading in this lane, which is similar to that in the *PstI* and *EcoRI* lanes. A possible explanation is that the signal in the *BamHI* lane is split between two (or more) bands, which reduces the signal to the level of the background on the blot. A second control probing with the AAT clone (reported in Chapter 4) did identify a single band in the *BamHI* lane, indicating that the DNA in this lane is intact, and the lack of signal seen with V-ATPase must be due to other factors.

D) Northern blot analyses

i) Northern blot of total RNA from *Manduca* Malpighian tubules, mouse brain and mouse liver

a) Probing with mssp11 and V-ATPase

Table 3.10 summarizes labeling information about these probes. The mssp11 probe did not identify any signal in the *Manduca* Malpighian tubule or mouse brain or liver lanes (not shown). The V-ATPase probing resulted in two bands running at 1.8 kb and 1.5 kb, the smaller band being more intense (Fig. 3.10A). This result is comparable to that of Dow *et al.* (1992) who found two bands running at 1.9 and 1.4 kb (again, the smaller band being more intense) in a Northern blot of poly-A⁺ RNA from *Manduca* midgut. No positive signal was discernible from the mouse liver or brain lanes.

b) Probing with CHP1 and mdr49/mdr65

Table 3.11 summarizes information about the probes. No signals could be discerned from the Northern blots in any lanes using these probes (not shown).

c) Probing with ubiquitin

Table 3.12 summarizes information about the probe. Probing with the ubiquitin resulted in a smear from ~1.9 kb to ~0.33 kb, with a distinct band at 1.9 kb and the heaviest signal running at ~0.8 kb (Fig. 3.10B). There is also a very faint signal running at ~3 kb. A smear was also detectable in the mouse brain, indicating cross-species hybridization of the *Manduca* probe to the mouse transcripts. The mouse brain lane also displays a distinct band running at 2.5 kb that the *Manduca* lane lacks. A very faint smear is also detectable in the mouse liver lane. Bishoff and Schwartz (1990) used this probe on Northern blots of many different *Manduca* tissues at different stages of pupal and post-pupal development. A 0.9-kb band was present in every sample tested, and other bands between 1.7 and 4.4 kb were variously present or absent at different stages of development.

ii) Northern Blot of mRNA from 4th and 5th instar *Manduca* larvae

a) Probing with mssp11

Table 3.13 summarizes information about the probe used in the standard hybridization. The probe used in the sandwich hybridization has exactly the same characteristics as shown in Table 3.8 above. Neither the standard nor sandwich hybridizations produced any signal from the blot with mssp11 (not shown). However, the integrity of the RNA was checked with a positive control, the AAT clone (work reported in Chapter 4) and AAT could be detected in only two of the five samples loaded on the gel. Since AAT is a ubiquitous and crucial enzyme involved in oxidative phosphorylation, it ought to be detected in all samples; the lack of signal may indicate

degradation of the RNA in three of the samples. The two samples in which the RNA was intact were the 4th and 5th instar gut fractions (*i.e.* midgut +Malpighian tubule tissue)

E) PCR of *Manduca* genomic DNA with primers to mssp11

Figure 3.11A shows the results of the PCR experiment after the second round of amplification. Multiple bands are seen in most of the experimental lanes, but a 390 bp band (or bands) stands out in each case. This is exactly the size of the band amplified from the mssp11 plasmid control, suggesting that the bands could be equivalent to mssp11. To test this, the genomic bands and plasmid band were each excised from the gel and purified, and half of each sample was digested with *EcoRV* a restriction enzyme that cleaves mssp11 into two smaller pieces (Figure 3.2). As seen in Figure 3.11B, the plasmid band was cut into two fragments of 160 and 230 bp, as expected; however, the genomic bands were not, indicating that they do not represent mssp11 faithfully amplified from genomic DNA.

Of the nine successful transfectants in the cloning of the PCR bands, none had sequence similarity to P-glycoprotein or to any other ABC transporter. However, the cloning efficiency was very low, and none of clones that were sequenced were siblings (*i.e.*, identical clones), suggesting that the major product represented by the 390 bp band in Figure 3.11 was not identified, and may be inherently difficult to clone.

Discussion

A) Dot-blot hybridizations

The dot-blot hybridizations demonstrated that cross-species hybridization is feasible (Figures 3.3, 3.4). DM13, a probe from *Drosophila* (fruit fly) hybridized to homologous DNA fragments from *Spodoptera* (army worm) and *Tetranychus* (mite) as

well as it hybridized to itself. Similar results were seen with the *Spodoptera* probe, SP01. This indicates a high degree of similarity in DNA sequence among species from different classes of the phylum Arthropoda in this region of the Pgp molecule (the Walker A and B sites and the region in between). The same *Drosophila* probe that bound very well to *Tetranicus* homologue TU19 bound a second homologue from the same species (TU15) 1/360 as well. Also, DM13 bound the *Manduca* homologue (mssp11) 1/125 as well as SP01, despite the fact both *Manduca* and *Spodoptera* are lepidopterans. A possible explanation for this observation is that DM13, SP01 and TU19 represent sequences equivalent to the same half of the same member of the Pgp gene family in all three species, while mssp11 and TU15 represent different gene family members and/or the other half of the gene. For example, if DM13, SP01 and TU19 represented the C-terminal half of an arthropod "class I" gene, then mssp11 and TU15 would represent either the N-terminal half of the "class I" gene or the N- or C-terminal half of a "class II" or "class III" gene.

Rodent Pgp genes have been successfully cloned using probes from sister Pgp genes of the same species (Endicott *et al.*, 1987; Ng *et al.*, 1989; Brown *et al.*, 1993). However, mammalian sister genes are less divergent than those in *Drosophila* (76% DNA sequence identity between human *MDR1* and *MDR3* versus 53% identity between *Drosophila* *mdr49* and *mdr65*; Wu *et al.*, 1991). The *Drosophila* divergence can be noted on the dot blots; hardly any cross-hybridization is seen between *mdr49* and *mdr65* (Figs. 3.3, 3.4) despite the probes' conserved Walker A sequences. Great divergence may also be a characteristic of other arthropod gene family members. In the two *Tetranicus* homologues TU19 and TU15, divergence could explain the difference in their hybridizations to DM13 and SP01 (Fig. 3.4).

These data suggest the possibility of cross-species grouping of Pgp gene family members beyond Class Mammalia. The evolutionary relationships of non-mammalian Pgp genes are the purview of the PhD project of M. Ell, from whom most of these probes were obtained.

It would be interesting to see where *mdr49*, *mdr65*, and *mdr50*, the three published *Drosophila* Pgp genes, fit into this arthropod scheme. As seen in Figures 3.3 and 3.4, there is little cross-hybridization to other arthropod DNA fragments seen with the *mdr49* and *mdr65* probes, despite the fact that *mdr49* overlaps the region of the gene coded by the other arthropod probes (Figure 2.4). However, this may be an indication that some or all of the arthropod probes are derived from the 3' half of the gene rather than the 5' half, like *mdr49* and *mdr65*. DNA sequence similarity between the 5' and 3' halves of the cDNA is 34-35% in the *Drosophila* cDNAs (Wu *et al.*, 1991). It is interesting to note that the probe SP01 does cross-hybridize to *mdr65* even though the two probes share only a very small region of overlap (Figure 2.4).

B) Plaque density experiments

A negative correlation of signal strength with increasing plaque density is very clearly demonstrated by these experiments. At the plating density used in the first round of screening of the cDNA library (325 pfu/cm² or 50 000 pfu per 140-mm plate; as suggested by Stratagene, 1993) the 100% homology probe produces only very weak signals (Figure 3.6). A lower homology probe, such as most of the probes used in the first round of screening, would be expected to give an even weaker signal.

The most reasonable explanation for this result is that dense crowding results in smaller plaques which have less DNA and therefore produce an attenuated signal. This result led to a decision to re-screen the library at a much lower density than that

suggested by Stratagene (1993). Given that this second round of screening did not lead to isolation of a clone, it might be worthwhile to probe the library at an even lower density still. The density of 96 pfu/cm² in the second round of screening was used (despite being twice the optimal density determined by the positive control experiment) because any lower would make it unwieldy to screen a large number of plaques. It was thought that screening a total number of plaques close to the titre of the primary library (550 000 pfu) was desirable to do a thorough screening, hence 500 000 plaques on 10 plates were screened in the first round, and 300 000 plaques on 20 plates were screened in the second round. However, Tijssen (1993) suggests a range of 100 to 200 000 plaques be screened for a cDNA library, depending on the expected degree of expression in the tissue, so it appears that the plaque numbers screened were more than adequate.

C) Southern blot analyses

The Southern blot experiments showed that one of the major problems with the cDNA library screening is the sensitivity of the probes. Specifically, it was determined that the hamster Pgp probe CHP1 is much less sensitive than the control probes V-ATPase and AAT. Both of the control probes clearly detected bands in the lanes of *Manduca* genomic DNA (V-ATPase: Figure 3.9; AAT, see Chapter 4), but only very faint bands were detected in the mouse genomic DNA lane with the hamster CHP1 probe (Figure 3.8). This result was seen despite the fact that the probes were all labeled to similar specific activities ($\sim 1 \times 10^9$ cpm/ μ g) and that the mouse DNA lane clearly has more DNA than the *Manduca* lanes, as judged by Figure 3.7 (Note: the CHP1 cDNA is 90% identical in nucleotide sequence to the equivalent region of the mouse class I coding sequence). The CHP1 sequence has a higher G+C nucleotide content (57%) than either V-ATPase (40%) or AAT (37%) which theoretically means it should make a more stable

hybrid (GC pairings are stabilized by three hydrogen bonds, versus two for AT pairings). The two control probes are 100% identical to the target sequence, versus 90% for CHP1 against mouse DNA, but it does not seem likely that this 10% mismatch could be entirely responsible for the weaker signal from CHP1, especially when probing at a low stringency. The reason for the discrepancy in sensitivity between CHP1 and the two control probes therefore remains unknown, but the problem may be due to some idiosyncrasy of the random primer labeling process. It would therefore be worthwhile to repeat the experiments using a variety of different probe production techniques and see if this difference in sensitivity still exists. Single-stranded DNA or RNA probes have the potential to be more sensitive than double-stranded DNA probes since there is no reannealing of complementary strands. As well, use of *Taq* or T4 polymerases can improve incorporation of radiolabeled nucleotide compared to Klenow polymerase (G. Drouin, pers. comm.).

The difference in sensitivity between the CHP1 probe and the V-ATPase probe seen in the Southern blot experiments parallels the observations from the plaque density experiments. In those experiments, it was shown that the CHP1A probe produced only very faint signals from positive plaques when the plaques were packed together at a high density equivalent to that used in the first round of cDNA library screening (320 pfu/cm², Figure 3.6). However, at this same density, the library was probed with V-ATPase, resulting in easily identifiable positive signals (Figure 2.8). Since, as mentioned in the previous section, the plaque density is a determinant of the amount of DNA in an individual plaque, by the same token it is likely that increasing the loading of DNA in the lanes of the Southern blot would result in a stronger signal using the CHP1 probe.

Poor DNA loading on the Southern gels may, in fact, be the reason why these experiments were unable to replicate the results of M. Ell (personal communication; see Chapter 1, section E) and identify bands in the *Manduca* genomic DNA lanes with the probe CHP1. As seen in Figure 3.7, there were different amounts of DNA seen on the gel following electrophoresis and prior to capillary transfer, despite the fact that equivalent amounts of DNA were originally digested with the restriction enzymes. This loss of DNA may have occurred during the ethanol precipitation step, which is done following the restriction digest to concentrate the DNA before loading it on the gel. This step is a standard part of the Southern blot procedure (Sambrook *et al.*, 1989), but Ell prepared gels with very large wells, and thus did not need to precipitate the DNA following digestion to reduce the solution volume. It is therefore possible that Ell had somewhat more DNA on his blots, enough to reach the threshold of detection.

Msspl I did not detect any bands in the *Manduca* genomic lanes, and this suggests two possibilities. The negative result could be due to a sensitivity problem similar to that seen with CHP1, or, a remote possibility is that msspl I is not a *Manduca* homologue of Pgp, but a homologue from some other organism (perhaps a *Manduca* parasite) whose DNA contaminated the preparation used in the original PCR done by Ell. Of these two scenarios, the former should be favoured, at least until the issue of probe sensitivity is satisfactorily resolved.

D) Northern blot analyses

The Northern blot of *Manduca* tubules, mouse brain, and mouse liver total RNA did not show any signal when probed with Pgp probes from *Manduca*, hamster and *Drosophila*. A blot of mRNA from *Manduca* gut tissue from both 4th and 5th instar larvae also did not show any signal when probed with msspl I and CHP1.

Unfortunately these results are confounded by the issue of probe sensitivity discussed in the above section on the Southern blots. Until this issue is resolved it is difficult to make any conclusions about these experiments.

It is difficult to predict what level of expression should be expected for a Pgp homologue in *Manduca sexta*. The high expression levels seen in drug-resistant mammalian tumour cells or pesticide-resistant insects like *Heliothis virescens* may not be comparable. In both of these situations the organism is exposed to a highly toxic man-made insult that selects for populations of cells or insects that by an accident of genetics happened to be expressing high levels of Pgp at the time. The *Manduca* situation is very much different, reflecting an evolutionary relationship between the hornworm and the tobacco plant that has developed over millenia.

It is possible the Pgp in *Manduca* has been “fine-tuned” to a degree where high levels of expression may not be necessary to provide adequate protection. There are at least three possible mechanisms by which the hornworm might optimize its use of P-glycoprotein. 1) Strategic expression of Pgp in tissues which are most likely to come into contact with toxins. 2) Subcellular localization of Pgp such that it is working in tandem with other transport or detoxification systems. 3) Alteration of the kinetics of substrate binding for more efficient transport of the xenobiotics that the hornworm is most likely to encounter, like nicotine. While a characteristic feature of P-glycoproteins is very broad substrate specificity, preferences for certain substrates over others have been previously demonstrated (Tang-Wai *et al.*, 1995).

E) PCR as an alternative cloning method

One of the limitations associated with this project has been the relative difficulty of obtaining tissues. *Manduca sexta* are expensive and time-consuming to raise, and the

tissues of interest, in this case, the Malpighian tubules and the abdominal nerve cord, represent very small fractions of the mass of the organism. Given these limitations, it would be useful if methods, such as reverse transcriptase PCR (RT-PCR) which require only small amounts of tissue, could be applied to study expression of Pgp. As a preliminary step toward this goal, PCR was attempted on *Manduca* genomic DNA using primers specific to the sequence of mssp11 (Figure 3.2). If this procedure were successful in regenerating mssp11 from genomic DNA, or at least in generating other Pgp homologues, it would be conceivable to try the same primers in RT-PCR.

Although these primers succeeded in amplifying a band of the expected 390 bp size (Figure 3.11A), this band is not mssp11 as it is not cleaved into two smaller pieces by the restriction enzyme *EcoRV* (Figure 3.11B). The possibility remains that the band could be another member of the Pgp gene family in *Manduca* or another ABC transporter. Unfortunately, the identity of the band remains unknown; attempts to clone the band were unsuccessful. Of the nine clones isolated after transfection, all were contaminating sequences as none resembled Pgp; however, none of the nine clones were siblings (*i.e.* identical clones), suggesting that the major constituent of the band (if there is one) was not isolated. The reason for this lack of success in cloning the PCR band is unknown but it deserves further investigation; it seems highly unlikely that the primers would amplify a random non-Pgp band that happens to be the exact molecular weight expected (390 bp). However, these unusual results caution against using these particular mssp11-directed primers for RT-PCR; other primers (perhaps degenerate primers for conserved Pgp sequences) may be more suitable.

Table 3.1: Probe labeling and radioactivity data for four Pgp-homologous probes, the *Manduca* genomic fragment mssp11, CHP1 from Chinese hamster, and mdr49 and mdr65 from *Drosophila melanogaster*.

Probe characteristics	Probe			
	mssp11	CHP1	mdr49	mdr65
probe template size (bp)	450	600	1800	1200
template DNA (ng)	40	30	45	20
newly synth. DNA (ng)	0.16	0.26	0.16	0.13
percentage incorporation	2.9%	4.7%	2.9%	2.5%
specific activity (cpm/ μ g)	4.3×10^7	6.2×10^7	3.0×10^7	4.5×10^7
activity in hybrid. solution (cpm/mL)	3.4×10^4	3.7×10^4	2.7×10^4	1.8×10^4
[DNA] in hybrid. solution (ng/mL)	0.80	0.60	0.90	0.40

Table 3.2: Probe labeling and radioactivity data for four Pgp-homologous probes, the *S. frugiperda* genomic clone SP01 and DM13, mdr49 and mdr65 from *Drosophila melanogaster*.

Probe characteristics	Probe			
	DM13	SP01	mdr49	mdr65
probe template size (bp)	490	485	1800	1200
template DNA (ng)	25	25	25	25
newly synth. DNA (ng)	0.88	5.6	0.74	1.2
percentage incorporation	4.0%	24%	5.1%	5.5%
specific activity (cpm/ μ g)	2.4×10^8	7.8×10^8	2.8×10^8	4.0×10^8
activity in hybrid. Solution (cpm/mL)	2.2×10^5	6.4×10^5	2.0×10^5	3.1×10^5
[DNA] in hybrid. Solution (ng/mL)	0.92	0.82	0.74	0.75

Table 3.3: Probe labeling and radioactivity data for the *Manduca* genomic fragment mssp11.

Probe characteristics	Probe
	mssp11
probe template size (bp)	450
template DNA (ng)	50
newly synth. DNA (ng)	36
percentage incorporation	41%
specific activity (cpm/ μ g)	4.8×10^8
activity in hybrid. solution (cpm/mL)	7.4×10^5
[DNA] in hybrid. solution (ng/mL)	1.5

Table 3.4: Results of second series of dot blot experiments.

Membrane-bound DNA	Probes ¹				
	DM13	SP01	mssp11	mdr49	mdr65
TU19	1	1	1/64	0	0
TU15	1/360	1/32	1/64	0	0
DM13	1	1	1/32	0	0
SP01	1	1	1/250	0	0
mssp11	1/125	1/64	1	0	0
CHP1A	1/250	1/1000	1/750	0	0
mdr49	0	0	1/1000	1	0
mdr65	0	1/250	1/1000	1/500	1

¹Degree of hybridization of the radioactive probe is presented as a fraction of self-hybridization. *e.g.* probe DM13 against membrane-bound DM13 gives a value of one, while DM13 hybridizes 1/125 as well to membrane bound mssp11.

Table 3.5: Probe labeling and radioactivity data for CHP1A (Trial 1, plaque hybridization experiment)

Probe characteristics	Probe
	CHP1A
probe template size (bp)	430
template DNA (ng)	120
newly synth. DNA (ng)	2.1
percentage incorporation	9.6%
specific activity (cpm/μg)	3.8×10^7
activity in hybrid. solution (cpm/mL)	4.6×10^4
[DNA] in hybrid. solution (ng/mL)	1.2

Table 3.6: Probe labeling and radioactivity data for CHP1A (Trial 2, plaque density experiment).

Probe characteristics	Probe
	CHP1A
probe template size (bp)	430
template DNA (ng)	20
newly synth. DNA (ng)	2.1
percentage incorporation	13%
specific activity (cpm/μg)	4.1×10^8
activity in hybrid. solution (cpm/mL)	9.5×10^4
[DNA] in hybrid. solution (ng/mL)	0.23

Table 3.7: Probe labeling and radioactivity data for two Pgp-homologous probes, the *Manduca* genomic fragment mssp11 and CHP1 from Chinese hamster (Southern blot, standard hybridization).

Probe characteristics	Probe	
	mssp11	CHP1
probe template size (bp)	450	600
template DNA (ng)	30	30
newly synth. DNA (ng)	5.5	4.4
percentage incorporation	25%	20%
specific activity (cpm/ μ g)	1.78×10^9	1.84×10^9
activity in hybrid. solution (cpm/mL)	1.0×10^6	1.0×10^6
[DNA] in hybrid. solution (ng/mL)	0.54	0.53

Table 3.8: Probe labeling and radioactivity data for two Pgp-homologous probes, the *Manduca* genomic fragment mssp11 and CHP1 from Chinese hamster (Southern blot, multiple sandwich hybridization).

Probe characteristics	Probe	
	mssp11	CHP1
probe template size (bp)	450	600
template DNA (ng)	40	40
newly synth. DNA (ng)	9.2	10
percentage incorporation	42%	46%
specific activity (cpm/ μ g)	1.74×10^9	9.34×10^8
activity in hybrid. solution (cpm/mL)	1.2×10^6	1.6×10^6
[DNA] in hybrid. solution (ng/mL)	0.70	1.7

Table 3.9: Probe labeling and radioactivity data for *Manduca* vacuolar-ATPase (Southern blot).

Probe characteristics	Probe
	V-ATPase
probe template size (bp)	1400
template DNA (ng)	30
newly synth. DNA (ng)	n/a
percentage incorporation	n/a
specific activity (cpm/ μ g)	1.83×10^9
activity in hybrid. solution (cpm/mL)	1.0×10^6
[DNA] in hybrid. solution (ng/mL)	0.52

Table 3.10: Probe labeling and radioactivity data for two *Manduca* probes, mssp11 and V-ATPase (Northern blot).

Probe characteristics	Probe	
	mssp11	V-ATPase
probe template size (bp)	450	1400
template DNA (ng)	160	80
newly synth. DNA (ng)	1.9	1.2
percentage incorporation	8.8%	5.6%
specific activity (cpm/ μ g)	8.1×10^7	7.7×10^7
activity in hybrid. solution (cpm/mL)	2.6×10^5	1.2×10^5
[DNA] in hybrid. solution (ng/mL)	3.2	1.6

Table 3.11: Probe labeling and radioactivity data for mdr49 and mdr65 (combined labeling) and CHP1 (Northern blot)

Probe characteristics	Probe	
	mdr49/65	CHP1
probe template size (bp)	1800/1200	600
template DNA (ng)	90 + 40	300
newly synth. DNA (ng)	5.5	1.1
percentage incorporation	25%	5.2%
specific activity (cpm/ μ g)	1.8×10^8	2.4×10^7
activity in hybrid. solution (cpm/mL)	4.9×10^5	1.4×10^5
[DNA] in hybrid. solution (ng/mL)	2.7	6.0

Table 3.12: Probe labeling and radioactivity data for *Manduca* ubiquitin cDNA probe (Northern blot).

Probe characteristics	Probe
	ubiquitin
probe template size (bp)	600
template DNA (ng)	20
newly synth. DNA (ng)	1.0
percentage incorporation	4.6%
specific activity (cpm/ μ g)	1.1×10^8
activity in hybrid. solution (cpm/mL)	1.2×10^5
[DNA] in hybrid. solution (ng/mL)	0.42

Table 3.13: Probe labeling and radioactivity data for the *Manduca* genomic fragment mssp11.

Probe characteristics	Probe
	mssp11
probe template size (bp)	450
template DNA (ng)	30
newly synth. DNA (ng)	15
percentage incorporation	68%
specific activity (cpm/ μ g)	2.00×10^9
activity in hybrid. solution (cpm/mL)	1.0×10^6
[DNA] in hybrid. solution (ng/mL)	0.50

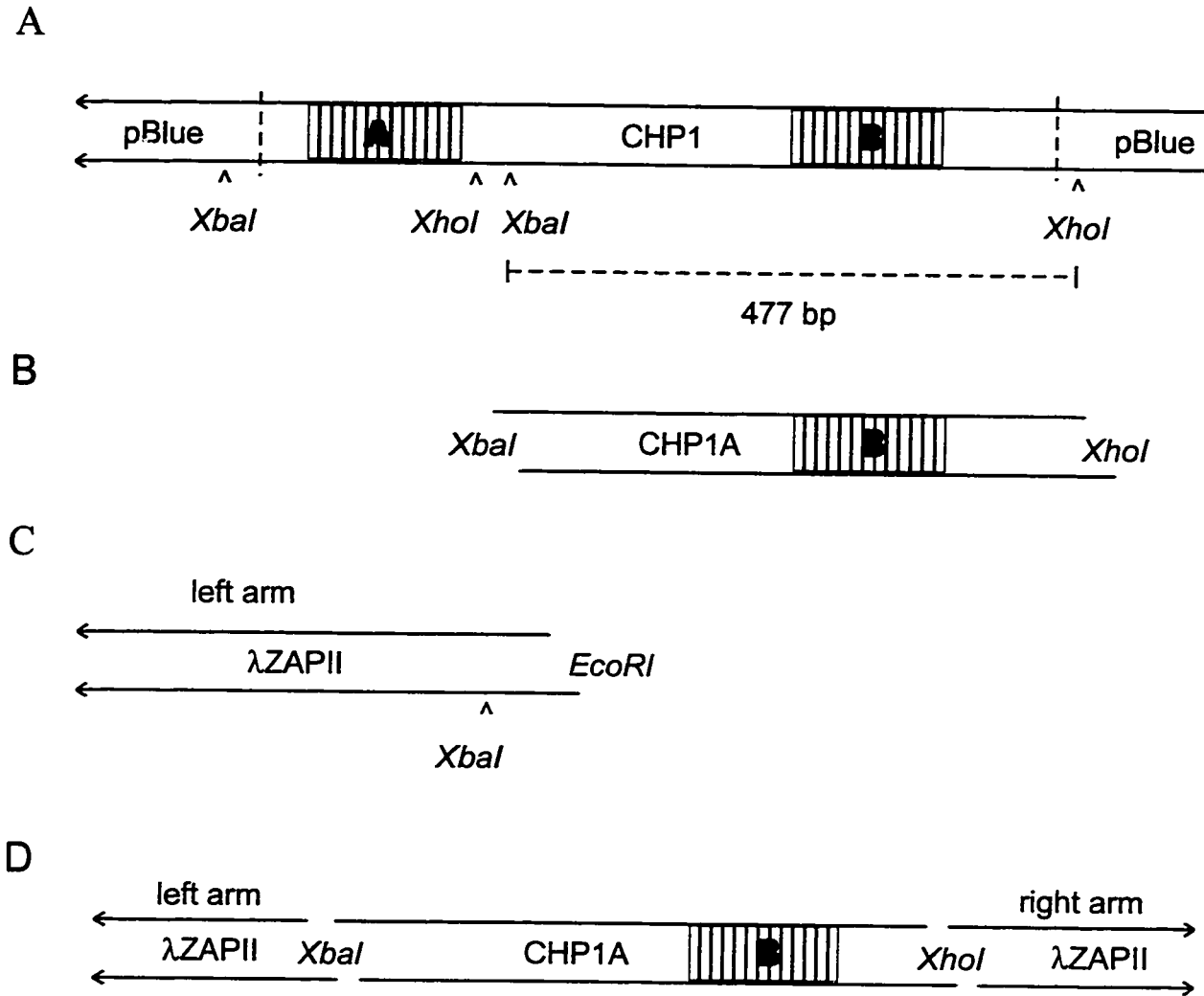


Figure 3.1: Preparation of CHP1A- λ ZAPII construct. A) The CHP1-pBluescript plasmid is digested with *XbaI* and *XhoI*. B) The 477 bp fragment (CHP1A) is purified. C) The left arm of the λ ZAPII vector was digested with *XbaI* to replace the *EcoRI* sticky end with an *XbaI* sticky end. D) CHP1A is ligated with the left and right λ ZAPII arms, and packaged as for the cDNA library (Stratagene, 1993). The Walker A and B consensus sites of the nucleotide binding domain are indicated.

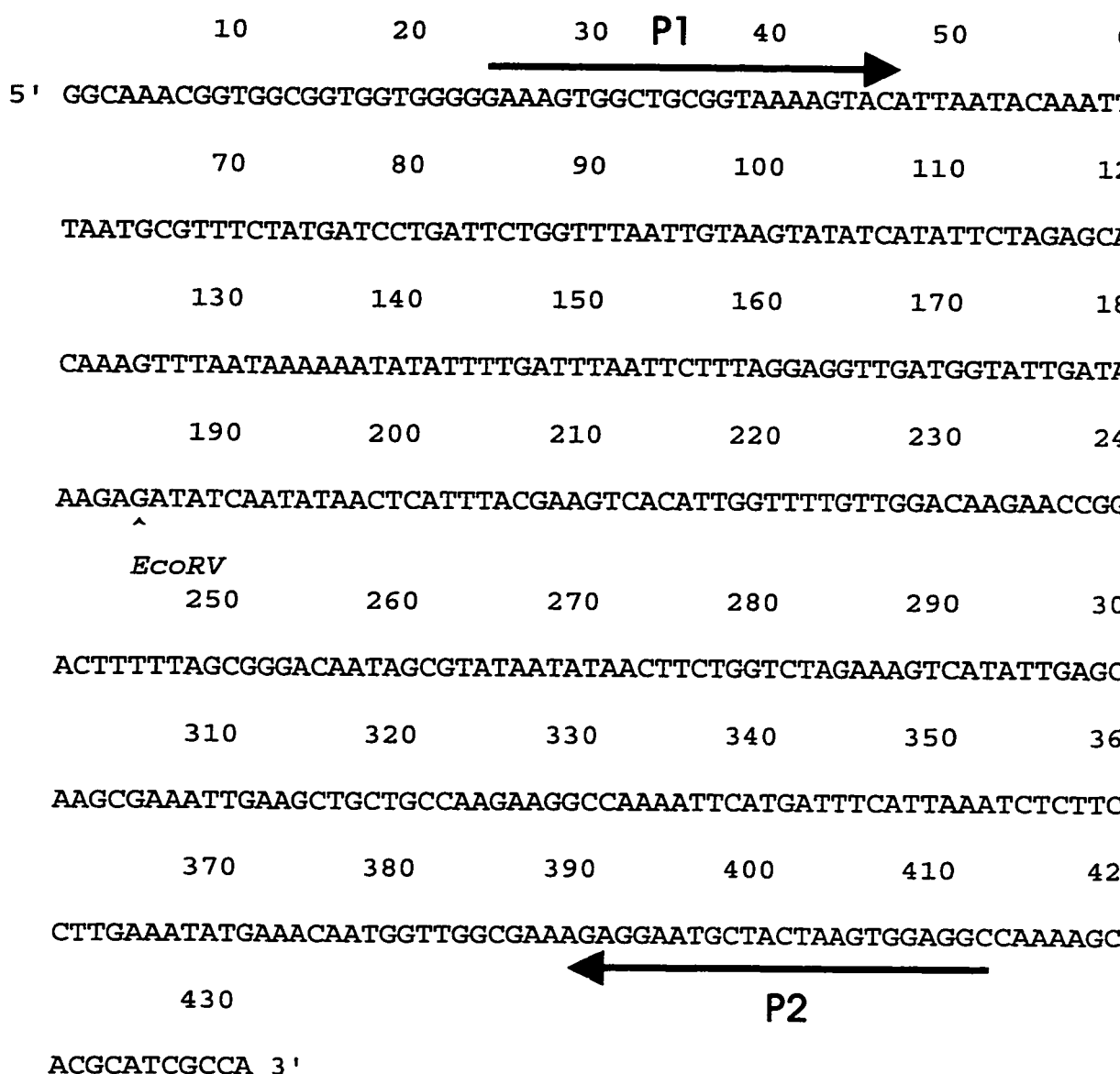


Figure 3.2: Placement of PCR oligonucleotide primers P1 and P2 in the sequence of mssp11. The primers were selected from the sequences just inside the original primers used to obtain mssp11 (Ell and Drouin, unpublished). The P2 primer is equivalent to the complementary sequence. A recognition site for the restriction enzyme *EcoRV* is also indicated.

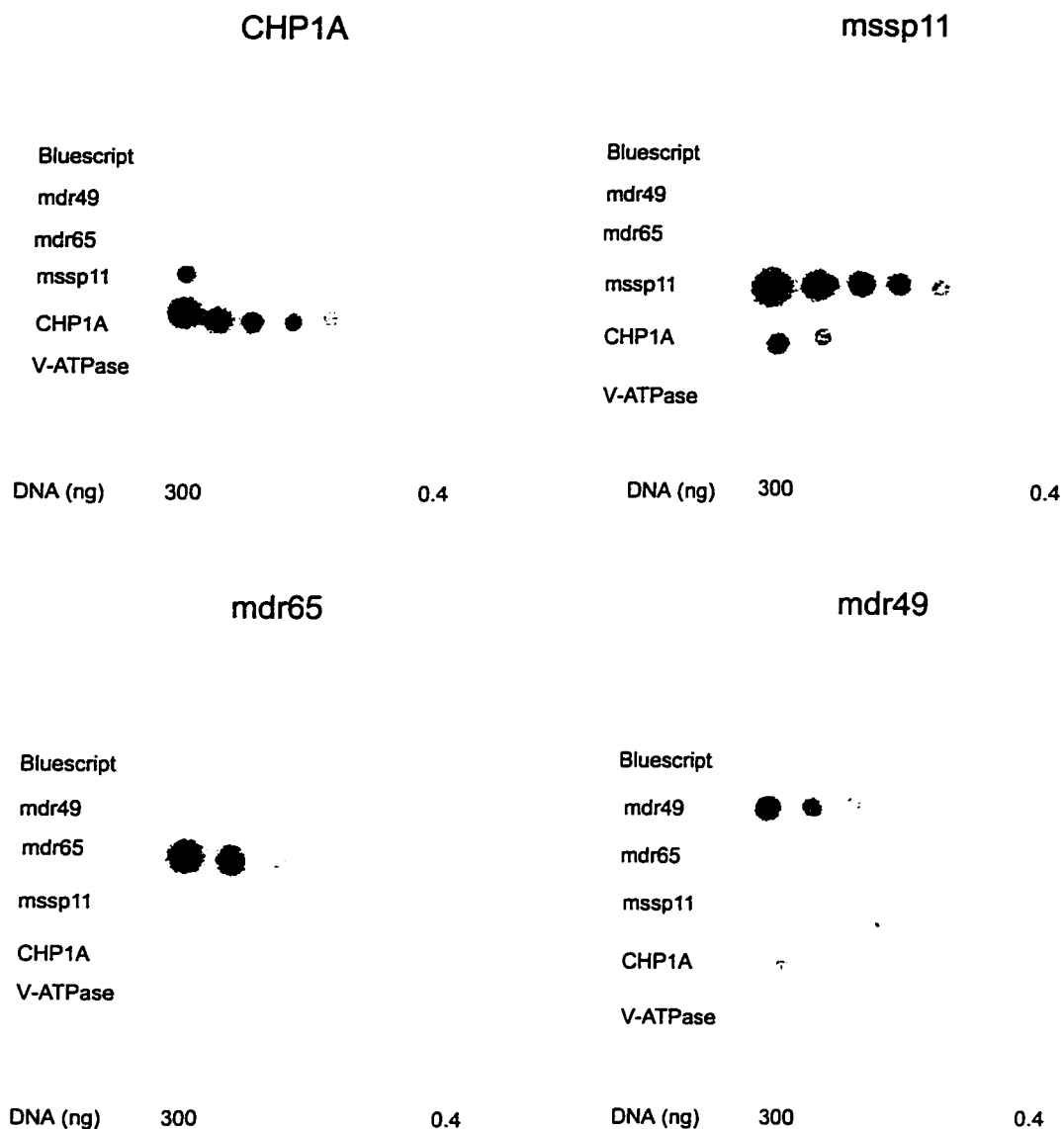


Figure 3.3: Phosphoimager record of the first dot blot hybridization experiment. The probe used in each case is indicated above the image. Seven threefold serial dilutions of plasmids were applied to the membranes. The mssp11 plasmid was diluted from 300 ng to 0.4 ng. The other plasmids were diluted to be equimolar to mssp11, taking into account the size of the inserts.

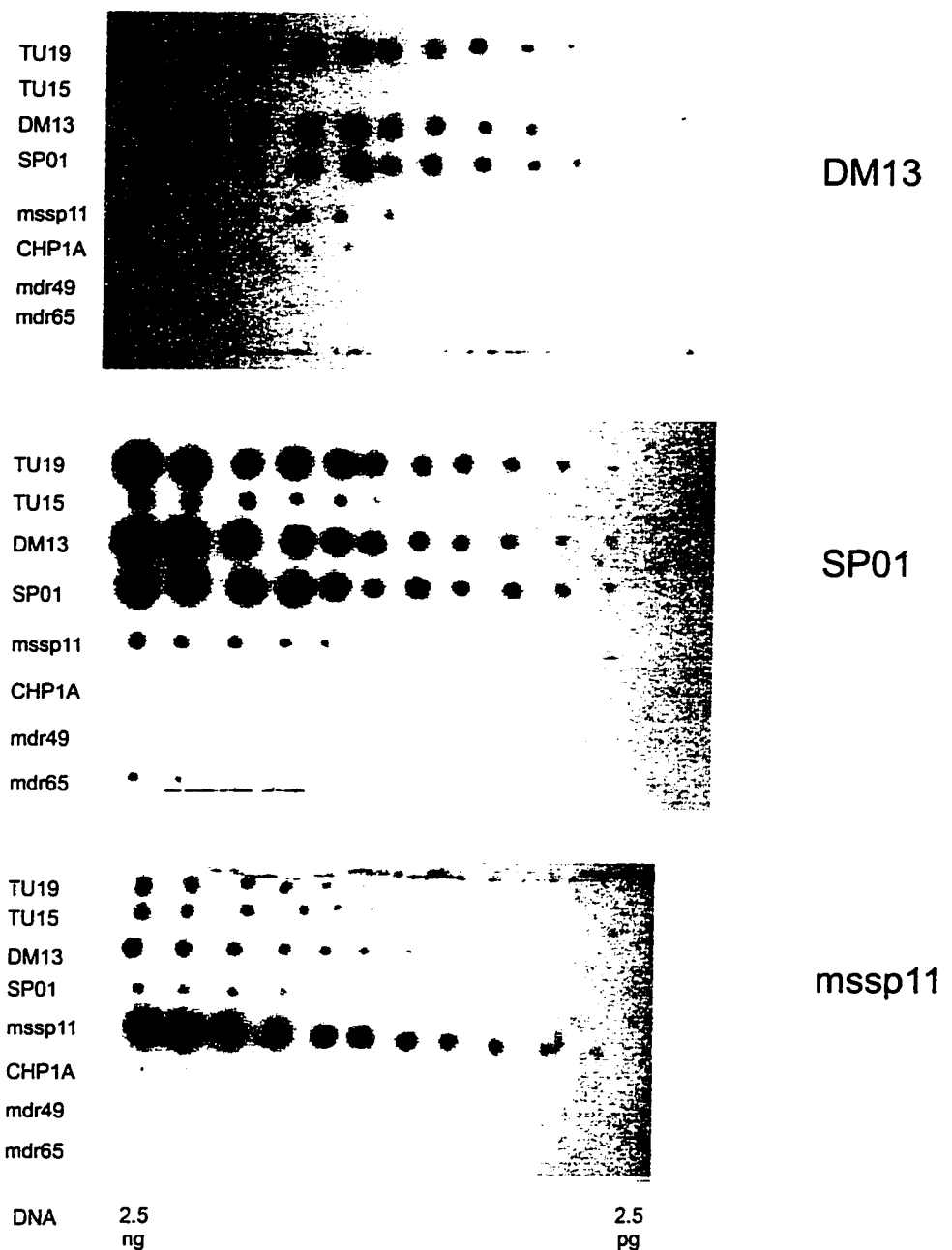


Figure 3.4: Autoradiographs of second series of dot blots. DNA fragments were applied in eleven twofold dilutions. Mssp11 was applied from 2.5 ng to 2.5 pg, and the other fragments were diluted to be equimolar to mssp11. The blots were hybridized with the probe indicated to the right of the image (see also next page). Note that the blots probed with SP01 and DM13 have similar hybridization patterns, while the blots probed with mdr49 and mdr65 (next page) show hybridization almost exclusively to the corresponding row.

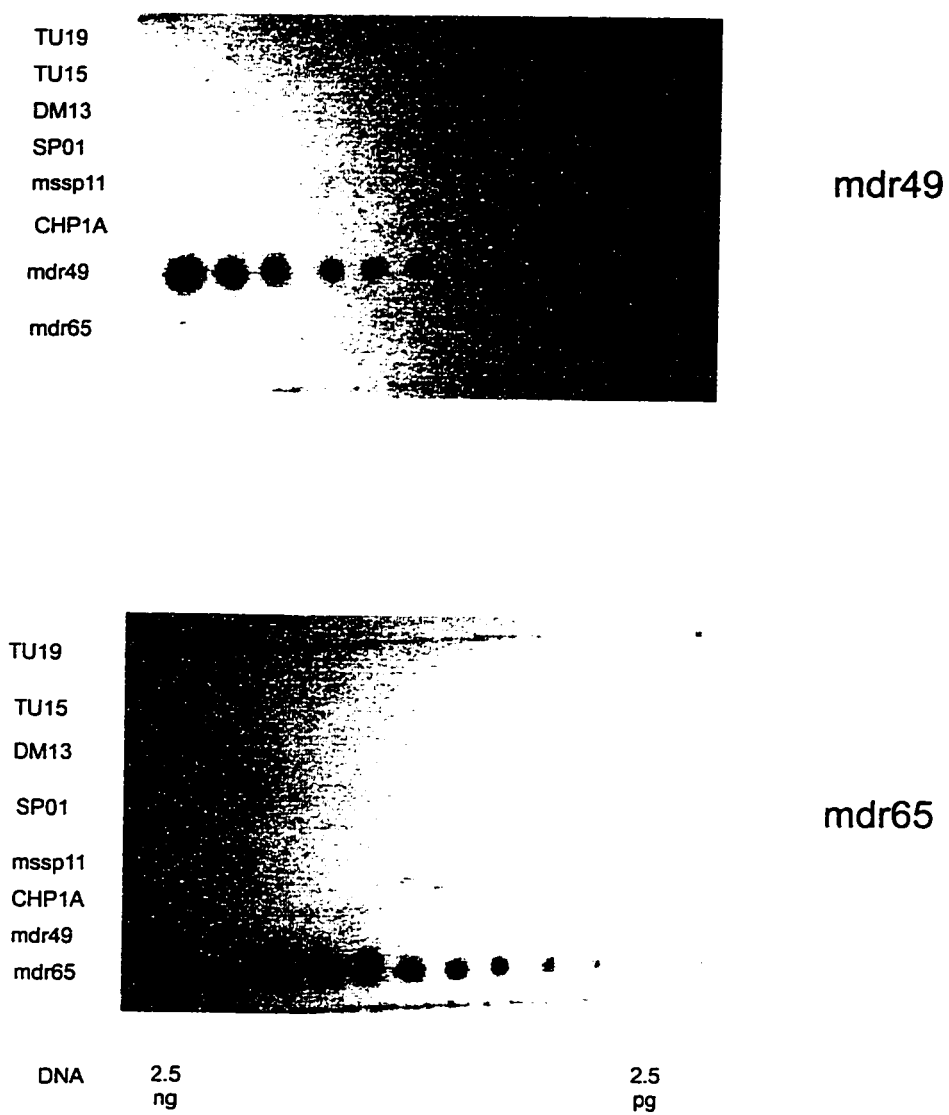


Figure 3.4 (cont.)

520 pfu/cm²



52 pfu/cm²



5.2 pfu/cm²



Figure 3.5: Phosphoimager recording (enhanced) of first plaque density experiment. Approximately 150 positive control plaques (CHP1A) were mixed with the *Manduca* cDNA library plated at three plaque densities: 5.2, 52 and 520 pfu/cm². Note that all the positive plaques are visible at the two lower densities, but the signal is almost completely attenuated at 520 pfu/cm².

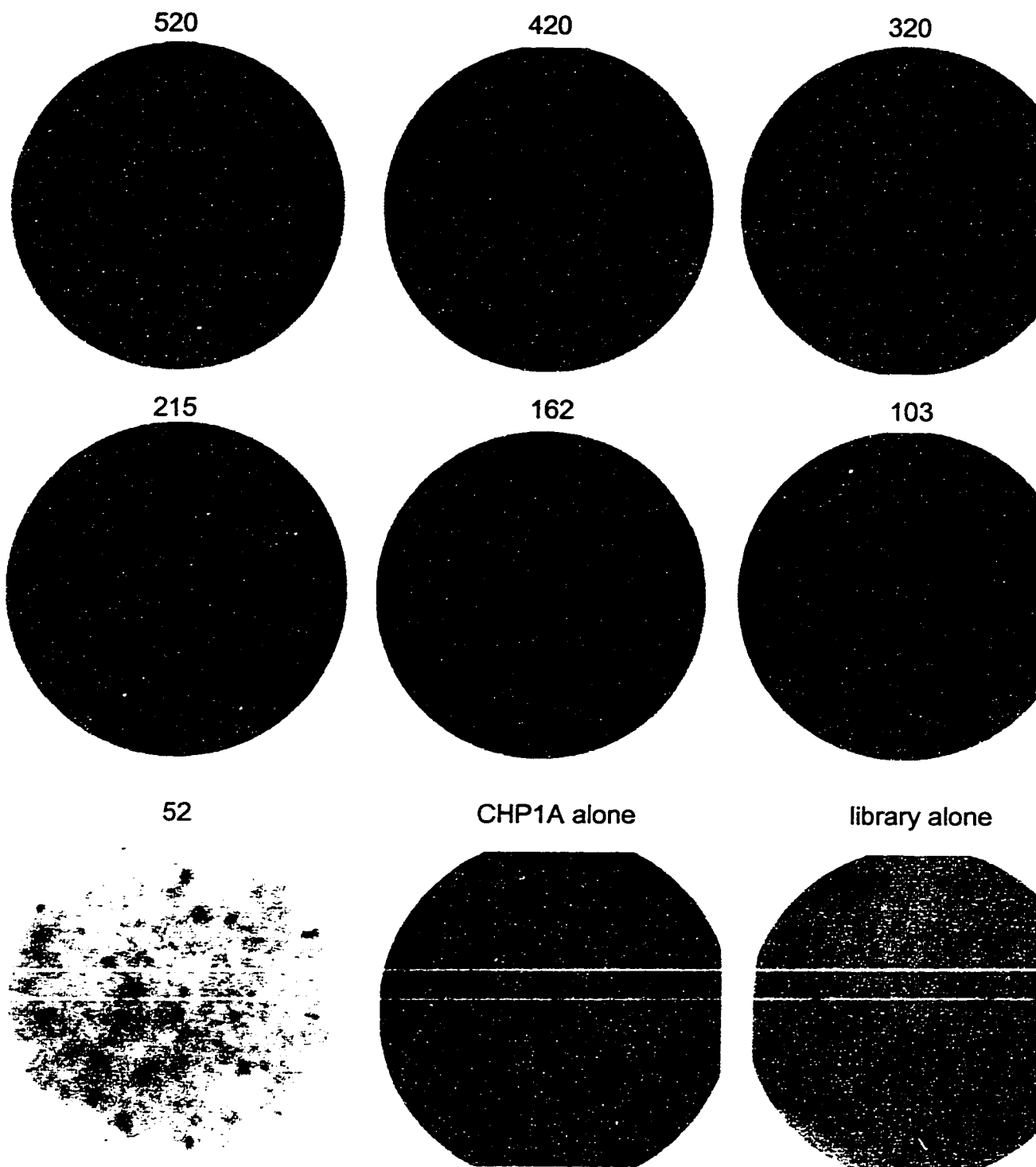


Figure 3.6: Autoradiograph of second plaque density experiment. Approximately 20 pfu of CHP1A positive control were plated with a range of plaque densities of the *Manduca* cDNA library (52-520 pfu/cm²). Also shown (bottom right) are CHP1A alone and the library alone. Note that the signal from the positive plaques is dramatically attenuated at plaque densities higher than 52 pfu/cm².

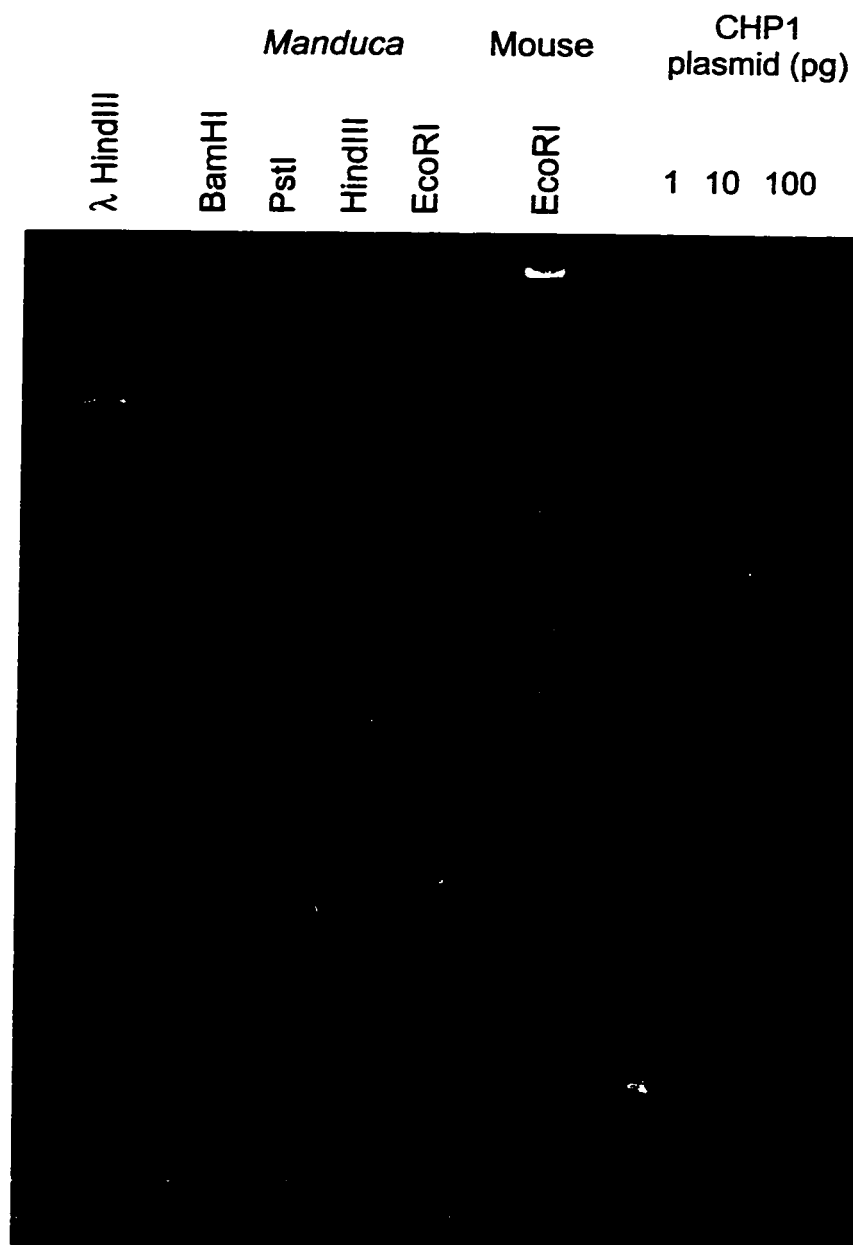


Figure 3.7: Photograph of 0.8% agarose gel prior to Southern transfer. Note that the mouse lane contains the most DNA and the *Manduca HindIII* lane contains the least (see text). Two such gels were prepared; the gels were identical except that the other gel contained mssp11 plasmid as a positive control, instead of CHP1.

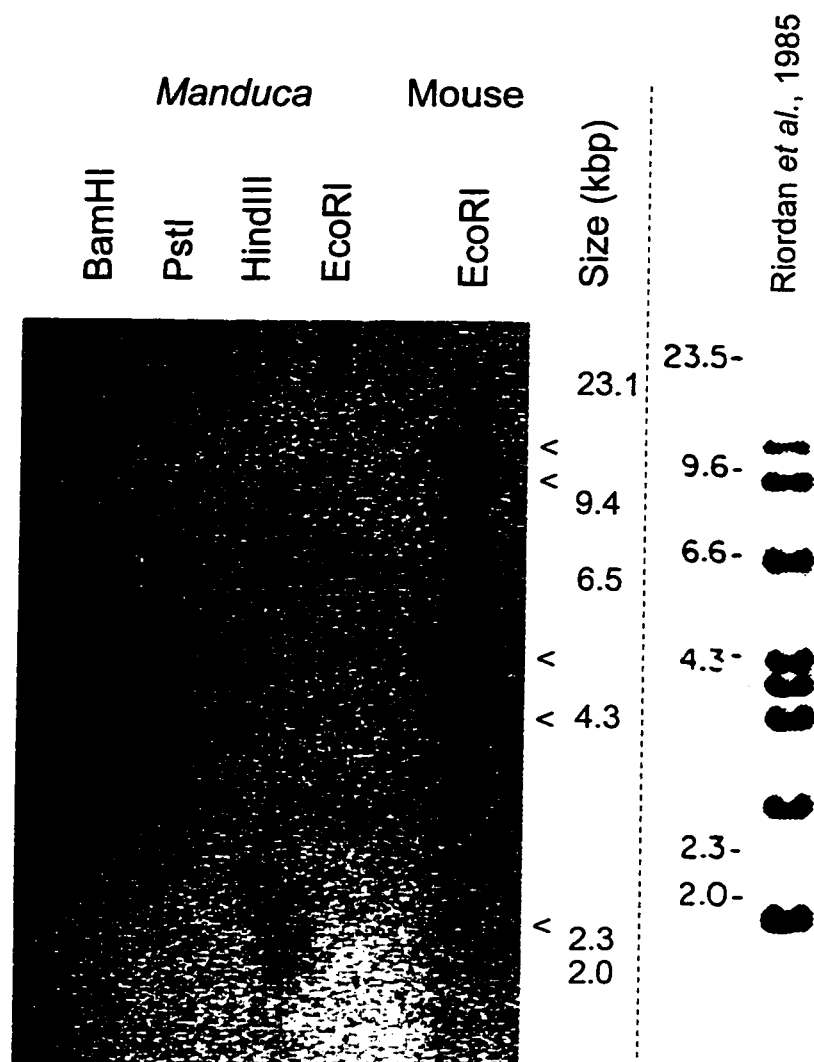


Figure 3.8: Southern blot of *Manduca* and mouse genomic DNA probed with CHP1, a probe derived from *pgp1*, the class I P-glycoprotein homologue of hamster. The genomic DNA was digested with the restriction endonucleases indicated. The blot was hybridized using the sandwich method (Wu et al., 1995), overnight at 42°C (50% formamide) and washed to 0.2x SSPE, 68°C. The blot was exposed to film for 5 days. The autoradiograph was digitized and contrast-enhanced (auto-equalization adjustment, Corel Photo-Paint) for this figure. To the right of the dotted line, a lane equivalent to the mouse lane is shown from an experiment by Riordan et al. (1985). The positions of the bands of the molecular weight markers for both experiments are shown (there is a small discrepancy). Only the five bands indicated by "<" were visible on the unenhanced autoradiograph. This difference in the stoichiometry of the bands may reflect uneven hybridization to different members of the mouse *Pgp* gene family.

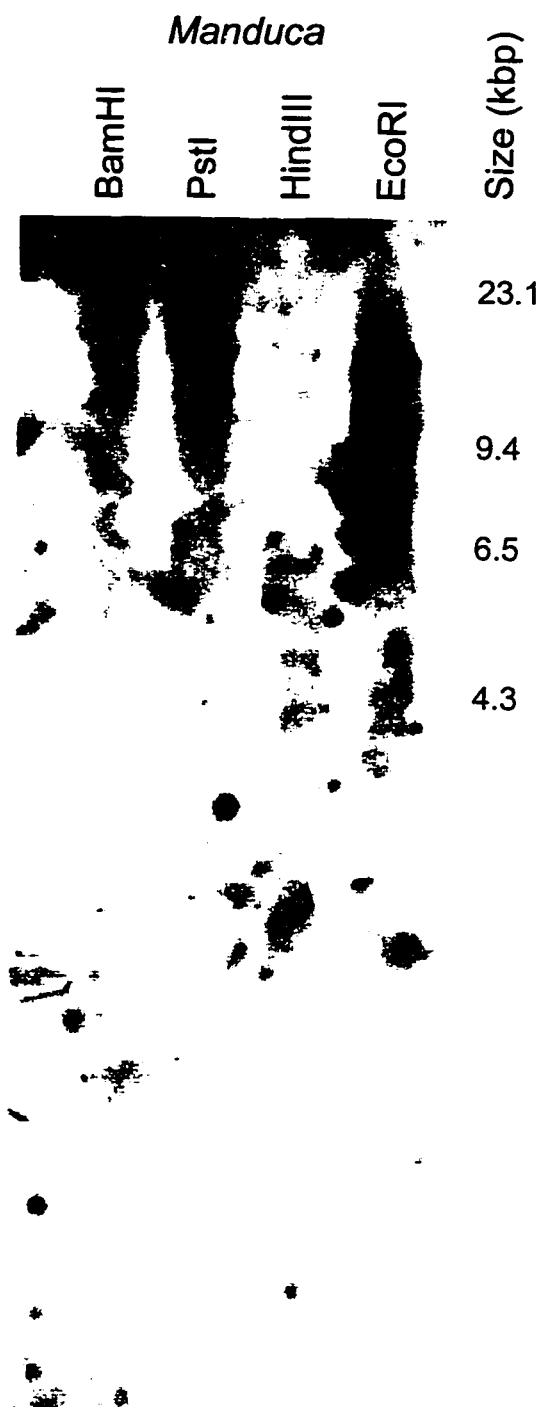


Figure 3.9: Southern blot of *Manduca* genomic DNA probed with *Manduca* V-ATPase probe. The blot was hybridized overnight at 45°C (no formamide) and washed to 2x SSC, 57°C. The blot was exposed to film for 4 days. The positions of the molecular weight marker bands are shown.

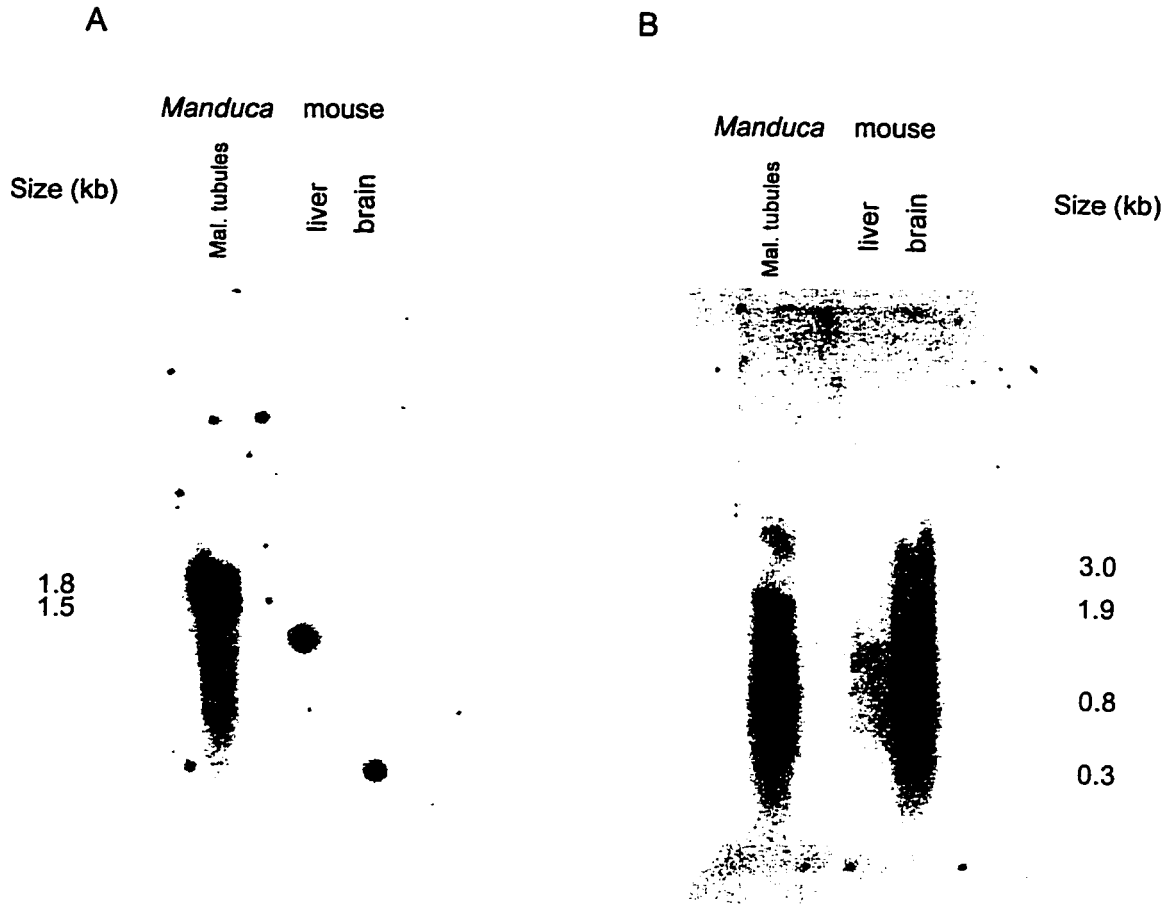


Figure 3.10: Autoradiographs of Northern blot of total RNA from *Manduca* Malpighian tubules, mouse brain and mouse liver, probed with *Manduca* V-ATPase (A) and *Manduca* ubiquitin (B). Molecular weights of blot features are indicated. A: The V-ATPase probe identifies two bands 1.9 and 1.4 kb in size in the *Manduca* Malpighian tubule lane. B: The ubiquitin probe identifies in mouse brain a 3.0 kb band, and a smear from 0.3 to 1.9 kb, with the heaviest signal appearing at 0.8 kb. The smear is also seen in *Manduca* Malpighian tubules. No signal is seen in the mouse liver lane, possibly indicating that the RNA is degraded. Sizes were determined by comparison to the ribosomal RNA subunits of mouse.

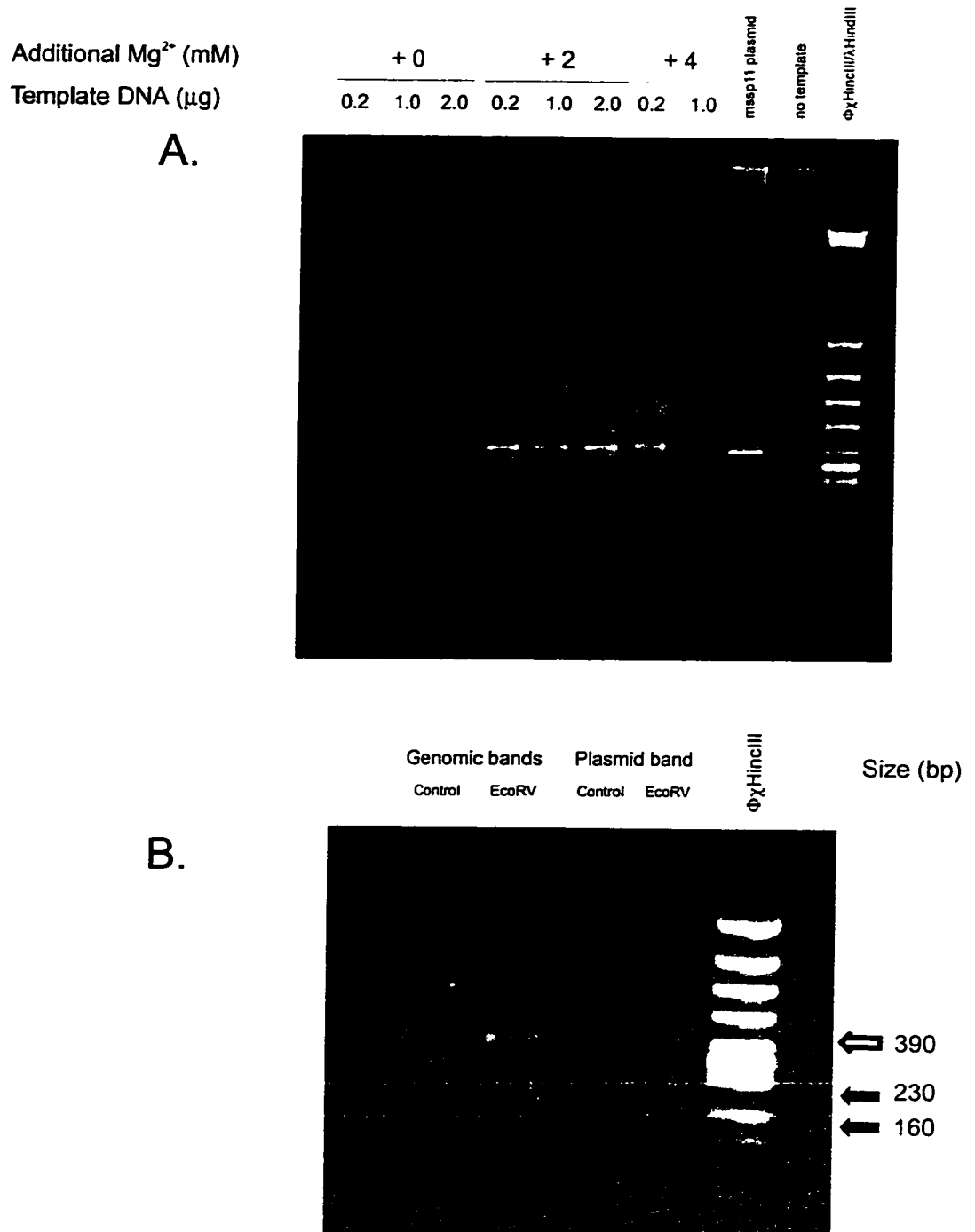


Figure 3.11: PCR of *Manduca* genomic DNA with primers specific to mssp11. **A.** Results of second round of PCR. Under all conditions (varied Mg²⁺ concentration and amount of template DNA) a band of approximately 390bp is seen. Amplification from mssp11 plasmid template also results in a 390bp band. The bands from the first six lanes of genomic PCR were excised from the gel (indicated by outline) and pooled. The band amplified from mssp11 plasmid was also excised. **B.** Restriction analysis of the excised bands. Half of each sample was digested with *EcoRV*. The band amplified from mssp11 plasmid (white arrow; 390 bp) is cut into two smaller fragments (black arrows, 230 and 160 bp), as expected; however, the pooled bands amplified from genomic DNA are not cut, indicating that they do not represent mssp11 faithfully amplified from genomic DNA.

Chapter 4: A cDNA clone of the *Manduca sexta* ADP/ATP translocase

Introduction

The ADP/ATP translocase (AAT) is an important metabolic enzyme involved in oxidative phosphorylation. It is present as a homodimer in the inner mitochondrial membrane and represents 10-15% of the protein in this membrane. It exchanges ATP produced in the matrix of the mitochondrion for ADP produced in the cytosol by ATP hydrolysis. This antiport process is electrogenic and is driven by the electrical potential of the inner mitochondrial membrane. The enzyme is a target of a number of secondary metabolites including bongkreikic acid (produced by *Pseudomonas cocovenenans* growing on coconut) and atractyloside (produced by the thistle *Atractylis gummifera*; *The Merck Index*, 1989).

Of the false positive clones that were sequenced at one point or another during the cDNA library screening (see Chapter 2), one cDNA clone was identified as an ADP/ATP translocase (a.k.a. ADP, ATP carrier protein), a sequence that had not been cloned from *Manduca*. It was identified as such by its sequence similarity to the equivalent sequences in *Drosophila*, *Rana* and other species.

This chapter describes the sequencing of this clone in its entirety, and Northern and Southern blot analysis which were done to partially characterize this cDNA.

Methods

A) Sequencing of ADP/ATP translocase clone

The sequence of a partial cDNA representing the *Manduca* homologue of ADP/ATP translocase was determined. This clone was originally identified as a false

positive in expression screening of the *Manduca* Malpighian tubule library with the mdr Ab-1 polyclonal antibody (work not reported). The sequence was determined using the Loeb Institute's automated sequencer (Li-Cor Inc.), on a 66 cm, 4% Long Ranger gel. Sequencing reactions were prepared with the Thermosequenase PCR-based kit, according to the manufacturer's protocol, using M13 forward and reverse primers.

B) Southern blot analysis

The Southern blot of *Manduca* genomic DNA (which was prepared according to Chapter 3, Methods, section C) was probed with the partial cDNA of the ADP-ATP translocase gene. This was done both as a second control for the previous experiments and for the intrinsic value of this original data. Hybridization conditions were the same as for the msspl1/CHP1 probing of the Southern blot (Chapter 3, Methods, section C-iii-a) with the exception of the hybridization temperature, which was 55°C. Following hybridization, the blot was washed in the following series of washes (all washes contained 0.1% SDS):

2 x SSC, 57°C, 30 min.
2 x SSC, 65°C, 25 min.

The blot was exposed to film after each step.

C) Northern blot analysis

The Northern blot of mRNA from 4th and 5th instar *Manduca* gut tissue (prepared in Chapter 3, Methods, Section D-ii) was also probed with AAT, both as a control for the quality of the RNA preparation, and for the intrinsic value of this original data. The blot was probed at the same time as the Southern Blot in section B above. Hybridization conditions are the same as reported in section B. The same series of washes were used as

well. The blot was stripped in 50% formamide, 2 x SSPE, 65°C, 1 hour ("gentle stripping", Sambrook *et al.*, 1989).

Results

A) Sequencing of AAT clone

The sequence of the AAT clone indicates that it is a 1106-bp segment of the *Manduca* cDNA consisting mostly of the 3'-untranslated region (Figure 4.1A). The 5' end of the molecule contains a 207 bp region which is highly similar to the C-terminal end of the coding region of the *Drosophila* (Figure 4.1B). Because the 5' end of the clone is missing, it is difficult to ascertain the proper open reading frame for this clone. If the sequence is arranged according to the open reading frame of the *Drosophila* gene, two premature termination codons result (Figure 4.1A,B). At least one of these premature termination codons could be attributed to a band compression (see Discussion). However the region in between these termination codons is undeniably homologous to the *Drosophila* gene. Figure 4.2 shows that the *Manduca* clone codes for the sixth transmembrane domain of AAT, and the region just N-terminal to this domain.

B) Southern blot analysis

Table 4.1 summarizes radioactive labeling data for the AAT probe. Figure 4.3 displays the autoradiograph of the Southern blot. A single band is identified in each lane. The band seen in the *HindIII* lane is weak compared to the other lanes, but this is expected due to the DNA loading on the blot (see Figure 3.7). The distinct band seen in the *BamHI* lane shows that the DNA in this lane is not degraded, thus the inability of the V-ATPase probe to detect a distinct band in this lane is likely due to other factors (see Chapter 3, Results, Section C-ii).

C) Northern blot analysis

The same probe was used for the Northern analysis as for the Southern analysis above; please refer to Table 4.1 for labeling data.

Figure 4.4 shows an autoradiograph of the Northern blot. The AAT probe identifies two mRNA species of approximate sizes 2.15 and 1.45 kb in both 4th and 5th instar gut tissue fractions (*i.e.* midgut + Malpighian tubules). As well, a light smear above the larger band is seen in the 5th instar gut, but not in the 4th instar gut. This probing was also a control for the quality of the RNA preparation, as AAT is essential for oxidative phosphorylation, it should be expressed in all tissues. No signal was detected in three other RNA samples that were loaded on the blot (4th and 5th instar body wall, and a second sample of 4th instar gut), indicating that the RNA in these samples was lost or degraded at some point in the purification procedure (likely during the poly A+ purification with Oligo-tex, as the total RNA from these samples was intact; not shown).

Discussion

A cDNA was isolated from the *Manduca sexta* Malpighian tubule library which has very high similarity to the ADP/ATP translocase gene from *Drosophila* and other organisms. Although, the sequence of this clone was determined, it must be considered preliminary; it represents only a small part of the C-terminal coding region and a putative open reading frame deduced from that of *Drosophila* results in two premature stop codons and a two base-pair insertion which distorts the reading frame (Figure 4.1). This sequence was determined twice (on one strand) to confirm the presence of these unusual features. A compression defect was seen both times on the sequencing gel in the region of the second premature stop codon; the codon could be GTA - valine, instead of a TGA

stop codon. Sequencing on the opposite strand might give more information about this peculiarity.

The AAT clone identified single bands on a Southern blot of *Manduca* genomic DNA (Figure 4.3), which suggests that there may be only a single gene in *Manduca*, however, the possibility that there are two or more genes closely linked one after the other, with no *EcoRI*, *PstI*, *HindIII* and *BamHI* sites in between, cannot be discounted. *Drosophila* is predicted to have two genes, based on Southern blot analysis and humans have at least three genes (Louvi and Tsitilou, 1992 and reports cited therein).

Two mRNA species were identified on a Northern blot of gut tissue from 4th and 5th instar *Manduca* larvae (Figure 4.4). Of the two, the cDNA clone likely represents the 1.45 kb species, as this would be the approximate size of the clone if the entire coding region were included. The larger 2.15 kb mRNA may represent the same gene with a longer 3'-UTR derived by alternate polyadenylation, or it may represent a closely related gene that is also expressed. The identity of the smear in the 5th instar gut lane is not known, however, it could represent general binding of poly-T rich probe fragments to the poly-A tails of other mRNAs .

Table 4.1: Probe labeling and radioactivity data for *Manduca* ADP/ATP translocase (AAT) partial cDNA (including 3'-untranslated region), Southern and Northern blots.

Probe characteristics	Probe
	AAT
probe template size (bp)	1100
template DNA (ng)	30
newly synth. DNA (ng)	5.1
percentage incorporation	23%
specific activity (cpm/ μ g)	7.97×10^8
activity in hybrid. solution (cpm/mL)	1.0×10^6
[DNA] in hybrid. solution (ng/mL)	1.25

```

1 G GCA CGA GGT CCG TAG CGC ATG ATG ATG CAG TCT GGC CGC GCT AAG AGG CAT ATC CT
59 TAC AAG AAC ACC CTG CAC TGC TGG GCG ACT ATC GCG CGG ACC GAA TGA CC TCG GC
117 TTC TTC AAG GGA GCC TTC TCC AAC ATC CTG AGA GGC ACA GGT GGC GCC TTC GTG CT
174 GTC CTC TAT GAC GAG ATC AAG AAG CTC CTC TAA

208 ATGTAA TGTAAGAATT GTTACCAGTT ACATCAATGT
241 GATTCCACAT CACCGCAGCC ACTCCCCTCA AATCATGTAT TATTAGACGC GAAAACGACT
301 CTAGTAAC TT ATTTTAGTGA AATAAAGCGT TATACGATGA TTCCATAATT TATTGTTTTA
361 TTAACATTAA AATTTAAGAG TTACATAATT ATGTGTGTCA CTAAGTGATG GTTAAATGTC
421 AAAGGTTACA GTTGTTAACT CTAATTAATT TACTGTCATC TAATGTGTCA TGTATTTCTG
481 TTCTTTATTTC TGTATTCAAA TTTATTTAAA CATATCTTTA CTGTATATTT AAATATAAGA
541 CAGTTGCATG CCCGGCCAGT GTTATGTAAAC CAACCATATT CTTTACACAT ACCGGAAGCC
601 AAGGCTGAAC AGTAAGATAT TATTTCTAGA CTATACATTA CCAACTGAAA TCTTTGCTAA
661 ATATATTGTG AACCAATGAT TTGGTGAAAA TTATGAATTT ACACCAAATT TTCTATCATA
721 AGGTGACATA AGGGAGTGGA CAAGACCATT GTTAGTTTTG TTACCATGTT TTCAATCTGT
781 ATAACGCTGA TAATAATCGC CGTAAATTGT TCGAAACCTT GTGATTATGA AGCTGTTATG
841 TAACAATGCA GATGTATGTA TATCGTGCTA TGTGGTGCGT TTGTAGATTA TTTTAGTTCA
901 CATTTAAGAG TGTGCGGATT CGCTGCGTAG TAAAGTTCCA CACATCTGTA CGTCTATCTA
961 GTAGGAAACG TTGCGTAGGA GGAAGACTAA GTACCTATGT GAACATCTTT GCTCGGGTGT
1021 GAGTTTTTTG TGAGAAAAAT TCACGCAATA CGAGTTGTAT TGATTAATAG GAAATTAAAT
1081 GTTAAAATAA AAAAAAAAAA AAAAAA

```

```

Manduca ARGP*RMMMQSGRAKRHILYKNTLHCWATIARTE*-AFFKGAFSNILRGTGGAFVLVLYDEIKKL*
          RMMMQSGR ++YKNTLHCWATIA+ E +FFKGAFSNILRGTGGAFVLVLYDEIKK+L
Drosoph. DTVRRRMMMQSGRKATEVIYKNTLHCWATIAKQEGSPSFFKGAFSNILRGTGGAFVLVLYDEIKKVL*

```

Figure 4.1: A. Complete nucleotide sequence of a partial cDNA of ADP/ATP translocase from *Manduca* Malpighian tubules. The first 207 bp are arranged in codons based on the coding sequence of the homologous gene in *Drosophila melanogaster*. Two premature termination codons (bold, underlined) are indicated; however these may be the result of sequencing artifacts (see text). A two base-pair insertion following the second premature stop codon throws the rest of the sequence out of frame. The rest of the sequence represents a large 3'-UTR with poly-A tail. Three putative polyadenylation signal sequences are double-underlined. B. Alignment of predicted amino acid sequence of the putative coding region of the *Manduca* clone with the equivalent part of the *Drosophila* coding region. Identical amino acids are in between the two sequences; functionally similar amino acids are indicated by "+". The stop codons are indicated by asterisks and the two base-pair insertion is indicated by a dash.

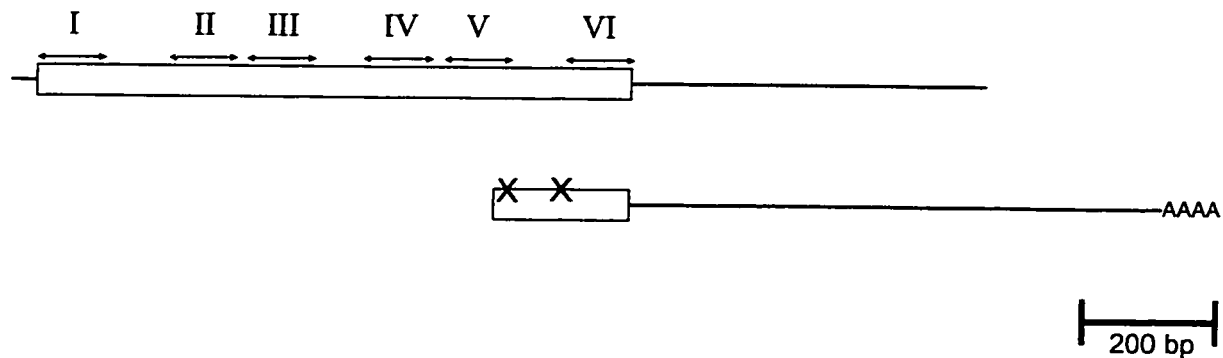


Figure 4.2: Cartoon comparing the *Manduca* AAT cDNA clone to the *Drosophila* cDNA reported in Louvi and Tsitilou (1992). The open reading frame is indicated by a white box; the black line indicates 5'- and 3'-untranslated regions. The regions identified by Roman numerals indicate the six predicted transmembrane domains of the *Drosophila* gene. The two "X" symbols indicate the premature termination codons in the *Manduca* clone. The poly-A tail of the *Manduca* clone is also shown.

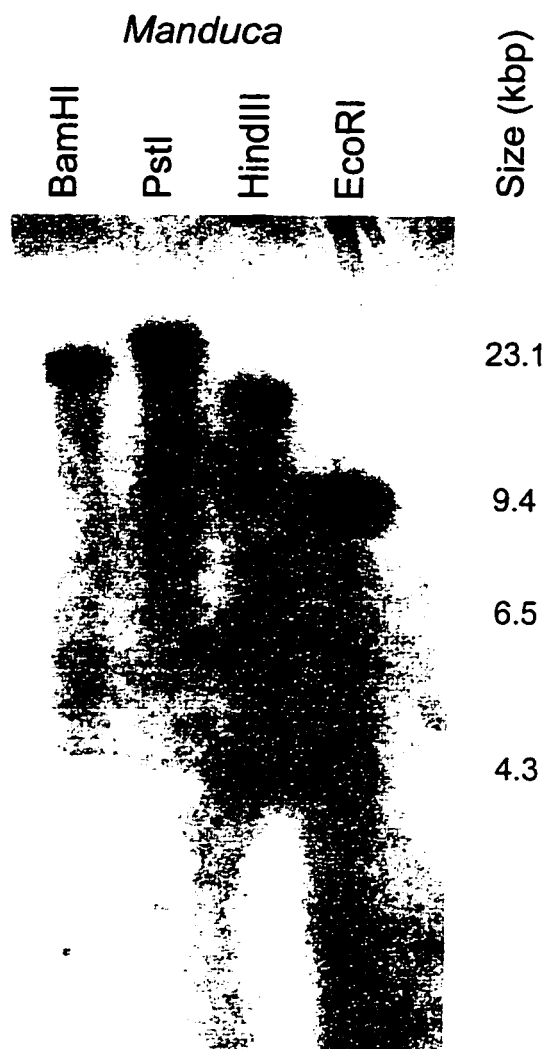


Figure 4.3: Southern blot of *Manduca* genomic DNA probed with a *Manduca* AAT probe. The blot was hybridized overnight at 55°C (no formamide) and washed with 2x SSC at 65°C. The blot was exposed to film for 6 days. The positions of the molecular weight marker bands are shown. Note that a single band appears in each lane.

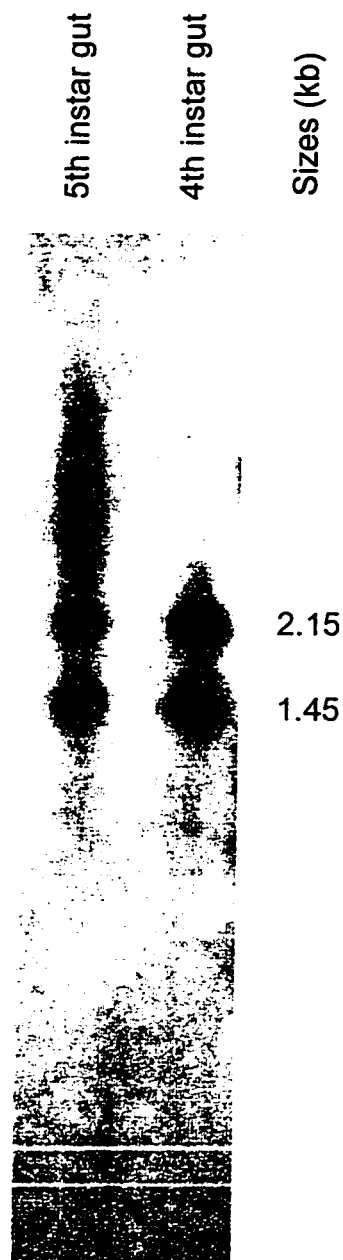


Figure 4.4: Northern blot of mRNA from gut fraction (*i.e.* midgut +Malpighian tubule tissue) of 4th and 5th instar *Manduca* larvae probed with *Manduca* AAT. The blot was hybridized overnight at 55°C in aqueous hybridization solution and washed with 2x SSC at 65°C. The blot was exposed to film for 6 days. The molecular weights of the bands were determined by comparison to an RNA molecular weight marker.

Chapter 5: General Discussion

Twenty years ago, the techniques of the fledgling science of molecular biology were almost exclusively used by those who were responsible for their development, the biochemists. The geneticists were the first biologists to take advantage of the rapidly advancing technology for use in their own research, and today biological sciences as disparate as taxonomy, ecology, and behavioural neuroscience commonly employ molecular methods. The reason for this growth is not hard to understand; examining biological phenomena at the DNA level can be likened to taking apart a complex appliance to examine the circuits and inner workings. The mechanical analogy is particularly appropriate to the use of molecular techniques in physiology, which is essentially the study of how living things work. Molecular physiology links macroscopic phenomena such as nerve impulses, muscle contraction and toxin excretion with their microscopic originators: ion channels, contractile filaments and transport proteins.

In the current research, the objective is to link the considerable physiological data pointing to a nicotine pumping mechanism in the Malpighian tubules and the CNS of *Manduca sexta* with the responsible protein(s). At this time, the most likely candidate appears to be P-glycoprotein, based on the following pieces of evidence:

- 1) P-glycoprotein-like immunoreactivity has been detected in *Manduca* tissues where nicotine pumping has been directly (Malpighian tubules) and indirectly (CNS) demonstrated (Morris, 1983a-c; Murray *et al.*, 1994; Murray, 1996). Pgp immunostaining is also seen in *Rhodnius prolixus* Malpighian tubules, which can transport a wide range of alkaloids (Murray, 1996; Maddrell and Gardiner, 1976)

2) P-glycoproteins expressed in epithelial layers (kidney, liver, intestine) and at the blood-brain barrier in other species transport a number of different alkaloid compounds and evidence suggests the protein plays a role in protection from xenobiotics (e.g. Schinkel *et al.*, 1994).

3) Alkaloid transport by *Manduca* Malpighian tubules is pharmacologically similar to drug transport by P-glycoprotein, in that the tubules pump the known P-glycoprotein substrate vinblastine, and both vinblastine and nicotine pumping activity are inhibited by the known Pgp inhibitor verapamil (Murray, 1996; L. Gaertner, personal communication).

4) At least one, and possibly two or more P-glycoprotein homologues exist in *Manduca sexta*, as judged by hybridization of hamster and *Drosophila* Pgp probes to *Manduca* genomic DNA; as well, a fragment of a putative *Manduca* Pgp homologue has been amplified from *Manduca* genomic DNA by PCR (M. Ell, personal communication).

That is what is known about P-glycoprotein in *Manduca sexta*. These are some of the things that were not known about P-glycoprotein in *Manduca sexta* prior to the commencement of this project:

1) It was not known for sure that the P-glycoprotein-like staining seen by Murray (1996) is truly representative of P-glycoprotein. Staining was seen with the monoclonal antibody C219 and the polyclonal antibody mdr Ab-1 (which was raised against a polypeptide containing the C219 epitope). This staining was specific in that it was abolished by pre-incubation with the epitope polypeptide. What is not yet known is whether these same antibodies will detect a protein of the approximate molecular weight of Pgp on a Western blot of *Manduca* tissues. It has also been shown recently that mAb

C219 cross-reacts with an unrelated protein in mouse brain capillaries (Jetté *et al.*, 1995). Lanning *et al.* (1996a) show that a Pgp-like molecule of approximately 150 kDa can be detected in tissues of the lepidopteran *Heliothis virescens* with three different Pgp antibodies, c219, mdr Ab-1 and c494. However the tissue expression in this organism is somewhat different than that seen in *Manduca sexta* immunohistochemistry (no Pgp-like expression is seen in the *Heliothis* midgut; Lanning *et al.*, 1996a), so direct comparisons are probably not appropriate.

2) Almost nothing was known about Pgp mRNA expression in insects. The only published data is a Northern blot of adult *Drosophila* RNA probed with a fragment of the *mdr49* gene (Wu *et al.*, 1991), which shows a 4.5 kb mRNA. Nothing was known about tissue-specific expression of Pgp mRNA in insects.

3) It was not known which gene family member the amplified *Manduca* fragment (mssp11) represented, or even if the concept of cross-species homologous Pgp gene family members is relevant outside the mammalian lineage.

Of these three items, the first was not addressed by my research, but is nonetheless an important consideration. Immunohistochemistry evidence is somewhat weak if it is not backed up with Western blot data demonstrating that the immunoreactivity seen can be attributed to the presence of a protein of the appropriate molecular weight. While the *Heliothis* Western blots suggest that at least one lepidopteran can express a Pgp-like protein of an appropriate size (Lanning *et al.*, 1996a), this needs to be demonstrated in *Manduca* as well.

The second point was addressed in my research, by the construction of a cDNA library from Malpighian tubule tissue. While it might be suggested that construction of

the cDNA library was premature before obtaining expression data via a Northern blot, it should be pointed out that unlike Northern analysis, cDNA library screening has a great potential to detect mRNAs that are expressed only at low levels. This is because, in a plating of a cDNA library, each transcript in the library is represented by a phage plaque, a concentrated dot containing a large number of identical cDNA copies of the transcript. This has the potential to produce a much stronger signal than a few mRNA molecules on a Northern blot. For example, if P-glycoprotein was represented by 1 in 100 000 mRNAs in Malpighian tubules, then there would be 5-6 copies in my cDNA library, which has a primary titer of 550 000 plaque-forming units. If I screened 550 000 plaques total during a screening, then I would have 5-6 chances of seeing a strong signal from a plaque. By contrast, on a Northern I would be looking for a very weak signal with that level of expression. I might need to use a lot more tissue to get a signal, and in this particular situation, the tissue is difficult to obtain.

If it was not premature to construct the cDNA library, I must admit that it was premature to begin screening the library before re-checking the sensitivity of the probes with a Southern analysis under the conditions I would use for the screening. An assumption was made that mssp11 would make a good probe because it was a fragment of a Pgp homologue that was amplified from *Manduca* genomic DNA, and an equivalent assumption was made about CHP1, mdr49 and mdr65 because Ell had done successful Southern analysis with these probes. As my own Southern analysis and plaque density experiments have shown (Figures 3.5, 3.6, 3.8, 3.9, 4.3) CHP1 is in fact much less sensitive than other probes such as V-ATPase and AAT, and the usefulness of mssp11 is questionable. The reason for the discrepancy between my results and Ell's is not entirely

clear, but may be due to the use of somewhat different electrophoresis and transfer equipment by Ell. As suggested earlier, it would be worthwhile to try different methods of radioactive labeling of probes, since the usefulness of the random primer method (used by both myself and Ell) appears to depend on particular laboratory conditions.

One method of investigating Pgp expression in *Manduca* which would not depend on having a sensitive probe would be RT-PCR. Preliminary testing of PCR primers derived from the sequence of msspl1 has not been promising, but RT-PCR remains an attractive option for future work. Degenerate primers to conserved regions of Pgp could be used instead. Another advantage of RT-PCR is that PCR products could be candidates for new probes for the cDNA library.

The third unknown mentioned above has been at least partially answered by my research. The dot blot hybridization experiments (Figure 3.4) suggest that homologous gene family members may exist cross-species in non-mammalian groups. The probes DM13 from *Drosophila*, SP01 from *Spodoptera frugiperda* and TU19 from *Tetranicus urticae* have remarkably similar hybridization patterns, which suggests that they have high nucleotide sequence similarity. Complete characterization of these sequences, as well as Southern blot evidence, will, however, be required in order to confirm or refute this hypothesis.

Conclusions

This research has laid an important foundation for further molecular characterization of P-glycoprotein in *Manduca sexta* as it pertains to this organism's nicotine resistance mechanism. Priorities for future work should be the identification of sensitive Pgp probes and thorough study of the expression of Pgp in the nicotine-

pumping tissues. This work has also provided some new information about the ADP/ATP translocase gene in *Manduca sexta*.

References

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Appendix A - Wheat germ based diet for *Manduca sexta*

(based on Yamamoto, 1969)

Ingredients:	Single batch
<i>Dry mix:</i>	Mass (g)
wheat germ	50.00
casein	22.50
sucrose	20.00
brewer's yeast	15.00
Wesson's salt mix	7.50
ascorbic acid	2.00
sorbic acid	1.50
methyl-p-hydroxybenzoate	1.50
cholesterol	0.25

total	120.25
<i>Vitamin mix:</i>	Mass (mg)
nicotinic acid	5.00
calcium pantothenate	5.00
riboflavin	2.50
thiamine HCl	1.65
pyridoxine HCl	1.65
folic acid	0.10
biotin	0.10

total	16.00
<i>Liquid ingredients:</i>	Volume (mL)
formaldehyde (40%)	2.50
raw linseed oil	2.50
distilled water	750.00
<i>Other:</i>	Mass (g)
agar	10.00
streptomycin sulfate	0.50

Instructions:

Bring water to a boil. Add formaldehyde and linseed oil to the food processor (or blender), then add boiling water. Add agar and blend until temperature of mixture drops below 74°C. Add 120.25 g dry mix, 16 mg vitamin mix and 0.50 g streptomycin sulfate. Blend until particles remain in suspension after food processor is stopped. Place mould (plastic squares) on wax paper on counter; pour mixture onto mould. Spread mixture evenly over mould. Allow mixture to cool for 1 hour, then push individual cubes of diet from mould. Store cubes in the refrigerator for no longer than 3 weeks. Do not leave the larvae on the same diet cube for more than 3 days to avoid bacterial and fungal growth.

I hereby grant permission to Chris McIntosh to reproduce the sequence of the clone "msspl1" in his M.Sc thesis *A P-glycoprotein homologue in Manduca sexta: steps toward cDNA cloning*.

Signed,

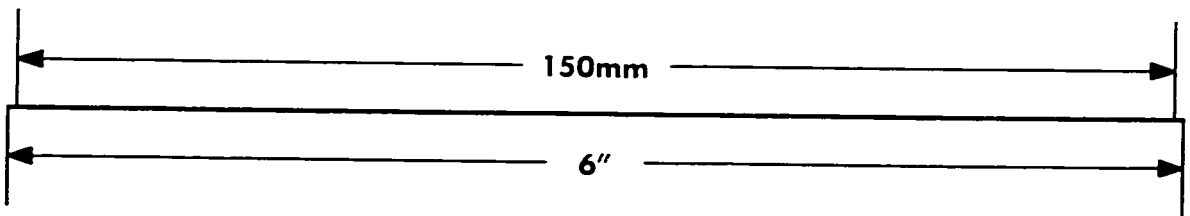
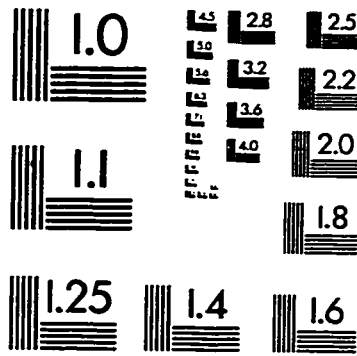
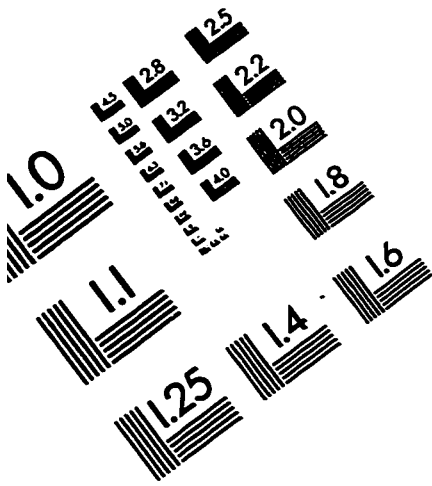
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