An investigation into the effects of the E5 family of transforming proteins on the vacuolar proton-translocating ATPase

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Abstract

There are a number of viruses known to be causative agents of cancer (zur Hausen, 1991b). Most of these viruses act through expression of proteins that interfere with the action of cellular proteins involved in regulating cell division. One such family of viruses are the papillomaviruses commonly associated with warts and other lesions of epidermal tissues. These viruses have in common a gene which encodes a small hydrophobic polypeptide termed E5, so called because it encoded by the E5 open reading frame. The E5 proteins are structurally related consisting of a hydrophobic N-terminal region but share little sequence homology. Nevertheless, they have transforming activity and part of this activity is thought to depend upon the binding to another hydrophobic cellular protein, ductin.

Ductin is a component of the vacuolar H^+-ATPase (V-ATPase) and the gap junction. Whilst gap junction communication is thought to be involved in cellular transformation (Stoker, 1967) the ability of the E5 proteins to perturb this cell-cell interaction does not correlate with their ability to bind to ductin (Ashrafi *et al.*, 2000). It is therefore thought that E5 proteins act through effects on the V-ATPase (Harada *et al.*, 1996) since two of the E5 proteins have been shown to perturb activities associated with the enzyme (Schapiro *et al.*, 2000; Straight *et al.*, 1995). This thesis tests this hypothesis by examining the effects of various forms of E5 proteins, including a related protein from a retrovirus (HTLV-1) using *Saccharomyces cerevisiae* as a model organism. Yeast was chosen as it can grow in the absence of V-ATPase activity, unlike higher eukaryotic cells, and much is known about its V-ATPase.

As *S. cerevisiae* is not the host organism of papillomaviruses and HTLV-1, the binding of the E5 proteins to yeast form of ductin was first examined. In an *in vitro* assay using co-translation and immunoprecipitation, all E5 proteins were found to bind to the *S. cerevisiae* form of ductin. When E5 proteins were expressed in *S. cerevisiae* using a vector known to give high expression of membrane proteins, all E5 proteins were detected in the vacuoles, a membrane which is the main site for the V-ATPase. Monitoring growth of the transformed yeast strains showed the expression of E5 proteins was not cytotoxic.

The effects of the E5 transforming proteins were next tested on the activity of the *S. cerevisiae* V-ATPase. This study was extended to include a mutant form of ductin in which a key glutamic acid residue (E137) had been changed to glycine and is known to act as a dominant negative (Hughes et al, 1996). This mutant prevented growth at pH 7.5 and in high extracellular Calcium, both restrictive growth conditions that require V-ATPase activity. However, none of the E5 proteins, despite binding to ductin, had any effect. The enzyme was then examined more directly by kinetic analysis of ATP hydrolysis. None of the E5 proteins perturbed the activity of the V-ATPase as measured by Km or Vmax.

Replacing glucose with galactose/raffinose in the growth medium of yeast reduces the V-ATPases activity (Kane, 1995). Repeating these experiments showed an initial complete loss of V-ATPase activity but the activity recovered to approximately 40% of the original value. This compromised system was therefore used to examine any effects of E5 transforming proteins. Whilst the dominant negative form of ductin again disrupted growth in the restrictive conditions, none of the E5 proteins had any effect.

The apparent lack of effect on V-ATPase activity by E5 proteins was further investigated by examining the locations of the V-ATPase and E5 proteins after separation by size fractionation on glycerol gradients after ultracentrifugation. This showed that a representative of the E5 transforming proteins, HPV-16 E5, did not co-purify with the V-ATPase indicating it became detached from the active enzyme. The separation explains why E5 transforming proteins, although capable of binding to yeast ductin, do not disturb V-ATPase function.

A partially disabled V-ATPase was used to discover if the E5 proteins could disturb activity. This form contained the Norway lobster (*Nephrops norvegicus*) ductin tagged with hexa-histidine tail at the C-terminus, known to markedly elevate the Km for ATP (Harrison et al, 1994). Again, the dominant negative form of ductin ablated growth at high pH, but none of the E5 proteins had any effect.

Therefore, the E5 transforming proteins do not affect V-ATPase activity and it seems likely that their transforming activity is not occurring through the V-ATPase as has been thought. A scheme is proposed which takes into account recent findings on the related enzyme F-ATPase by which the binding to ductin is part of the mechanism of targeting E5 transforming proteins from the Endoplasmic Reticulum to other cellular compartments.

Declaration

Unless stated, all the work in this thesis is my own. Sequence analysis of prepared DNA samples was performed by Robert Mcfarlane in the central sequencing facility. The work undertaken was performed under the supervision of Dr. Malcolm Finbow. No part of this work has been submitted for consideration for any other degree or award.

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Table of contents

Contents **Page**

List of figures

Figure **Page following**

Introduction

Results. Generation of epitope-tagged E5 protein pYPMA constructs: binding to yeast ductin and correct expression in *S. cerevisiae*

Results: Analysis of the E5 proteins using *S. cerevisiae* supported on glucose

List of tables

Table **page**

List of abbreviations

A-ATPase	Archaebacterial proton-translocating adenosine 5' trisphosphatase
ADP	Adenosine 5' diphosphate
AEBSF	4-(2-Aminoethyl)-benzenesulphonylfluoride
AIDS	Acquired autoimmune deficiency syndrome
AP-2	Adapter protein complex 2
ATP	Adenosine 5' trisphosphate
ATPase	Adenosine 5' trisphosphatase
bp	Base pairs
BPV	Bovine papillomavirus
cAMP	Cyclic adenosine 5' monophosphate
cDNA	Complementary deoxyribonucelic acid
DCCD	N'N-Dicyclohexidecarbodiimide
DNA	Deoxyribonucleic acid
ECV	Endosomal carrier vesicles
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
ER	Endoplasmic reticulum
F-ATPase	ATP synthase
GJIC	Gap junctional intercellular communication
HAAP	Human T-cell lymphotropic virus associated arthropathy
HAM	Human T-cell lymphotropic virus associated myelopathy
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HTLV	Human T-cell lymphotropic virus
IL-2	Interleukin 2
kDa	Kilodaltons
Km	Michaelis constant
LCR	Long control region
LTR	Long terminal repeat

MES	2-[N-Morpholino]ethanesulphonic acid
min	Minutes
MOPS	3-[N-morpholino]propanesulphonic acid
mRNA	Messenger ribonucelic acid
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NEM	N-ethylmaleimide
ORF	Open reading frame
PCR	Polymerase chain reaction
PDGF	Platelet dervived growth factor
PDGF-R	Platelet dervived growth factor receptor beta
Pi	Inorganic phosphate (HPO_4^{2-})
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	Seconds
SRP	Signal recognition particle
TE	Tris buffered ethylenediaminetetraacetic acid
TPA	12-O-tetradecanoyl phorbol-13-acetate
Tricine	N-Tris-[hydroxymethyl]methylglycine
Tris	Tris-[hydroxymethyl]aminomethane
TSP	Tropical spastic paraparesis
TWEEN	Polyoxyethylenesorbitan monolaurate
V-ATPase	Vacuolar proton-translocating adenosine 5' trisphosphatase
V_1	Soluble ATP-hydrolysing portion of the V-ATPase
Vmax	Maximal rate of enzyme catalysis
Vo	Membrane-bound proton translocating portion of the V-ATPase

1. Introduction

1.1 General introduction

Cancer is thought ultimately to be the result of the accumulation of genetic lesions that lead to permanent stimulation of cell growth and division (Nowell, 1976). Such lesions can be caused by a number of agents (carcinogens), all of which act by either causing DNA damage directly or by stimulating cell division, increasing the probability that naturally-occurring mutations will disrupt the function of proteins involved in growth control. Carcinogens are often thought to be toxic chemicals, but this is not always the case since some hormones are carcinogenic through their ability to stimulate cell division (Henderson *et al.*, 1982).

Similarly, certain viruses are carcinogenic through their ability to stimulate cell division. This carcinogenicity can be traced back to individual proteins expressed by the viruses, which disrupt the normal scheme of growth regulation in the cell. Some of these proteins that cause cell transformation are derived from, or interact with cellular proteins involved in growth control (Collett *et al.*, 1978; Downward *et al.*, 1984). However, there is a small number of transforming viral proteins whose mode of action is not known, such as the E5 group of proteins from the papillomaviruses and the type 1 human T-cell lymphotropic viruses.

1.1.1 Bovine Papillomaviruses

Bovine papillomaviruses are double stranded DNA viruses responsible for benign papillomas in cattle (Jarrett, 1985). These papillomas normally regress, but there is good evidence to show that some can progress to cause cancer in cattle fed on bracken, which contains a number of carcinogens (Campo *et al.*, 1992; Jarrett *et al.*, 1978). The six strains of the virus are grouped according to the cell type which they infect. Fibropapillomaviruses (types 1, 2 and 5) infect subepithelial fibroblasts to produce skin, teat and penis fibropapillomas whereas epitheliotropic papillomaviruses (types 3, 4 and 6) infect epithelial keratinocytes to produce papillomas of the skin, teat and alimentary canal (Jarrett, 1985).

Viral infection is followed by expression of the early-expressed viral proteins, causing transformation of the basal cells to produce a localised thickening, and eventually a wart with the normal pattern of cell differentiation (Jarrett, 1985). The expression of viral proteins is regulated in line with cell differentiation so that viral genome replication is carried out in the intermediate spinous and granular cell layers, and capsid proteins are made only in the squamous cells, from which the mature virus is released (Anderson *et al.*, 1997). It is this dependence upon cell differentiation that has hindered *in vitro* studies, but sequencing of the viral genomes has permitted molecular and genetic analysis, with types 1 and 4 being used as candidates for each group in this discussion.

1.1.1.1 Bovine papillomavirus type 1

Subcutaneous injections of bovine papillomavirus type 1 (BPV-1) cause tumours in pika, domestic rabbits and hamsters (Breitburd *et al.*, 1981; Koller and Olson, 1972; Puget *et al.*, 1975). Murine fibroblasts transformed by the virus are tumourigenic in nude mice (Mantyjarvi *et al.*, 1988), yet in the natural host, infection only causes benign papillomas of the skin, teat frond or penis. This supports the idea that the cell transformation is an integral part of the life cycle of papillomaviruses but carcinogenesis is confined to instances when the interrelationship between virus and host is disrupted. This could either be due to the presence of external carcinogens, as mentioned previously, or by integration of the viral DNA into the host genome, disrupting the tight transcriptional regulation of the virus that normally prevents cell transformation from overprogression (Couturier *et al.*, 1991).

1.1.1.1.1 Organisation of the BPV-1 genome

A map of the genome is shown in Figure 1.1. Sequence analysis has identified the position of open reading frames (Chen *et al.*, 1982) and found similarities to the organisation of the human papillomavirus type 1 genome, later expanded to a common plan for all papillomaviruses (Danos *et al.*, 1984), supporting the idea of a common ancestor and more importantly, a similar mode of cell transformation.

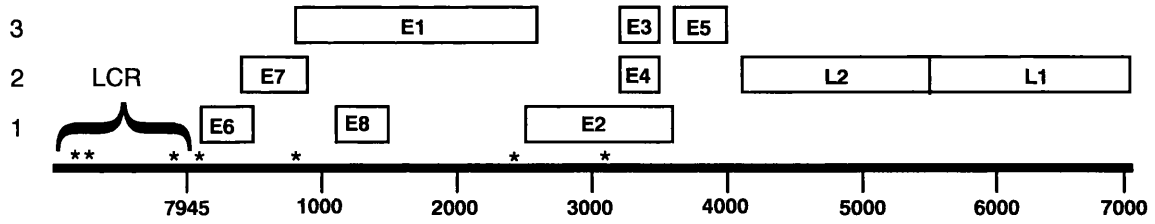


Figure 1.1 Organisation of the BPV-1 genome

Reading frames are indicated in the leftmost column. The long control region (LCR) controls the expression of early (E) and late (L) proteins, with promoters denoted by asterisks. Adapted from (Campo, 1988).

The early-expressed (E) proteins are those involved in cell transformation and viral replication whereas the two late proteins are the capsid proteins for packaging the virus before release. The long control region (LCR) between late and early open reading frames contains the origin of replication, and regulatory elements that co-ordinate the expression of the viral proteins via proximal and distal promoters. The majority of mRNA species are initiated at promoters inside or near to the LCR, with RNA splicing used to direct translation rather than individual promoters, reviewed by Lambert *et al.*, (1988).

1.1.1.2 Bovine papillomavirus type 4

Bovine papillomavirus type 4 (BPV-4) is a representative of the group B papillomaviruses (Jarrett, 1985), causing 'true' epitheilotropic papillomas by infection of basal cell keratinocytes. It shares many of the features of BPV-1. Both size and organisation of the genome are very similar (Patel *et al.*, 1987), the only major difference being the substitution of two major transforming proteins: E5 and the E6 for an E5 homologue, E8. This feature is shared with the other two

members of the viral subtype and may account for the differences between the two groups. In addition, whilst BPV-1 is capable of transforming primary fibroblasts independently (M S Campo, personal communication), BPV-4 requires an activated form of *ras* (a GTPase involved in signal transduction from growth factor receptors), reviewed by Wittinghofer, (1998) to cause transformation (Jaggar *et al.*, 1990).

1.1.2 Human papillomaviruses

The human papillomaviruses have a similar genetic organisation to BPV-1 (Figure 1.1) and are divided into two main groups: those that infect cutaneous epithelia to produce skin warts, and those that infect mucosal epithelia, which give rise to anogenital warts. In addition, these groups are subdivided into high risk and low risk subtypes, according to the chance of malignant transformation, see (zur Hausen and de Villiers, 1994). For the purposes of cancer research, it is the mucosal viruses (particularly the high risk types, 16 and 18) that are given the most attention, these being found in the majority of all cervical carcinoma biopsies (Walboomers *et al.*, 1999).

Further evidence for the causal role of HPV in human cancer comes from the discovery of transcripts for the major viral transforming proteins, E6 and E7 in most cervical tumours (Schwarz *et al.*, 1985) and that the transformation potency of these proteins correlates with the risk category of their virus (Munger *et al.*, 1989). However, the most convincing evidence comes from the observation that these transformed cells become malignant after extended cultivation *in vitro* (Pecoraro *et al.*, 1991), analogous to the proposed *in vivo* scheme of HPV (and

BPV) initiated carcinogenesis where infection is followed by a period of progression to the malignant phenotype (zur Hausen, 1991a).

1.1.3 Functional aspects of the papillomaviral proteins

The similarities in the genome organisation of the papillomaviruses suggests that they cause cell transformation by similar mechanisms. All of the viruses possess an open reading frame (ORF) for a small hydrophobic transforming protein, which together form the 'E5' family of proteins. Their mode of cell transformation is unknown, but reviewing what is known about the products from the other viral ORFs places the E5 proteins in context and aids their investigation.

1.1.3.1 The E1 ORF

The E1 ORF of the human papillomavirus is thought to be required for viral replication (Gopalakrishnan and Khan, 1994), the E1 ORF of BPV-1 coding for two products involved in viral replication (Lusky and Botchan, 1986); the E1 modulator (E1-M) and the E1 replicator (E1-R). E1-R is the helicase responsible for replication of the genome (Thorner *et al.*, 1993) whereas E1-M is thought to act as a negative regulator of replication (Berg *et al.*, 1986). One difference between the E1 proteins of bovine and human papillomaviruses is that although BPV-1 E1 is involved in transcriptional regulation (Sandler *et al.*, 1993), a similar activity has not been found for E1 of the human papillomaviruses.

1.1.3.2 The E2 ORF

The product of the E2 ORF binds to E1 in the replication complex (Benson and Howley, 1995; Frattini and Laimins, 1994). As well as participating in replication, E2 has been shown to regulate transcription (Bernard *et al.*, 1989; Jackson and Campo, 1991; Phelps and Howley, 1987). In BPV-1 this is due to the translation of two shorter E2 transcripts which act as repressor proteins (Lambert *et al.*, 1987), further analysis revealing phosphorylation sites on all three types (McBride *et al.*, 1989), implying cellular regulation of their activity. The complexity of E2-mediated transcriptional regulation is expanded by the discovery that the three types possess different nuclear localisation signals (Skiadopoulos and McBride, 1996) and that cellular transcription factors also compete for binding sites on the LCR (Jackson and Campo, 1995). Evidence for REDOX regulation has also been found (McBride *et al.*, 1992), somewhat reminiscent of V-ATPase regulation discussed in section 1.3.6.4, but the functional significance of this is not known.

1.1.3.3 The E4 ORF

Disruption of the BPV-1 E4 reading frame has no effect on transactivation, replication, or cell transformation (Neary *et al.*, 1987). From its abundance in HPV-1a infected cells during capsid synthesis (Doorbar *et al.*, 1986) it was inferred to be involved in the late gene expression. One possibility is that the E4 products directly regulate the late expression promoter, supported by observations that the E4 products are zinc-binding proteins and are liable to phosphorylation (Grand *et al.*, 1989; Roberts *et al.*, 1994).

1.1.3.4 The E6 ORF

The product of the human papillomaviral E6 ORF has been characterised the most extensively. Its transforming ability was discovered by viral expression studies in human primary fibroblasts (Watanabe *et al.*, 1989). Although E7 is also required to effect this transformation in human cell lines (Munger *et al.*, 1989), in murine cells persistent E6 expression is capable of causing not only transformation, but tumour formation *in vivo* (Song *et al.*, 1999).

E6 is thought to bind to and promote degradation of p53 (Scheffner *et al.*, 1990; Werness *et al.*, 1990), which normally protects the cell from mutations by causing cell cycle arrest or apoptosis in the event of DNA damage. In agreement with this mechanism of cell transformation, chromosome damage has been found in E6-transformed cells (Coursen *et al.*, 1997), and E6 expression has been shown to abrogate checkpoints in the cell cycle in a manner dependent upon p53 binding and degradation (Thompson *et al.*, 1997). However, dominant negative p53 mutations do not fully substitute for E6 (Sedman *et al.*, 1992), suggesting that it has other functions.

E6 has also been shown to act as a transcription factor (Akutsu *et al.*, 1996; Etscheid *et al.*, 1994; Patel *et al.*, 1999), in line with its predicted structure, possessing two DNA-binding zinc finger domains at its N-terminus (Barbosa *et al.*, 1989). It has also been shown to activate telomerase (Klingelhutz *et al.*, 1996), a chromosome repair enzyme that is typically upregulated in immortal cell lines.

BPV-1 E6 has also been studied, and appears to act in a different manner to that of HPV-E6, being involved in, but not essential for cell transformation (Neary and DiMaio, 1989). It is capable of causing cell transformation *in vitro* (Androphy *et al.*, 1985), but through a mechanism involving both transcriptional activation. (Chen *et al.*, 1997; Ned *et al.*, 1997; Vousden *et al.*, 1989) and binding to cytoskeletal and vesicular proteins (Tong *et al.*, 1998; Tong *et al.*, 1997).

1.1.3.5 The E7 ORF

Early studies in NIH 3T3 cells established the product of the E7 ORF as the major transforming protein of HPV-16 (Vousden *et al.*, 1988). Unlike E6, retrovirally-expressed E7 is capable of immortalising human fibroblasts (Halbert *et al.*, 1991) albeit more efficiently in the presence of E6.

Three domains have been identified which give clues as to its mode of action: a retinoblastoma protein (Rb) binding domain (Dyson *et al.*, 1989), two zinc fingers (Barbosa *et al.*, 1989) and substrate sites for protein kinase C and caesin kinase II (Armstrong and Roman, 1995; Firzlaff *et al.*, 1989). The Rb-binding activity is thought to contribute towards cell transformation by preventing Rb from binding to transcription factors such as elongation factor 2 (Zerfass *et al.*, 1995), which is implicated in the regulating the transcription of a number of genes required for cell cycle progression (Degregori et al., 1995). Rb binding potential correlates with the transforming ability of E7 and even the risk classification of the virus (Heck *et al.*, 1992) but additional activities are required to effect transformation by the protein

(Banks *et al.*, 1990), one possibility being the involvement of Cys-X-X-Cys zinc finger motifs.

DNA-binding has been demonstrated for E7 (Chinami *et al.*, 1996) but its role in transformation is unclear (Braspenning *et al.*, 1998; McIntyre *et al.*, 1993). The involvement of E7 phosphorylation sites seems to correlate with transforming ability (Armstrong and Roman, 1995; Firzlaff *et al.*, 1991), probably due to activation of other domains of the protein. Other binding partners involved in transcriptional regulation and cell proliferation have also been found: AP1 transcription factors (Antinore *et al.*, 1996), the TATA box binding protein (Massimi *et al.*, 1997) and members of the cyclin system (Massimi *et al.*, 1997). So it seems that the E7 has a complex mode of cell transformation.

Although the majority of work has been carried out on the human papillomaviral E7, sequence similarities between the bovine and human types suggesting similar functions. Presence of zinc finger motifs in BPV-1 E7 (Barbosa *et al.*, 1989) is consistent with a mode of action via DNA binding, however despite the requirement for E7 in cell transformation by BPV-1 (Neary and DiMaio, 1989), BPV-1 E7 is believed to be involved in maintaining the viral copy number rather than transformation *per se* (Jareborg *et al.*, 1992; Lusky and Botchan, 1985).

In BPV-4, E7 is the major transforming protein, capable of causing morphologically transformation in the presence of activated *ras* (Pennie *et al.*, 1993) and thought to act via DNA-binding zinc fingers and a Rb-binding domain (Jaggar *et al.*, 1990). However, this remains to be proven.

1.1.3.6 The L1 and L2 ORFs

These reading frames encode the two capsid proteins that are expressed before the virus is shed in the squamous layer (Zhao *et al.*, 1998). Although not important for transformation, they have been useful for production of a BPV-4 vaccine (Chandrachud *et al.*, 1995), and may prove useful for construction of vectors for gene therapy (Zhao *et al.*, 1998).

1.1.3.7 Summary

The papillomaviruses are double stranded DNA viruses which encode at least one transforming protein in addition to an E5 protein. A similar situation is also found in an otherwise unrelated retrovirus, the human T-cell lymphotropic type 1 virus.

1.1.4 Human T-cell lymphotropic virus type 1

The human T-cell lymphotropic type 1 (HTLV-1) virus is a enveloped double-stranded RNA retrovirus which infects T-lymphocytes to produce a range of diseases, for review see (Uchiyama, 1997). It is concentrated in Africa, central America and Japan, which is probably a consequence of the proposed mode of establishment by initial transmission from simians to humans in Africa and subsequent dispersion of population by the slave trade (Gallo *et al.*, 1983). The diseases themselves are more of a burden than a threat, but understanding the biology of the virus may aid research into the type III lymphotropic virus, the

human immunodeficiency viruse (HIV) which is believed to cause acquired immune deficiency syndrome (AIDS).

Despite infecting lymphocytes, it is rare for the associated leukaemia to develop into a pathological form. Adult T-cell leukaemia only develops in around 0.1% of carriers after a long latency period (average age at onset is 55) (Tajima, 1990). This is reminiscent of papillomaviral-induced cancer, and indeed of any productive relationship of a virus with its host. The amount of biochemical interference by the virus appears sufficient to maximise production of the virus, without causing death of the host and affecting viral propagation.

Lymphocyte disorders in the cerebrospinal fluid are thought to cause HTLV-1-associated myelopathy (HAM), also called tropical spastic paraperesis (TSP) (Osame *et al.*, 1986). This is a neurological disorder reducing control and sensory functions in the lower half of the body. As well as being debilitating, an increased risk of infections and cancer accounts for an average morbidity period of ten years (Osame *et al.*, 1990).

Joint disorders in infected patients led to the proposition of HTLV-1-associated arthropathy (HAAP) (Nishioka *et al.*, 1989). In much the same way, a form of uveitis has also been attributed to HTLV-1 (Mochizuki *et al.*, 1992). There is also evidence to suggest a link between HTLV-1 and other diseases (Kimura *et al.*, 1986; LaGrenade *et al.*, 1990; Morgan *et al.*, 1989; Ohshima *et al.*, 1992). None of these are associated with morbidity, but some bear symptoms similar to those of other autoimmune diseases such as chronic arthritis and atopic dermatitis, so it may be possible to combine the lessons learnt from both fields.

1.1.4.1 HTLV-1 genome organisation and protein function

The viral genome is illustrated in Figure 1.2. The viral RNA is 9 kilobases long, with 2 long terminal repeats (LTRs), 6 main coding domains (gag, env, pol, pro, tax, rex) and a short 3' 'end region' that encodes three more proteins with unknown functions (Seiki *et al.*, 1983). The retroviral life cycle has been reviewed by Varmus, (1988), it being distinct from that of DNA viruses in that the RNA genome must be reverse transcribed into a DNA 'provirus' before any viral proteins can be synthesised. To this end, the viral RNA is already primed with both DNA template and reverse transcriptase, the enzyme being activated when the viral capsid is exposed to the cellular cytoplasm. An integrase then inserts the provirus into the host genome and the viral genes are finally transcribed under the direction of the LTRs.

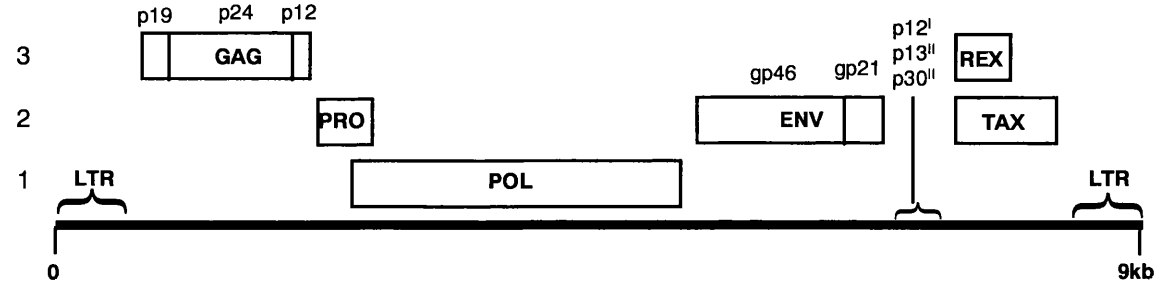


Figure 1.2 Genome organisation of HTLV-1

Reading frames indicated on the left. The long terminal repeats (LTRs) contain the viral promoters, gag, pro, pol and env encode structural and enzymic proteins, tax and rex encoding the major transforming proteins. Adapted from (Franchini, 1995).

1.1.4.1.1 The gag, pro, env, pol ORFs

The gag/pro ORF encodes the two capsid proteins, p19 and p24 as well as the p12 protease which is involved in viral protein maturation (Hatanaka and Nam, 1989). Env encodes the two envelope glycoproteins, gp21 and gp46 (Hattori *et al.*, 1984), which are most likely to be the cell surface receptors although their target is not known. Pol encodes the viral reverse transcriptase and the integrase (Netzer *et al.*, 1993).

1.1.4.1.2 The Tax and Rex ORFs

These ORFs encode two proteins involved in transcriptional regulation, tax and rex. Two reviews deal with them thoroughly (Franchini, 1995; Uchiyama, 1997) and they will be dealt with briefly here.

Tax and rex are phosphoproteins that regulate viral transcription via the LTRs (Sodroski *et al.*, 1984) to produce sporadic expression patterns that have been proposed to hinder immune detection (Franchini, 1995). Tax interacts with many cellular transcription factors to upregulate the transcription of a number of cellular proteins, most of which being directly implicated in cell proliferation, e.g. the interleukin 2 receptor and the c-fos transcription factor (Franchini, 1995). Unlike rex, tax has been shown to immortalise mitogen-activated human T-cells (Grassmann *et al.*, 1989). The most likely reason for this is that rex seems only to regulate viral transcription; it upregulates gag, pol and env transcription as well as

genomic RNA synthesis, but downregulates tax and rex transcription (Yoshida *et al.*, 1994), in line with the sporadic expression patterns of the virus.

1.1.4.1.3 The pX region

Tax and rex are partly coded by a region denoted 'pX'. This also encodes three other proteins: p30", p13" and p12'. Very little is known about these products; p30" and p13" have been detected in the nucleus of transfected cells, but do not affect tax or rex transcriptional regulation (Roithmann *et al.*, 1994). The only evidence on their function comes from the high nonsense mutation frequency of p13" and p30", implying that they are of limited importance. $p12^{I}$ bears similarities to the papillomaviral E5 proteins and is discussed below.

1.1.5 The E5 proteins: a structurally related family of proteins with a conserved cellular binding partner

All of the viruses described previously encode at least one transforming protein. They also encode a small hydrophobic transforming protein which acts by an unknown mechanism. These proteins: BPV-1 E5, BPV-4 E8, HPV-16 E5 and HTLV-1 $p12^{I}$ (hereon termed 'E5 proteins') have structural similarities and bind to ductin, (a proteolipid component of the V-ATPase, mediatophore and gap junction), suggesting a similar mode of cell transformation.

1.1.5.1 BPV-1 E5

This encodes a 7kDa hydrophobic transforming protein (Figure 1.3). Although E6 is also involved in cell transformation (section 1.1.3.4), mutational analysis of E5 showed that it is essential for cell transformation by the virus (DiMaio *et al.*, 1986). Subsequent studies showed it alone to be capable of transforming a number of different cell lines (Bergman *et al.*, 1988; Leptak *et al.*, 1991; Schiller *et al.*, 1986). In searching for its mechanism of cell transformation, four binding partners have been identified: the platelet derived growth factor receptor beta (PDGF-R) (Petti and DiMaio, 1992), the epidermal growth factor receptor (EGF-R) (Cohen *et al.*, 1993a), an alpha adaptin homolog, p125 (Cohen *et al.*, 1993b) and ductin, a proteolipid component of the vacuolar ATPase, gap junction and mediatophore (Goldstein *et al.*, 1991).

Association with the PDGF-R causes receptor dimerisation and activation in the absence of growth factor (Drummond-Barbosa *et al.*, 1995; Lai *et al.*, 1998), with transformation depending upon this activation (Nilson *et al.*, 1995; Riese and DiMaio, 1995). Mutational analysis has shed light on the significance of the carboxy terminal domain, with crucial residues being conserved across papillomaviruses from four animal species (Horwitz *et al.*, 1988), and also to a limited extent with the extracellular domains of two forms of PDGF-R (Meyer *et al.*, 1994). Of particular note was the dependence upon an aspartic acid residue, presumed to participate with a lysine residue in PDGF-R to mediate binding (Klein *et al.*, 1999; Petti *et al.*, 1997) and also upon two cysteine residues which permit E5 (and therefore PDGF-R) dimerisation (Horwitz *et al.*, 1988). However, the carboxy-terminal domain also mediates binding to the alpha adaptin homologue

(Cohen *et al.*, 1993b), which could contribute to cell transformation through effects on vesicle trafficking.

More elusive is the role of the transmembrane domain of E5. This mediates E5 binding to ductin and PDGF-R (Goldstein *et al.*, 1992a; Petti *et al.*, 1997), but neither of these activities correlated completely with transforming ability (Sparkowski *et al.*, 1996). The glutamine residue at position 17 is thought to mediate the interaction with ductin via the active glutamic acid residue in the fourth helix of ductin (Figure 1.7) (Andresson *et al.*, 1995; Goldstein *et al.*, 1992b). Disruption of this interaction by mutation of the glutamine tended to reduce the transforming potential of E5 in NIH 3T3 cells (Sparkowski *et al.*, 1994), but expression in C127 cells revealed a number of hypertransforming mutants, all of which had polar residues of a similar size to glutamine, suggesting a role for this residue in forming hydrogen bonds (Sparkowski *et al.*, 1994). This was confirmed by more extensive mutagenesis studies, which demonstrated the correlation between hydrogen bonding capacity, PDGF-R binding and activation, and C127 cell transformation (Klein *et al.*, 1998).

However, earlier studies on E5 truncations showed the glutamine to have only a minor effect on transforming activity, the majority being confined to the carboxy-terminal (C-terminal) region (Rawls *et al.*, 1989). Also, partial substitution of the hydrophobic domain with that of a receptor tyrosine kinase, *neu* (Coussens *et al.*, 1985) did not abolish transforming activity despite the normally disruptive substitution of glutamine 17 for leucine (Meyer *et al.*, 1994).

Alanine scanning mutagenesis was performed to correlate the activity of transmembrane residues with respect to mediating the binding activities of E5, the phosphorylation of PDGF-R, and the transformation of NIH 3T3 cells. (Adduci and Schlegel, 1999). The residues involved in mediating the binding of E5 to itself and PDGF-R aligned to form distinct regions on a helical wheel projection, supporting the assumption that the transmembrane domain is α-helical in structure, and demonstrating the specificity of the interactions of E5. Mutations that prevented either binding activity prevented PDGF-R phosphorylation, but had no consistent effect on the transforming potential (Adduci and Schlegel, 1999). One proposal was that phosphorylation-defective E5 mutants transformed cells through their ability to form tetrameric membrane pore complexes (Adduci and Schlegel, 1999).

The role of ductin in the mode of action of E5 has not been investigated to the same extent. Ductin binds to E5 and PDGF-R (Goldstein *et al.*, 1992a; Goldstein *et al.*, 1991) and was suggested to mediate the binding of the proteins to each other, since the ability of E5 to bind ductin and PDGF-R were related, and a stable complex of the three proteins was found (Goldstein *et al.*, 1992a). Ductin mutants have also demonstrated an indirect link between E5 binding and cell transformation; mutation of the active glutamic acid not only preventing the binding to E5, but converted ductin into a transforming protein (Andresson *et al.*, 1995).

BPV-1 E5 may act in the Golgi, since its retention in the endoplasmic reticulum (ER) prevented cell transformation (Sparkowski et al., 1995), and it has also been shown to inhibit acidification of the Golgi apparatus in a manner that corresponded with its ability to transform NIH 3T3 cells (Schapiro *et al.*, 2000). Despite its

similarity to the influenza virus M2 and the adenovirus E3 channel-forming proteins, which exhibit some of the cellular effects of E5 (Henkel *et al.*, 1999; Hoffman and Carlin, 1994), BPV-1 E5 did not affect the permeability of the Golgi membrane to protons (Schapiro *et al.*, 2000), suggesting that it dissipated Golgi pH by a different mechanism. In the light of this, and data dissociating PDGF-R activation from cell transformation (Leptak *et al.*, 1991; Sparkowski *et al.*, 1996), it was suggested that E5 inhibited the V-ATPase through its interaction with ductin (Schapiro *et al.*, 2000).

1.1.5.2 HPV-16 E5

HPV-16 E5 was identified as an oncogene by its ability to induce anchorage-independent growth upon NIH 3T3 cells (Leechanachai *et al.*, 1992). It does not transform cells to the same extent as E6 or E7 (Rho *et al.*, 1996), appearing to carry out more of an accessory role in transformation of primary cells (Stoppler *et al.*, 1996). Just like the bovine papillomaviral E5 and E8 proteins, HPV E5 proteins are highly hydrophobic with conserved cysteines (only 1 in the case of HPV-16 E5) in the C-terminal domain (Bubb *et al.*, 1988). There are important differences though; the HPV E5 proteins are predicted to possess an additional transmembrane domain and lack the active amido group found at position 17 of BPV-1 E5 and BPV-4 E8 (Bubb *et al.*, 1988).

HPV-16 E5 has been shown to bind to a number of growth factor receptors (Hwang *et al.*, 1995); EGF-R and PDGF-R, as for BPV-1 E5, but also colony stimulating factor receptor, which regulates phagocyte proliferation (Nagata and

Fukunaga, 1993) and p185*neu*, a proto-oncogene involved in modulating the affinity of EGF-R for EGF (Stern *et al.*, 1988; Wada *et al.*, 1990). There is evidence to suggest that the interaction with EGF-R may contribute to cell transformation (Crusius *et al.*, 1998; Pim *et al.*, 1992), however the involvement of PDGF-R is uncertain, since another study showed that only the low risk type HPV-6 E5 could interact with this receptor (Conrad *et al.*, 1994).

HPV-16 E5 also binds to ductin (Conrad *et al.*, 1993). This binding has been shown not only to inhibit gap junctional communication independently of connexin phosphorylation (Oelze *et al.*, 1995), but to inhibit activities reliant on the V-ATPase; endosome acidification and EGF-R degradation (Straight *et al.*, 1995). However, whether this is due to a direct inhibition of the enzyme remains to be shown.

1.1.5.3 BPV-4 E8

Despite being found where the E6 ORF would be expected, the E8 ORF encodes a protein with homology to the BPV-1 E5 protein (Figure 1.3) (Jackson *et al.*, 1991). E8 has the same cellular localisation pattern as BPV-1 E5 (Anderson *et al.*, 1997), and is capable of conferring anchorage independent growth upon primary bovine fibroblasts containing activated *ras* (Pennie *et al.*, 1993). However, it is not found in the differentiated cell layers in which BPV-1 E5 is found (Burnett *et al.*, 1992) and there are no data to suggest that it binds to or activates growth factor receptors.

Mutational studies on E8 show both similarities and differences between the two proteins, for example the glutamine at position 17 in BPV-1 E5 is asparagine in E8. These residues share the same functional group but mutation of the BPV-1 E5 glutamine to asparagine disrupts the function of the protein (Sparkowski *et al.*, 1994) and mutation of the E8 asparagine reveals different requirements to that of E5; small hydrophobic residues as opposed to medium sized polar residues (O'Brien *et al.*, 1999). Domain swaps between the two proteins showed that only the hydrophobic domain of BPV-1 E5 can substitute for that of E8 (O'Brien *et al.*, 1999). Suggesting that not only is the C-terminal domain of E8 implicated in its mechanism of transformation, but that it acts in a way distinct from that of BPV-1 E5; a conclusion supported by poor C-terminal sequence homology, lack of growth factor receptor binding by E8 and the observation that, unlike BPV-1 E5, E8 upregulates cyclin A and its associated kinase (O'Brien and Campo, 1998).

In fact, the only evidence that they may have a similar mechanism for cell transformation comes from the fact they both bind to ductin and inhibit gap junctional intercellular communication (Ashrafi *et al.*, 2000; Faccini *et al.*, 1996). However, the transforming abilities of BPV-4 E8 mutants did not fully correlate with their effects on communication (Ashrafi *et al.*, 2000) so it is unlikely that this plays a major part in the mechanism of cell transformation. Another possibility is that E8 transforms cells by upregulating Cyclin A (O'Brien and Campo, 1998), since this protein is involved in cell cycle checkpoints and has been implicated in carcinogenesis (Desdouets *et al.*, 1995).

1.1.5.4 HTLV-1 p12^{I}

p12^{I} is implicated in the maintenance of viral infection (Collins *et al.*, 1998). Its precise role is not known although it has been shown to bind to and disrupt the targeting of the interleukin-2 (IL-2) receptor (Mulloy *et al.*, 1996) as well as binding to ductin (Franchini *et al.*, 1993). Its interaction with the IL-2 receptor could lead to hyperproliferation and therefore positive selection for infected cells, but evidence to suggest that its interaction with ductin could be involved comes from studies showing that not only does it exhibit sequence similarities with BPV-1 E5 (Figure 1.3), but also co-operates with it in cell transformation (Franchini *et al.*, 1993; Garry, 1995). However, the mode of binding to ductin appeared to be different to that seen for BPV-1 E5, and co-operation of two proteins does not indicate whether they act by similar mechanisms.

1.1.5.5 Ductin binding may represent a common mode of cell transformation

The structural similarities of the E5 proteins have been documented previously (Bubb *et al.*, 1988; Franchini *et al.*, 1993; Garry, 1995), and are illustrated by hydrophobic cluster analysis (Gaboriaud *et al.*, 1987) in Figure 1.3. The α-helical prediction is supported by genetic and physical structural analysis of BPV-1 E5 (Adduci and Schlegel, 1999; Surti *et al.*, 1998), and despite the overall lack of sequence homology, the presence of 'LLF' motifs in similar positions in the proteins would support the belief that they have similar structures.

Despite the fact that most of the E5 proteins have been found to bind to growth factor receptors, (Goldstein *et al.*, 1992a; Hwang *et al.*, 1995; Mulloy *et al.*, 1996), this activity does not always correlate with their transforming potential (Nilson and DiMaio, 1993; Sparkowski *et al.*, 1996), so may not represent the conserved mode of action suggested by the structural conservation. However, all of the proteins bind to ductin (Conrad *et al.*, 1993; Faccini *et al.*, 1996; Franchini *et al.*, 1993; Goldstein *et al.*, 1991), which as mentioned above is a proteolipid component of gap junctions and the vacuolar ATPase. Both of these have been implicated in growth control. (Harada *et al.*, 1996; Stoker, 1967), but it is not known if they are involved in the mechanism of cell transformation by the E5 proteins.

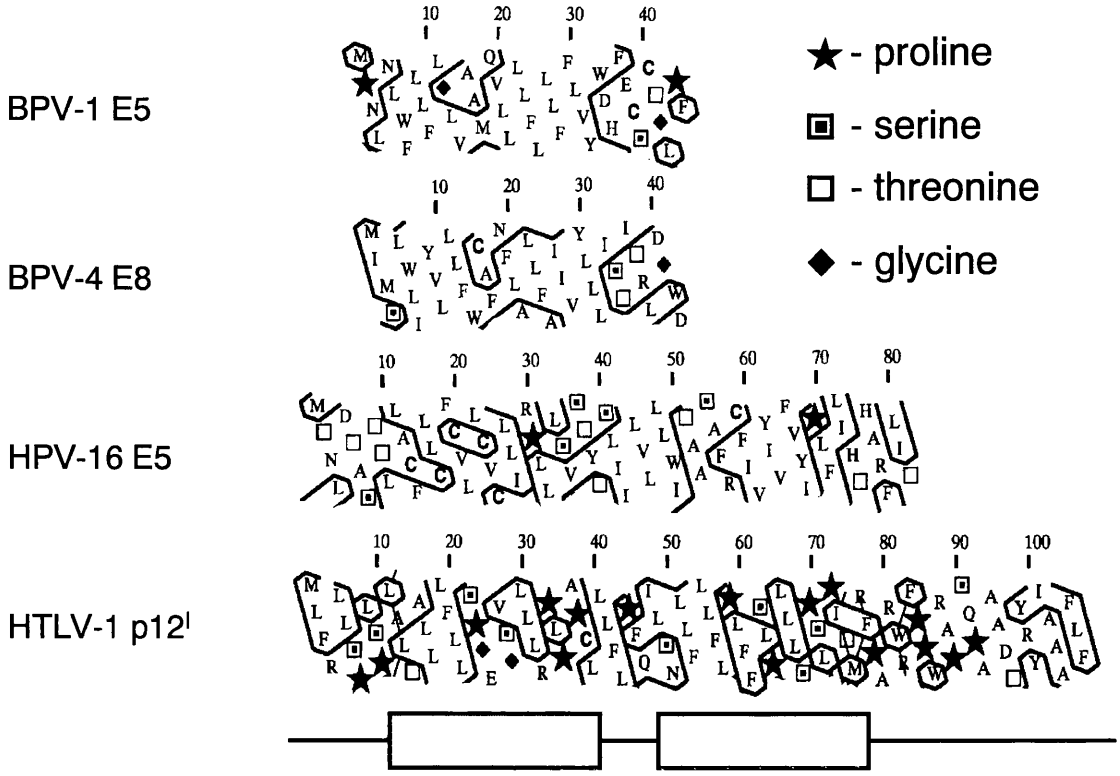


Figure 1.3 Structural similarities of the E5 proteins

The protein sequences of the E5 proteins were analysed by hydrophobic cluster analysis (Gaboriaud et al., 1987), which arranges the residues an an α-helical array and outlines any hydrophobic regions. The sequences were aligned according to the position of predicted transmembrane domains (denoted by grey boxes, underneath).

1.2 Ductin is a multifunctional pore protein and a possible tumour supressor protein

Because the E5 proteins bind to ductin, it suggests that it is involved in their mechanism of cell transformation. Evidence for this comes from experiments showing that not only does ductin expression suppress transformation by BPV-1 E5, but it is capable of transforming NIH 3T3 cells when expressed in a dominant negative form (Andresson *et al.*, 1995). However, just how it is involved is unclear, since ductin is found in three cellular structures, the vacuolar ATPase (V-ATPase), the gap junction, and the mediatophore, (Brochier and Morel, 1993; Finbow *et al.*, 1983; Mandel *et al.*, 1988). Both the gap junction and the V-ATPase are implicated in growth control, and so suggest potential mechanisms for the action of the E5 proteins.

1.2.1 Gap junctional intercellular communication and tumour suppression

Metazoan tissue function relies on the co-operation of its constitutive cells and this is achieved in part by gap junctional intercellular communication (GJIC); the passive diffusion of low mass solutes (<1.5kDa) through transmembrane channels known as gap junctions (Finbow and Pitts, 1981). Intercellular communication through gap junctions is required during embryonic development (Allen *et al.*, 1990), and co-ordinates cellular activities such as cardiac muscle contraction (Oyamada *et al.*, 1994) and cell proliferation (Pitts *et al.*, 1988).

Electron microscopy of isolated gap junctions revealed that the constituent proteins are arranged in hexagonal arrays to form hemi-channels which appose each other to form the complete channel (Hoh *et al.*, 1991). A number of proteins have been characterised from preparations of gap junctions, the largest and best characterised group being the connexins, reviewed by Goodenough *et al.*, (1996). These are predicted to be integral membrane proteins with 4 transmembrane alpha helices, with both termini facing the cytoplasm (Goodenough *et al.*, 1996). Ductin is another component of gap junctions (see section 1.3.5.3.4 for structural information), which unlike connexins is found in both vertebrates and invertebrates (Finbow, 1997). A pure preparation of ductin has the microscopic structure of the gap junction (John *et al.*, 1997), and antisera raised against ductin inhibit GJIC in mollusc, insect and mammalian cells (Bohrmann, 1993; Finbow *et al.*, 1993; Serras *et al.*, 1988), but there is no genetic evidence to prove that it is an active component of the gap junction. Another group of proteins have also been proposed as components of the invertebrate gap junction, the innexins (Phelan *et al.*, 1998). These are predicted to be of similar structure to connexins, but have no sequence homology to them or any other vertebrate protein.

A role for gap junctions in the control of cell proliferation was first suggested by (Lowenstein and Kanno, 1966), later reinforced by observations of antiproliferative effects of connexins expressed either inside transformed cells or in those adjacent to transformed cells (Rose *et al.*, 1993). One suggestion is that GJIC disperses second messengers that appear in a cell after stimulation with growth factors, (Loewenstein, 1979), this dilution attenuating the response of the individual cell to stimulation. So if a cell loses its ability to communicate with its neighbours then its sensitivity to growth stimuli will be enhanced.

Indeed, reduced GJIC is observed in many tumours, (Lowenstein and Kanno, 1966), and many tumour promoters have been shown to reduce GJIC *in vitro* (Enomoto *et al.*, 1981; Murray and Fitzgerald, 1979). However, although tumour cells may communicate poorly with non-tumour cells, communication within the tumour may be more efficient (Mesnil and Yamasaki, 1996). This suggests that tumours act like separate tissues, being separate communication compartments rather than simply a mass of non-communicating cells.

BPV-1 E5, BPV-4 E8 and HPV-16 E5 have all been shown to inhibit GJIC (Ashrafi *et al.*, 2000; Faccini *et al.*, 1996; Oelze *et al.*, 1995), but mutational analysis of BPV-4 E8 showed that this ability did not correlate cell transformation (Ashrafi *et al.*, 2000; O'Brien *et al.*, 1999). Similarly, the effect of the tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on junctional communication was different when measured *in vivo* and *in vitro* (Kam and Pitts, 1988; Pasti *et al.*, 1988), suggesting that the relationship between GJIC and cell transformation is complex. It also appears that GJIC is regulated by a number of mechanisms (Azarnia *et al.*, 1988; Jongen *et al.*, 1991; Kanemitsu and Lau, 1993), further complicated by the number of proteins implicated in the formation of gap junctions. Because of this complexity, it seems that GJIC is unsuitable for studying the role of ductin binding in the mechanism of cell transformation by the E5 proteins.

However, ductin is known to be an essential component of the vacuolar ATPase (Umemoto *et al.*, 1990), which is directly linked to growth regulation through its maintenance of the endocytic pathway (Mellman, 1992), which downregulates cell stimulation by growth factors. Measuring disturbances of the V-ATPase may

provide a simpler way to investigate the significance of ductin binding by the E5 proteins in their mode of cell transformation

1.2.2 The vacuolar ATPase and the regulation of cell signalling by endocytosis and degradation of growth factor receptors

Growth stimulation in eukaryotes begins with the binding of a growth factor to its receptor. There are various mechanisms that translate this binding into a cascade of biochemical events, (commonly protein phosphorylation), which end in the specific redirection, or maintenance of cellular activity ascribed to the growth factor (van der Geer *et al.*, 1994). However, the cell surface receptors must be continually refreshed in order to respond to the dynamic environment of growth factors, which is achieved by endocytosis and subsequent processing of the receptors. This has been reviewed extensively (Gruenberg and Maxfield, 1995; Mellman, 1992; Sorkin and Waters, 1993) and shall be covered briefly here.

1.2.2.1 Structure of growth factor receptors

The epidermal growth factor receptor (EGF-R) is one of the most popular models (van der Geer *et al.*, 1994). It consists of four key domains:

- A cytoplasmic tyrosine kinase domain (Chinkers and Brugge, 1984) that is activated by EGF binding, which phosphorylates cellular proteins including other growth factor receptors (Yarden and Schlessinger, 1985).
- A transmembrane domain, which anchors the protein in the membrane.

- A heavily glycosylated extracellular EGF-binding domain (Lax *et al.*, 1990; Lax *et al.*, 1989) which presumably changes conformation upon binding in such a way as to activate the kinase domain.
- A cytoplasmic internalisation domain involved in inducing endocytosis of the receptor upon epidermal growth factor (EGF) binding (Chang *et al.*, 1993).

1.2.2.2 Receptor mediated endocytosis

The phenomenon of 'growth factor-induced receptor downregulation' was first reported in 1976 (Carpenter and Cohen, 1976), where EGF addition to cells caused an 80% decrease in their subsequent binding capacity. This was attributed to receptor internalisation and degradation, eventually leading to the term of 'receptor-mediated endocytosis'.

The scheme of receptor mediated endocytosis and receptor processing is shown in Figure 1.4. As well as triggering a cascade of protein phosphorylation, binding of EGF to its receptor is also thought to activate the internalisation domain, with autophosphorylation contributing to this (Sorkin *et al.*, 1992). Once activated, the receptor complexes are recruited to clathrin-coated pits in the membrane via the association of the internalisation domain with a clathrin-binding protein complex called 'adaptor-protein complex 2' (AP-2) (Sorkin *et al.*, 1995). These pits bud off and fuse with other vesicles to form early endosomes (Figure 1.4 A), and receptor processing begins.

1.2.2.2.1 Sorting in the early endosomes

The early endosomes are tubo-vesicular membrane structures with an internal pH of 6.2 (Tooze and Hollinshead, 1991) which is generated by the action of the vacuolar ATPase (V-ATPase) (Galloway *et al.*, 1983). Both of these features have been implicated in its role of sorting proteins for degradation or recycling to the cell surface (Linderman and Lauffenburger, 1988). Acidic pH causes dissociation of ligands from their receptors (Davis *et al.*, 1987) and the tubular constrictions might be responsible for size-fractionation (Geuze *et al.*, 1987), Figure 1.4 B. However, current thinking is that many ligands remain bound until late in the endocytic pathway, and that acidification may serve another purpose, such as in vesicle targeting from the early endosome (Johnson *et al.*, 1993).

Vesicles budding from the 'sorting endosome' are either recycled via 'recycling endosomes' (Figure 1.4 C). These are slightly more tubular than sorting endosomes, have a slightly more alkaline pH and contain material not destined for degradation, e.g. α-2 macroglobulin (Yamashiro *et al.*, 1984). Material destined for degradation is segregated into endosomal carrier vesicles (ECVs), Figure 1.4 D, (Gruenberg *et al.*, 1989), which require an active V-ATPase (Clague *et al.*, 1994), and commit their contents to degradation by their being unable to fuse with early endosomes (Gruenberg *et al.*, 1989).

1.2.2.2.2 Degradation of growth factor receptors

As well as driving the recruitment of clathrin-binding protein complexes, the activated kinase domain also targets the receptor for degradation inside the cell. (Felder *et al.*, 1990). This is presumably to avoid the futile cycling of loaded receptors back to the cell surface, and maybe to increase the efficiency of downregulation. The ECVs fuse with acidic (pH 5.5) late endosomes, which in turn mature to form lysosomes (Figure 1.4 E). These have a pH of around 5.0 (Murphy *et al.*, 1984), which causes dissociation of all ligands from their receptors and activates the lytic enzymes in the vacuole. (Their low isoelectric point is required to prevent autolysis during synthesis or in the event of lysosome rupture.)

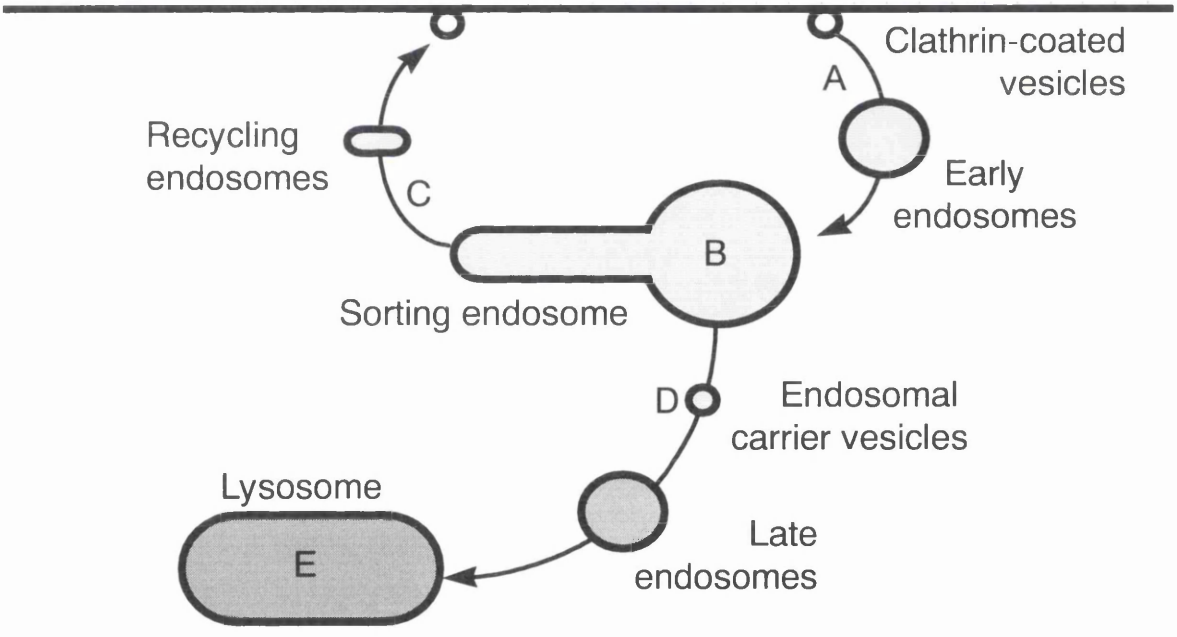


Figure 1.4 Receptor mediated endocytosis of growth factor receptors

Growth factor receptor binding triggers a signalling cascade through its receptor, which is then recruited into clathrin-coated pits and internalised by endocytosis. The receptor is either recycled to the cell surface, or degraded in lysosomes, the decreasing pH of the lytic pathway represented by shading.

1.2.2.3 The vacuolar ATPase is required for receptor mediated endocytosis

As well as dissociating ligands and activating lysosomal enzymes, the acidic pH generated by the V-ATPase is required for correct vesicle trafficking along the endocytic pathway. Bafilomycin A_1, a specific V-ATPase inhibitor, not only inhibited degradation of EGF in the lysosome (Yoshimori *et al.*, 1991), but reduced the delivery of a number of proteins to this compartment (van Deurs *et al.*, 1996; van Weert *et al.*, 1995). V-ATPase inhibition also inhibited the recycling pathway as well as the formation and fusion of acidic endosomes (Clague *et al.*, 1994; Yamamoto *et al.*, 1998), consistent with suggestions by (Johnson *et al.*, 1993). They observed that bafilomycin A_1 only affected the exocytosis of EGF receptors possessing a wild-type internalisation domain and suggested that the pH of these compartments regulates vesicle trafficking via effects on the conserved 'tyrosine β-turn' motif in the internalisation domain of internalised molecules. In support of this, the membrane binding of the protein which initiates vesicle formation in the Golgi, (ADP ribosylation factor) has been shown to be dependent upon upon this low internal pH (Zeuzem *et al.*, 1992).

In summary, the binding of a growth factor to its receptor causes a conformational change that not only starts a signalling cascade, but causes the recruitment of clathrin-binding proteins to the receptor, resulting in its eventual endocytosis. Internalised receptors are sorted in the early endosomal compartments, with activated receptors being targeted for degradation rather than recycling. The V-ATPase progressively acidifies the vesicular environment, mediating vesicle targeting, and terminating signalling by the receptor by causing ligand dissociation; the low pH eventually activating enzymes that degrade the receptor.

This disruption of the endocytic pathway through direct inhibition of the V-ATPase illustrates its pivotal role in downregulation of activated growth factor receptors. The effects of V-ATPase inhibition are similar to those observed in E5-expressing cells (Straight *et al.*, 1993; Waters *et al.*, 1992). This suggests a common mechanism of transformation by the E5 proteins through binding to ductin and inhibiting the V-ATPase.

1.3 The vacuolar proton-translocating adenosine 5' trisphosphatase (V-ATPase)

The search for the vacuolar ATPase (V-ATPase) was started by the discovery of a novel potassium excretion mechanism in insect epithelia (Ramsay, 1953). In contrast to the Na^+/K^+ antiporter, this was not inhibited by ouabain and vanadate, and despite its inhibition by the mitochondrial (F-type) ATPase inhibitor, N'N-dicyclohexidecarbodiimide (DCCD), it was not affected by other F-ATPase inhibitors, such as sodium azide and oligomycin, (Wieczorek *et al.*, 1986). These properties defined a new type of proton pump being discovered in chromaffin granules (Kirshner, 1962), lysosomes (Schneider, 1979) and clathrin-coated vesicles (Stone *et al.*, 1983). In all cases it functioned as an ATP-driven proton pump, but its eventual role depended upon its cellular location. The various activities will be dealt with briefly here, but for reviews see (Harvey and Wieczorek, 1997) and (Wieczorek *et al.*, 1999a).

1.3.1 Intracellular pH regulation

Proton transport creates a proton gradient across the membrane, which in turn creates a potential difference. If chloride channels are present in the membrane, this potential difference can drive the co-transport of chloride ions, forming hydrochloric acid and generating a transmembrane pH gradient. V-ATPases have been detected in the plasma membrane of some cells, and this has been proposed as a mechanism of intracellular pH regulation in corneal cells and macrophages (Swallow *et al.*, 1990; Torres-Zamorano *et al.*, 1992). This is possibly required to compensate for lactate production in the anaerobic environment of the corneal epithelium and the high level of lysosomal acidification in macrophages. The importance of regulating cytoplasmic pH is highlighted by the discovery that a plasma membrane V-ATPase in cardiomyocytes protects against myocardial infarction by attenuating cytoplasmic acidification that would otherwise lead to apoptosis (Gottlieb *et al.*, 1996).

1.3.2 Extracellular pH regulation

Extracellular pH regulation is crucial for many tissues. In kidney tubules, it ultimately regulates the pH of the blood (Gluck *et al.*, 1982), and in the lumen of the epididymis, the low pH is required for sperm maturation and as such, the V-ATPase has been suggested as a target for modulating fertility (Breton *et al.*, 1996). Low pH is used by osteoclasts to dissolve bone matrix (Sasaki *et al.*, 1994) and in a similar manner by ameloblasts to dissolve tooth enamel, although the pH was suggested to activate lytic enzymes rather than directly solubilising the

inorganic substrate (Lin *et al.*, 1994). Chondrocytes are also thought to use acidic pH to denature collagen prior to digestion (Creemers *et al.*, 1998).

1.3.3 Active transport

In mammalian cells, the combination of the Na^+/K^+ plasma membrane ATPase and the K^+ channel generates a sufficient electrochemical gradient to drive most transport processes, e.g. glucose via the Na^+/glucose symporter (Hediger and Rhoads, 1994). Despite only one occurrence of V-ATPase-driven plasma membrane energisation in mammals (Simon *et al.*, 1992), there are examples in other animals: sodium uptake in frog skin (Lacoste *et al.*, 1993), and V-ATPase induced *alkalinisation* of the Tobacco hornworm midgut by proton-motivated active transport of potassium ions (Wieczorek *et al.*, 1991).

Inside the cell, the V-ATPase drives a number of other active transport processes. These range from activities secondary to their endosomal locations, e.g. osmoregulation and amino acid accumulation in plant vacuoles (Sze *et al.*, 1992), to dedicated roles such as in neurotransmitter accumulation. (Kirshner, 1962). Interestingly, the method of transport depends on the type of neurotransmitter, both the pH gradient and the electrochemical gradient being used to transport catecholamines (Njus *et al.*, 1986) but only the electrochemical gradient being used to transport glutamate (Cidon and Sihra, 1989). In all cases, it is the 'context' of the enzyme in the cell that defines its function; the enzyme itself always acts as an ATP-driven proton pump.

1.3.4 Structure of the V-ATPase

1.3.4.1 The structure of the V-ATPase is similar to bacterial ATPases

When V-ATPases were first observed by electron microscopy (Gupta and Berridge, 1966) the projections from the membrane surface were reminiscent of 'ball and stick' structures seen on mitochondrial inner membranes. Subsequent structural analysis confirmed this similarity with the ATP synthase (F-ATPase) (Bowman *et al.*, 1989) and genetic analyses revealed varying degrees of sequence identity between F-type and V-type subunits (Mandel *et al.*, 1988; Zimniak *et al.*, 1988). The F-ATPase has been studied more extensively, but the similarities between the two enzymes has led to structural and mechanistic predictions of the V-ATPase based on the working models of the F-ATPase.

The biochemistry of the F-ATPase has been reviewed (Boyer, 1997), describing how the enzyme consists of a membrane-bound proton-translocating 'Fo' complex, coupled by 'stalk' subunits to the hydrophilic 'F_1' ATP-synthesising complex (Figure 1.5). This structure has since been partially resolved by crystallisation of the whole enzyme (Stock *et al.*, 1999), showing that the Fo consists of a ring of 10-12 (Jones *et al.*, 1994) small proton-conducting proteolipids (subunit c) coupled to six large ATP-binding proteins (subunits α and β) in the F_1. In addition to structural information on the enzyme, studies on the sub-components of the enzyme have revealed aspects of the catalytic mechanism.

Structural analyses of subunit c by Nuclear Magnetic Resonance (Rastogi and Girvin, 1999), have shown that the deprotonation of the active glutamic residue causes a conformational change capable of driving the rotation of the γ subunit. This rotation has been correlated to conformational changes in the catalytic β subunits, which are in turn related to the nature of their bound nucleotide (Abrahams *et al.*, 1994), Figure 1.5, demonstrating a means of coupling the proton-driven rotation of the Fo-associated stalk to catalysis in the F_1. By fusing actin to the γ subunit it was even possible to observe rotation in response to ATP addition (Noji *et al.*, 1997). This has also been shown for another component of the stalk complex, the ε subunit (Kato-Yamada *et al.*, 1998), and also for subunit c (Sambongi *et al.*, 1999), suggesting that the stalk and subunit c arrangement act as the rotor in the proposed scheme of rotational catalysis (Figure 1.5).

This rotational model for catalysis by the F-ATPase can be extended to the V-ATPase in light of similarities between the two enzymes. Further evidence for the evolution of the V-ATPase from the F-ATPase comes from the discovery of two 'intermediate' forms of enzyme which share characteristics of both types; the prokaryotic V-type sodium-translocating ATPase (Na^+-ATPase) from *Enterococcus hirae* and the archaebacterial proton-translocation ATPase (A-ATPase).

The Na^+-ATPase was discovered as an ATP-driven sodium pump in *Streptococcus faecalis* (Heefner and Harold, 1980) and later in *E. hirae* (Kakinuma and Igarashi, 1989). Sensitivity to nitrate and N-ethyl maleimide (NEM) suggested its similarity to the V-ATPase (Kakinuma and Igarashi, 1990), which was supported by sequence comparisons of the enzyme subunits revealing 15-50%

sequence identity to subunits of the *S. cerevisiae* V-ATPase (Takase *et al.*, 1994). In addition the enzyme was found to contain a 16kDa proteolipid similar to ductin, with an active glutamic acid, as opposed to the 8kDa protein bearing the aspartic acid seen in the F-ATPase (Kakinuma *et al.*, 1993; Takase *et al.*, 1999). However, the enzyme did not posses the regulatory cysteine found in the V-ATPase subunit A (section 1.3.5.2.1) and was insensitive to inhibition by bafilomycin A_1 and concanamycin A, reviewed by Kakinuma *et al.*, (1999), demonstrating its similarity to the F-ATPase.

The archaebacterial A-ATPases are ATP synthases with structural features of V-ATPases. Their properties vary between organisms and have been reviewed by Muller *et al.*, (1999). Of greatest interest is the observation that A-ATPases possess many subunits not seen in F-ATPases (Schafer and Meyering-Vos, 1992), and that some species express duplicate or triplicate forms of the 8kDa proton-translocating proteolipid (Muller *et al.*, 1999; Ruppert *et al.*, 1999), suggesting mechanisms for the origin of the duplicate form (ductin) seen in the V-ATPase. In contrast to the V-ATPase, which has 6 active acidic residues in the proteolipid complex the A-ATPase maintains at least 8 and usually 12 (Muller *et al.*, 1999; Ruppert *et al.*, 1999) and is also distinct in that it is capable of performing ATP synthesis. This led to the suggestion that the restriction of the V-ATPase to proton pumping is due to the number of acidic residues per enzyme, rather than the size of the proteolipid (Ruppert *et al.*, 1999), and is discussed further in section 1.3.5.3.4.

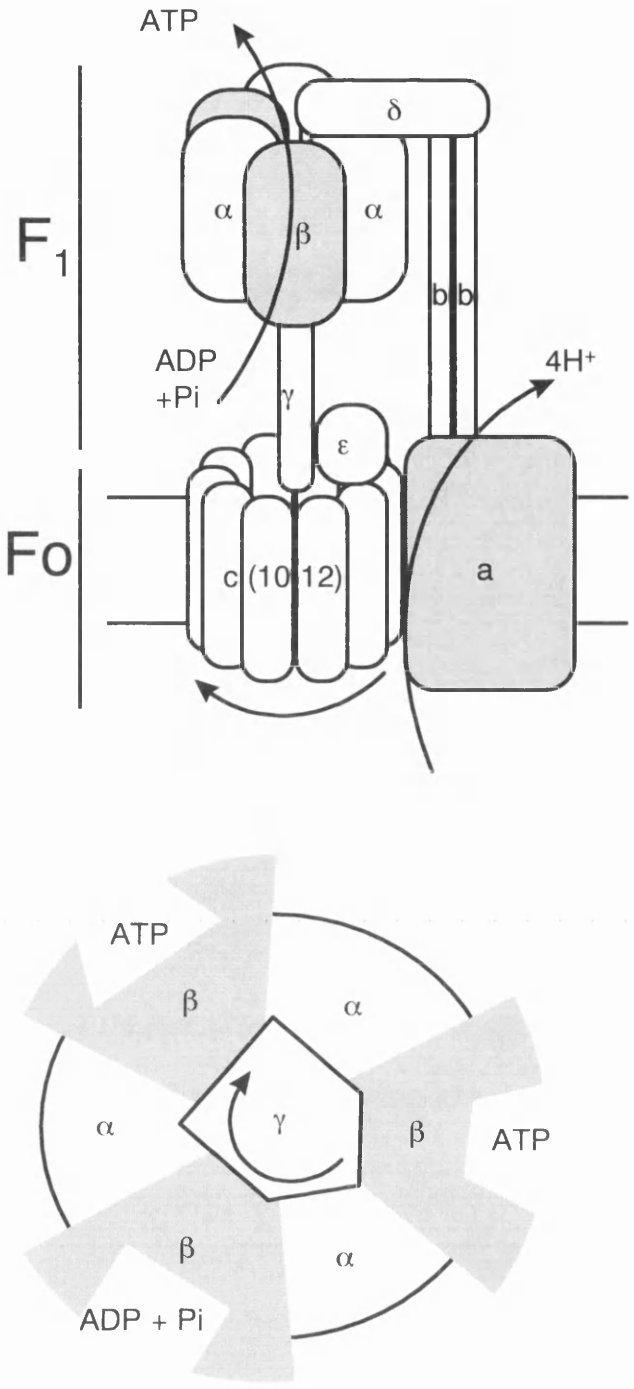


Figure 1.5 Tertiary structure of the F-ATPase

The enzyme uses the potential energy of a transmembrane proton gradient to drive ATP synthesis. Proton translocation through the membrane-bound proton-translocating 'Fo' complex drives the rotation of the γ subunit, which sequentially changes the conformation of the catalytic F1 subunits, driving ATP synthesis. Adapted from (Boyer, 1999).

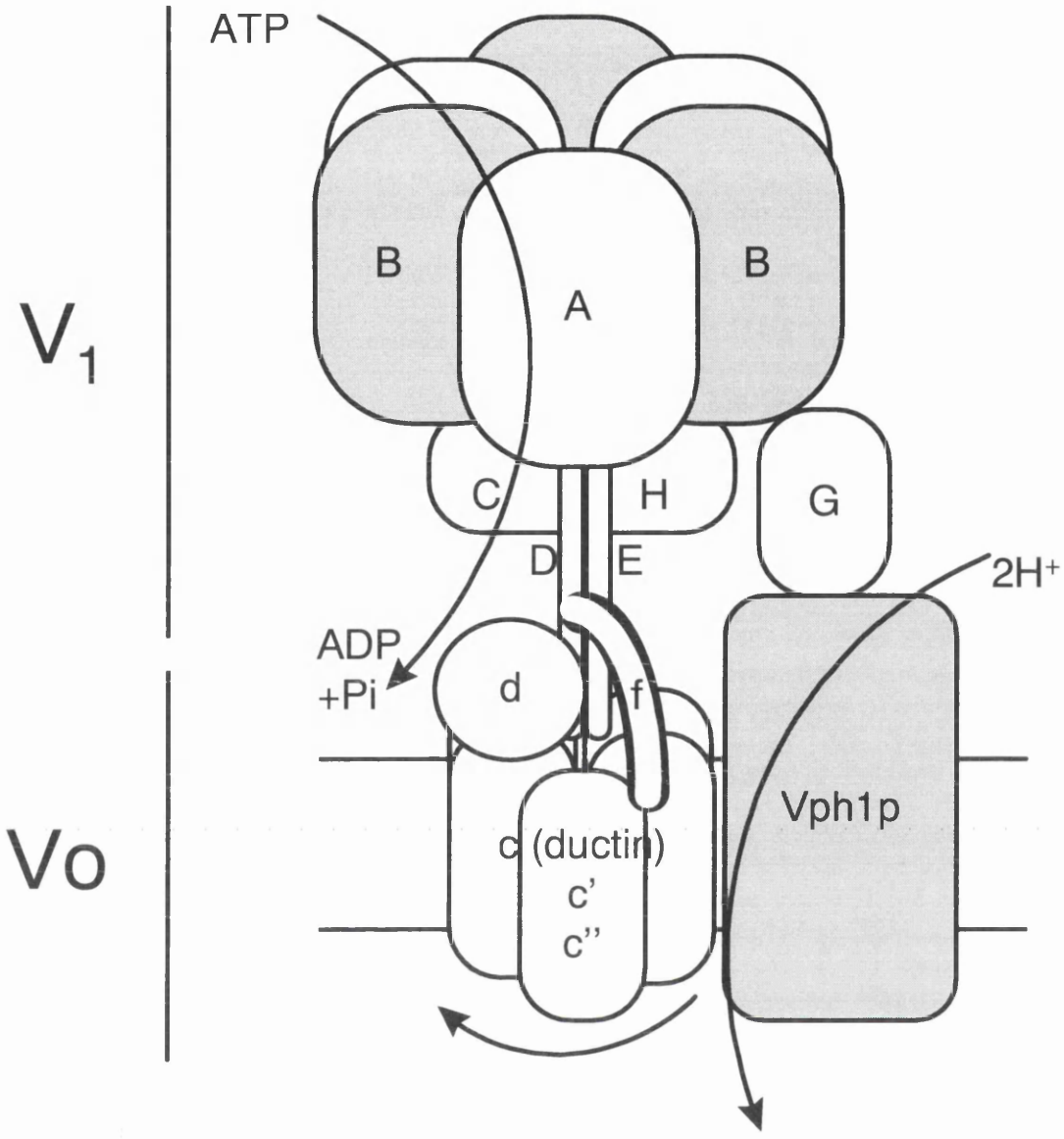


Figure 1.6 Subunit organisation of the V-ATPase

ATP-hydrolysis in the V_1 sector drives proton translocation by Vo sector by the same rotor-stator scheme of catalysis as seen in the F-ATPase. However, the V-ATPase contains additional subunits and cannot reverse its action to synthesise ATP.

Table 1.1 Properties of the *S. cerevisiae* V-ATPase subunits

The location of the subunits in the V-ATPase is indicated along with their molecular mass, suggested F-ATPase homologue, and function in the enzyme. Adapted from Finbow and Harrison, (1997).

Subcomplex	Subunit designation	S. cerevisiae gene	Mass (kDa)	Stoichiometry	F-ATPase homologue	Proposed function
V_1	A	VMA1	69	3	β	ATP catalysis
	B	VMA2	57	3	α	ATPase regulation
	C	VMA5	42	1	-	?
	D	VMA8	32	1	γ	V1-Vo coupling
	E	VMA4	27	1	γ	V1-Vo coupling
	F	VMA7	14	1	ε/σ	Regulatory? Coupling proteolipids to stalk
	G	VMA10	13	3	b	Anchors A/B complex to subunit a
	H	VMA13	54	1	-	?
Vo	a	VPH1/STV1	95	1	a	Proton hemi-channel Anchors stator to membrane
	c (ductin)	VMA3	17	6?	c	Proton hemi-channel
	c'	VMA11	17	?	-	Proton hemi-channel
	c''	VMA16	23	?	-	Proton hemi-channel
	d	VMA6	40	?	-	Vo stability
(Vo)	-	VMA12 VMA21 VMA22	25 8 21	-	-	Molecular chaperones

1.3.5 Gross structure

Figure 1.6 shows the proposed structure of the V-ATPase. In line with the ball and stick structures seen by electron microscopy, the enzyme is believed to follow the pattern of F-ATPases (Pedersen and Amzel, 1993). A hydrophilic catalytic head complex, termed V_1 associates with the proton-conducting membrane-bound complex, termed Vo. However, the V-ATPase has more subunits, and areas of poor sequence homology between the two types leaves gaps in the allocation of homologues. In fact, only subunits A, B, D and c (ductin) show notable homology with F-ATPase subunits.

1.3.5.1 Subunit structure and function

The yeast V-ATPase is the best characterised form, and will be considered here. Genome sequencing programmes have revealed homologous V-ATPase genes in a number of organisms (Manolson *et al.*, 1988; Nishigori *et al.*, 1998) suggesting a similar conservation of structure and function. The information on the yeast V-ATPase subunits is summarised in Table 1.1.

1.3.5.2 Catalytic V_1 sector

1.3.5.2.1 Subunit A

This is the homologue of the F-ATPase β subunit, showing 25% protein sequence homology (Bowman *et al.*, 1988b). Protection studies identified the presence of the suggested ATP hydrolysis site by using ATP analogs azido-ATP and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole chloride which inhibit the enzyme and label subunit A in an ATP-protectable manner (Arai *et al.*, 1987b; Uchida *et al.*, 1988a; Zhang *et al.*, 1995). The sulphydryl reagents, N-ethyl maleimide (NEM) and omeprazole behave identically despite their non-resemblance to ATP (Arai *et al.*, 1987b; Nadler *et al.*, 1998), which led to the discovery of a regulatory cysteine (C254) at the ATP binding site (Feng and Forgac, 1992). In the V-ATPase, C254 is the 'X' in the conserved GXGKTV Walker consensus 'A' sequence identified as the nucleotide-binding 'p-loop' (Walker *et al.*, 1985), and is thought to allow regulation of the V-ATPase in response to the REDOX state of the cell by disulphide bond formation with cysteine-532, as discussed in section 1.3.6.4.

1.3.5.2.2 Subunit B

This has 25% homology with subunit A, and with the regulatory α subunit of the F-type enzyme (Manolson *et al.*, 1988). It also contains the p-loop sequence (Nelson *et al.*, 1989) and labels with ATP-analogs (Manolson *et al.*, 1985). However, from structural information inferred from the F-ATPase α-subunit (Abrahams *et al.*, 1994) and demonstrations of the resistance of enzyme activity to

mutations of the ATP binding site of this subunit (Liu *et al.*, 1996), it is thought that subunit B is responsible for allosterically regulating the activity of the enzyme in response to ATP (Arai *et al.*, 1989).

1.3.5.2.3 Subunit C

Not to be confused with the proteolipid, subunit c, the precise function of this subunit is unknown. It has no homology with any subunit from the F-ATPase, but studies in *Saccharomyces cerevisiae* suggest it may play a role in V_1 assembly (Beltran *et al.*, 1992; Ho *et al.*, 1993a). However, it is not always seen in preparations from *Neurospora crassa* (Bowman *et al.*, 1992) and the form seen isolated from bovine clathrin-coated vesicles does not seem to be essential for activity (Puopolo *et al.*, 1992b). Interestingly, despite poor sequence conservation, the yeast V-ATPase can tolerate extensive bovine subunit C chimeras (Beltran *et al.*, 1992), so it seems that the structure rather than the sequence may well be conserved. Further characterisation of this subunit will be useful in highlighting the differences between the F- and V-type ATPases.

1.3.5.2.4 Subunit D

This has only weak homology with an F-ATPase subunit; the γ-subunit, which acts as the central spindle coupling the F_1 and Fo (Nelson *et al.*, 1995). There is no firm evidence to suggest that it performs this role in the V-ATPase, since it is dispensable in the bovine enzyme (Crider *et al.*, 1997) and only weakly associated in *N. crassa* (Margolles-Clark *et al.*, 1999). However its isoelectric point is similar

to the γ-subunit of the F-ATPase, and the conservation is concentrated around areas implied to interact with A-B subunit complex involved in rotation (Nelson *et al.*, 1995).

1.3.5.2.5 Subunit E

This also has a similar size and structure prediction to the γ-subunit (Bowman *et al.*, 1995). It has no homology to any other protein but its pattern of conservation across species closely matches that of the γ-subunit (Margolles-Clark *et al.*, 1999) suggesting that, assuming the two have similar structures, their functional domains are in the same place. Unlike subunit D, subunit E is indispensable for bovine V-ATPase function (Crider *et al.*, 1997) and remains tightly associated with the *N. crassa* enzyme, although its isoelectric point is 3 units lower than that of the γ-subunit. One suggestion is that the stalk is composed of both subunits D and E (Tomashek *et al.*, 1996), which may also account for the extra length of the V-ATPase stalk (Dschida and Bowman, 1992).

1.3.5.2.6 Subunit F

This has no significant homology to any of the proteins in the F-ATPase. However, its size and isoelectric point are very similar to that of the ε-subunit of *E. coli* F-ATPase and the σ-subunit of the mitochondrial F-ATPase (Nelson *et al.*, 1994), which themselves are poorly conserved over species (Walker *et al.*, 1985). On these grounds it was suggested to be the V-ATPase counterpart of these subunits, linking the Vo sector to the stalk (Nelson *et al.*, 1994). Interestingly, this

study also demonstrated that addition of antibodies to subunit F caused inhibition whilst retaining coupling, indicating that it may be involved in regulating activity. Helical wheel projection identified a distinct region of sequence conservation, which along with the high content of charged residues suggested a bridging function for the protein.

1.3.5.2.7 Subunit G

Despite exhibiting homology to the F-ATPase Fo subunit b, this subunit has no predicted transmembrane domain (Supekova *et al.*, 1996). Its designation as part of the V_1, on the basis of cold-induced dissociation from the membrane in *Manduca sexta* (Lepier *et al.*, 1996) is contradicted by its retention to the Vo in yeast (Supekova *et al.*, 1995). Because the V-ATPase is a two-part enzyme, fickleness of the proteins at the interface is not unexpected, supporting the theory that subunit G performs the same role as subunit b, anchoring the catalytic complex to the membrane complex to form the stator. The findings that helical wheel projections show high conservation on one face of the helix (Hunt and Bowman, 1997) and that it contains a high proportion of charged residues are consistent with it performing a bridging function.

1.3.5.2.8 Subunit H

This has no homology to other proteins, and has no counterpart in the A- and F-ATPases. The yeast protein and nucleic acid sequences fail to find matches in the Genbank database despite the discovery of proteins of similar mass in plant and

bovine material (Parry *et al.*, 1989; Puopolo *et al.*, 1992b). Despite its requirement for yeast growth at neutral pH (Ho *et al.*, 1993b) it was found to be involved in enzyme stability rather than assembly (Puopolo *et al.*, 1992b).

1.3.5.3 The Vo sector

1.3.5.3.1 Vma12p, Vma21p and Vma22p

These proteins are responsible for guiding the assembly of the Vo sector rather than being active components of the final V-ATPase complex. Vma12p is predicted to be a two-pass transmembrane protein in the endoplasmic reticulum (ER) required for Vph1 stability and correct Vo assembly and targeting to the vacuole (Hirata *et al.*, 1993; Jackson and Stevens, 1997). Vma22p is similar in many ways in that it is an ER resident protein that prevents Vph1p degradation in the ER and is required for assembly of the V-ATPase (Hill and Stevens, 1995). Although Vma22p is a hydrophilic protein, it is associated to the membrane via its interaction with Vma12p (Graham *et al.*, 1998), this complex also interacting with Vph1p, and so being implicated in chaperoning its assembly into the Vo (Graham *et al.*, 1998).

Vma21p is not found in the Vma12/22p complex (Graham *et al.*, 1998). It is similar to Vma12p in that it is a small ER-resident protein with two predicted transmembrane domains that is required for Vph1p stability (Hill and Stevens, 1995). However, its exact function is not known; it was suggested that it might be a part of the Vma12/22p complex or that it may even be involved in regulating vesicle trafficking of the V-ATPase to the vacuole (Graham and Stevens, 1999).

1.3.5.3.2 Vph1p/Stv1p

Two complementary genes code for this protein in yeast; VPH1 and STV1 (Similar To VPH1) resulting in an incomplete vacuolar disruption (Manolson *et al.*, 1992). The reason for this is not known, but STV1 is expressed at lower levels than Vph1p, to which it is only 55% identical and may contain Golgi retention signals. Suggesting that the two are location-specific isoforms (Manolson *et al.*, 1994). Both proteins are predicted to be 6-7 pass transmembrane glycoproteins (Adachi *et al.*, 1990; Manolson *et al.*, 1992), required for assembly of the Vo complex (Kane *et al.*, 1992), mutational studies attributing this function to the carboxy terminus (Leng *et al.*, 1998). The presence of crucial charged residues in the transmembrane domains (Leng *et al.*, 1998; Leng *et al.*, 1996) and a bafilomycin A_1 binding site (Bowman *et al.*, 1988a) suggest a role for these proteins in proton translocation, consistent with the proposed role of their homologue (subunit a) in the F-ATPase (Jiang and Fillingame, 1998).

1.3.5.3.3 Subunit d

Unlike the other Vo subunits, this subunit can be stripped from the membrane by strong chaotropic agents (Bauerle *et al.*, 1993), which along its hydrophilicity and lack of any putative transmembrane domains, suggests that it is associated to, rather than integrated into the membrane. Its absence destabilises the Vo, preventing the V_1 from docking (Bauerle *et al.*, 1993), but its role aside from being an assembly factor has not been probed.

1.3.5.3.4 Subunit c (ductin), and related proteins

Ductin is the 16kDa protein found in preparations of gap junctions, mediatophores and V-ATPases (Brochier and Morel, 1993; Finbow *et al.*, 1983; Mandel *et al.*, 1988); its role as a channel protein leading to it being called ductin (duct protein) (Holzenburg *et al.*, 1993). Its multifunctional nature is illustrated by the ability of *Nephrops norvegicus* ductin, discovered in gap junction preparations, to complement the function of *S. cerevisiae* ductin in the V-ATPase (Harrison *et al.*, 1994). One of the reasons it is capable of forming so many structures is that it is inserted into the lipid bilayer in both orientations (Dunlop *et al.*, 1995); the environment of the hydrophilic loops determining the binding activity and eventual function, reviewed by Finbow *et al.*, (1995). The number of functions (and hence the number of residues susceptible to mutation) may also explain why ductin is the most conserved integral membrane protein known, with 65% sequence conservation between all species.

Structural predictions, and sequence comparisons with the F-ATPase subunit c suggested that ductin forms a 4-pass α-helical bundle, with an active glutamic acidic residue in the middle of the fourth helix, reminiscent of a dimeric form of the subunit c (Figure 1.7), (Finbow *et al.*, 1992). Further sequence comparisons between the two proteins suggested that they evolved from a common ancestor, with gene duplication accounting for their size differences (Figure 1.8), (Mandel *et al.*, 1988). This was supported by the finding that the *E. coli* subunit c can be functionally substituted by a dimeric or trimeric construct of the protein (Jones and Fillingame, 1998), however, the V-ATPase cannot synthesise ATP, so it was suggested that the number of active acidic residues per enzyme determines the

type of activity (section 1.3.4.1), (Cross and Taiz, 1990; Ruppert *et al.*, 1999). This would agree with the thermodynamic differences between the two enzymes, with the ratio of ATP to proton binding sites corresponding to a high substrate:product ratio in both enzymes, which would be expected to increase their processivity of the enzymes by increasing the equilibrium constant.

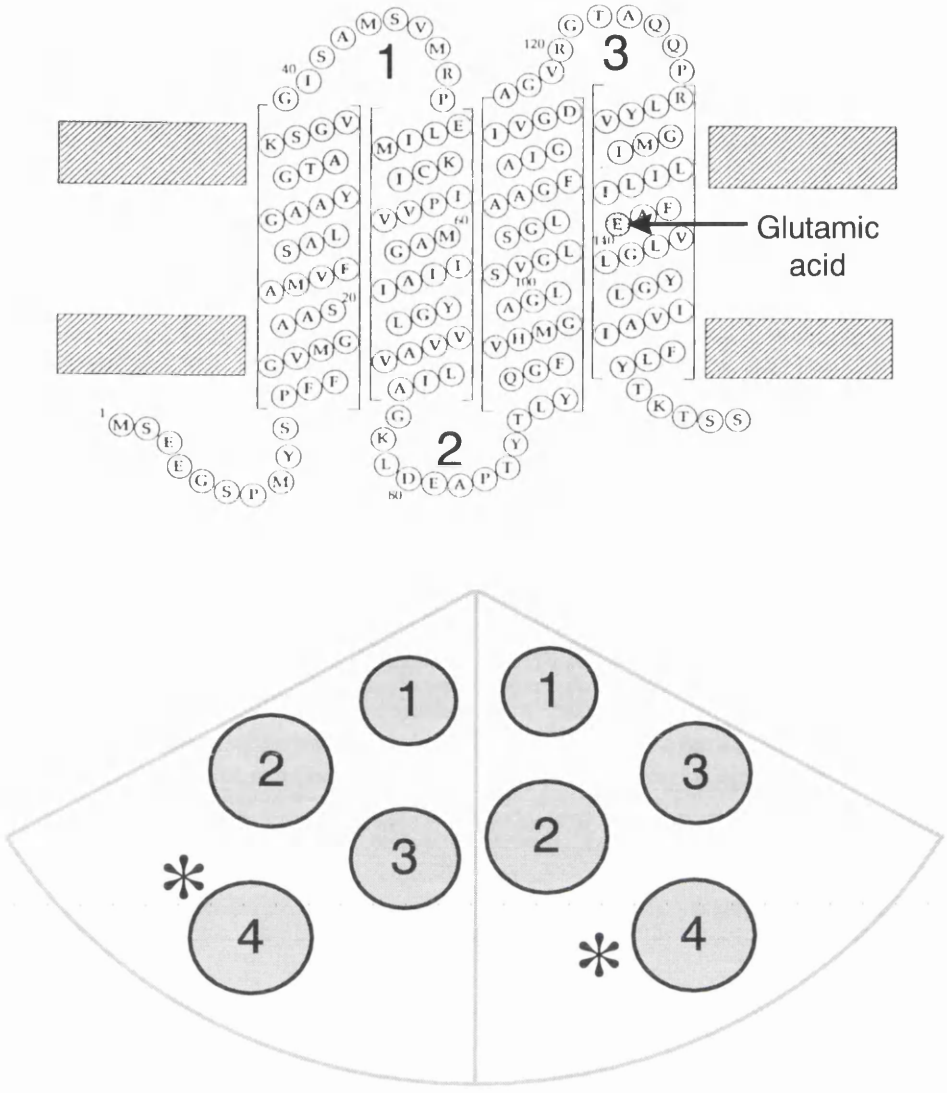
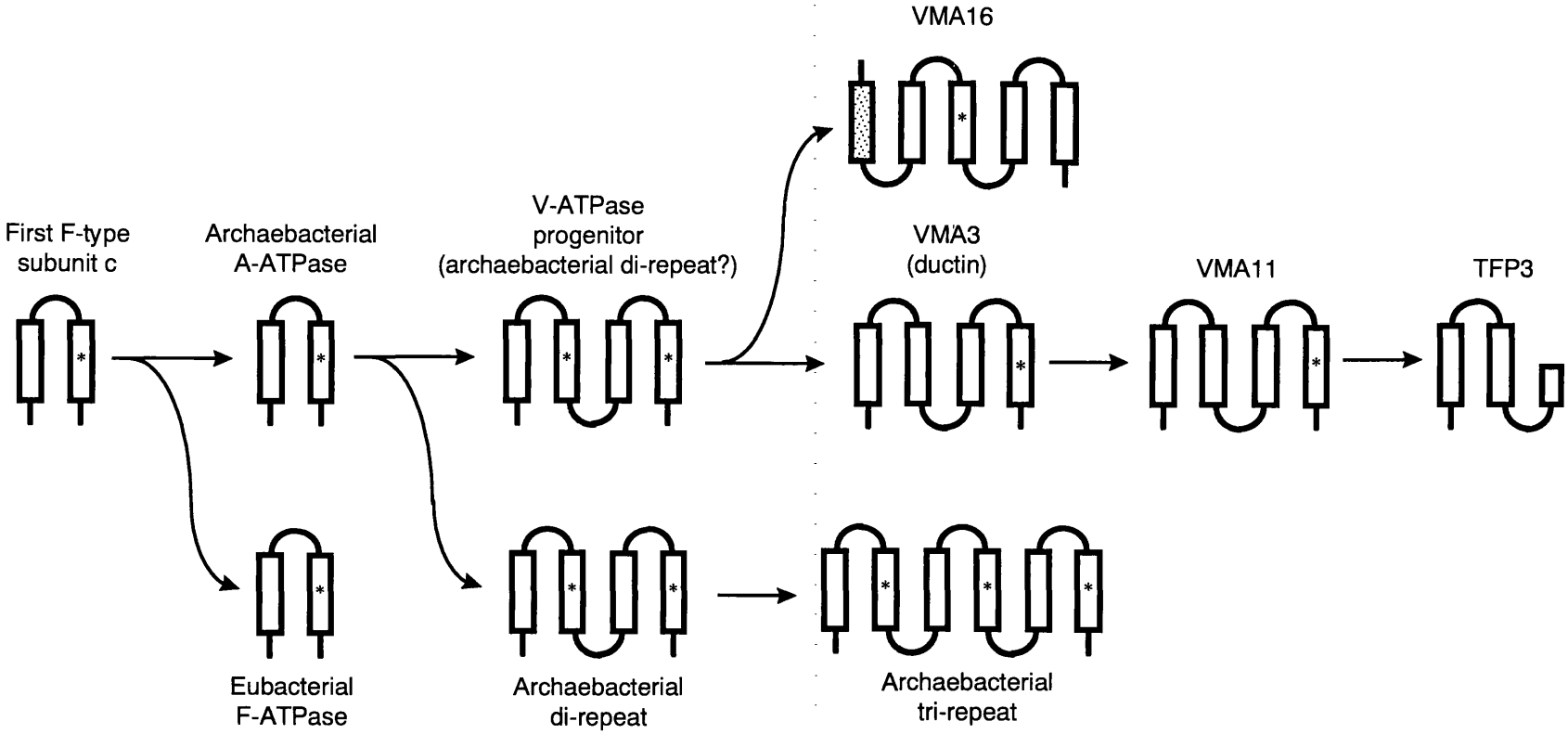


Figure 1.7 Membrane disposition of ductin and organisation in the Vo

Membrane disposition of Nephrops norvegicus *ductin, adapted from (Finbow et al., 1995), showing the transmembrane location of the active glutamic acid and the disposition in the membrane, with loops 1 and 3 believed to be apposed in gap junctions, and face the V_1 portion of the V-ATPase consistent with the dual orientation of ductin. The arrangement of the four helices in the Vo is shown as determined by cysteine mutagenesis crosslinking experiments (Harrison et al., 1999). The active glutamic acid residue is indicated by an asterisk and is situated on the exterior face of the complex, analogous to the aspartate in subunit c of the F-ATPase (Girvin et al., 1998).*

Figure 1.8 Proposed evolution of ductin and related proteolipids

Transmembrane domains are indicated by rectangles, critical acidic residues by asterisks, adapted from Finbow and Harrison, (1997). The sequence similarities between the proteins indicate their similar roles in the F-, A-, and V-ATPases, however, the function of TFP3 is not known. VMA16 is thought to be involved in proton translocation, but has an additional transmembrane domain, and a different placement of its critical glutamate. The functional significance of VMA11 is also unknown since it has only been discovered in fungal sources (Lyne et al., 1999; Pounder and Bowman, 1999; Umemoto et al., 1991).


VMA16
First F-type subunit c
Archaebacterial A-ATPase
V-ATPase progenitor (archaebacterial di-repeat?)
VMA3 (ductin)
VMA11
TFP3
Eubacterial F-ATPase
Archaebacterial di-repeat
Archaebacterial tri-repeat

The active glutamate is specifically labelled by N'N-dicyclohexidecarbodiimide (DCCD) (Arai *et al.*, 1987a; Finbow *et al.*, 1992), a reagent which labels acidic residues in non-polar environments. This labelling corresponded with inhibition of the enzyme, showing that the residue is not only exposed to the lipid bilayer, but is implicated in catalysis. The lipid-exposure of the glutamate was confirmed by structural analysis employing cysteine mutagenesis and N-(1-pyrenyl)maleimide labelling to identify proximal and lipid-exposed residues respectively (Harrison *et al.*, 1999). This study also revealed similarities to the structure of the F-ATPase subunit c (Jones *et al.*, 1998), with even numbered helices forming the exterior face, as shown in Figure 1.7.

Proton permeability assays on vesicles containing reconstituted Vo sectors confirmed the role of ductin, with it alone being able to form a proton channel for the V-ATPase, and the other Vo subunits merely enhancing the permeability (Zhang *et al.*, 1994). This contradicted previous results showing that the Vo does not act as a pore for passive proton diffusion (Zhang *et al.*, 1992). The reasons for this are not known, but it was suggested that although ductin is capable of forming a proton channel *in vitro*, its permeability depends upon other factors (Zhang *et al.*, 1994). From these results it was thought that the ductin was the only proton-conducting component of the V-ATPase, however, other related proteolipids have since been discovered, Vma11p and Vma16p (Figure 1.8) which are related to ductin which may be involved in proton translocation (Apperson *et al.*, 1990; Hirata *et al.*, 1997; Umemoto *et al.*, 1991).

Vma11p also called subunit c' is the same size as, and has 54% sequence identity to ductin (Umemoto *et al.*, 1991). Although only a minor component of the Vo

(Hirata *et al.*, 1997), gene inactivation resulted in significant loss of V-ATPase activity (Umemoto *et al.*, 1991), and like ductin its function is also dependent upon a glutamic acid in the fourth transmembrane domain (Hirata *et al.*, 1997). The VMA11 gene knockout can be partially rescued by overexpression of ductin (M. Harrison, unpublished observations), however any single helix replacements of Vma11p to those of ductin ablate the activity of the V-ATPase, (A. Ashby, unpublished results) so it does appear to have a distinct function. Interestingly, Vma11p has only been discovered in fungal V-ATPases (Lyne *et al.*, 1999; Pounder and Bowman, 1999; Umemoto *et al.*, 1991) so it may represent a functional adaptation specific for these organisms.

Vma16p, also called subunit c" has only 35% homology to ductin and 30% cross species identity, it having been identified in *Arabidopsis*, *Caenorhabditis*, yeast, human and mouse. Again, inactivation of the VMA16 gene disrupts vacuolar function, suggesting that it is an essential component of the V-ATPase Structurally, it is thought to be very similar to ductin with the exception that has an extended N-terminus and retains a glutamic acid in its second helix rather than the fourth (Figure 1.8) (Apperson *et al.*, 1990). Despite this similarity, Vma16p does not bind to BPV-4 E8 (Faccini *et al.*, 1996), and it appears that the glutamic acid on the second helix is involved in proton translocation rather than that on the fourth (Hirata *et al.*, 1997), a finding supported by the relatively high level of conservation in this helix across species. The N-terminal extension is predicted to be a transmembrane α-helix, but although it is required for activity, its purpose is not known. It might be that it occludes the pore in the middle of the Vo (Cadwallader and Finbow, unpublished results), and perhaps explains the

differences in permeabilities between different Vo preparations (Zhang *et al.*, 1994; Zhang *et al.*, 1992).

The function of Tfp3p, so named because its overexpression confers resistance to trifluorperazine in yeast (Shih *et al.*, 1990), remains elusive. It has not been identified as a formal V-ATPase subunit, so it is likely that despite its homology to ductin, it has a separate function, perhaps as another type of membrane channel.

1.3.5.4 Summary

The parallels between the V-ATPase and the F-ATPase allow information to be inferred about the catalytic mechanism, however, the V-ATPase has additional subunits, different patterns of sequence conservation, and a different pattern of inhibitor sensitivity than the F-ATPase (Bowman, 1983; Drose and Altendorf, 1997; Uchida *et al.*, 1988b), suggesting that there are fundamental differences between the two types. One way in which this manifests itself is through the differences in the ways the enzymes are regulated; an unsurprising feature since the enzymes effectively perform opposite roles and operate in different cellular locations, albeit using the same blueprint for catalysis.

1.3.6 Regulation of the V- ATPase

The activity of the V-ATPase needs to be regulated around the ATP budget of the cell and also to meet the specific demands of the compartment upon which it resides. For example, early endosomes require only a slight pH gradient

compared to lysosomes (Gruenberg and Maxfield, 1995). To this end, the enzyme is regulated at many stages within the cell, the effects varying in timescale and consequence.

1.3.6.1 Transcriptional regulation

Neurospora crassa V-ATPase gene promoters are thought to resemble consititutively-expressed housekeeping genes (Wechser and Bowman, 1995), but observations of cyclic adenosine monophosphate (cAMP)-dependent upregulation of subunit B expression during monocyte to macrophage differentiation (Lee *et al.*, 1995), suggest that the situation may be different in higher organisms. Studies on *Manduca sexta* subunits reviewed by Wieczorek *et al.*, (1999b) support this claim, with both V_1 and Vo subunits possessing upstream ecdysone response elements and exhibiting ecdysone-induced downregulation. V_1 subunit sequences also contain what appear to be cAMP-responsive elements and are upregulated in response to cAMP (Merzendorfer, 1998). This differential regulation of the V_1 and Vo subunits is probably related to the fact that the two halves of the enzyme are often present in different amount in the cell (Myers and Forgac, 1993a).

1.3.6.2 mRNA stability

The role of the 3' untranslated region (3'-UTR) in mRNA stability is reviewed by Decker and Parker, (1994). cDNA sequencing has revealed variation in the sequences of 3' untranslated regions of Vo and V_1 subunits (Dow *et al.*, 1992; Graf *et al.*, 1994), coupled with differential expression in one case (Puopolo *et al.*,

1992a). Unpublished results reviewed by Merzendorfer *et al.*, (1997a) support this suggestion by the observation of size differences in transcripts of *M. sexta* subunit B corresponding to two different polyadenylation sites. The discovery of signal recognition particle (SRP) independent localisation of ductin mRNA also led to the suggestion that the 3' untranslated regions were involved in subcellular targeting (Jager *et al.*, 1996).

Regulation by way of antisense RNA transcription has also been suggested, reviewed by Knee and Murphy, (1997). It is thought that formation of double stranded RNA renders the transcript vulnerable to degradation by double-stranded ribonucleases. Evidence for this type of regulation for the V-ATPase comes from the identification of antisense transcripts of the *M. sexta* M40 subunit by reverse transcriptase polymerase chain reaction (RT-PCR) (Merzendorfer *et al.*, 1997b) but whether this is significant, or even whether antisense regulation actually occurs in eukaryotes has yet to be proven.

1.3.6.3 Protein Targeting

The AP-2 adapter protein complex, responsible for recruiting EGF receptors to clathrin-coated pits is also implicated in activation of the V-ATPase. The 50kDa subunit of the AP-2 complex was found to associate with the V-ATPase and phosphorylate subunit B (Myers and Forgac, 1993b). These results were expanded upon by the finding that this 50kDa subunit is actually required for activity and assembly of the enzyme (Liu *et al.*, 1994). The interrelationship between endocytosis and the V-ATPase makes this mechanism of regulation attractive, since it ensures an even number of V-ATPases per internalised vesicle.

1.3.6.4 Cytoplasmic REDOX conditions

The role of the conserved cysteine residue at the catalytic site in regulating enzyme activity in response to REDOX conditions has been discussed in section 1.3.5.2.1. As well as inhibition and specific labelling by sulphydryl reagents there are genetic data to show that this is more than just a casual feature of the enzyme.

CYS4 encodes cystathione β-synthase, a key enzyme in cysteine and subsequent glutathione synthesis, but a CYS4 mutant has also been shown to disrupt the V-ATPase (Oluwatosin and Kane, 1997), implicating either the enzyme itself, the cellular levels of cysteine, or its downstream products in the regulation of the V-ATPase. Addition of glutathione to the medium restored activity, so it was suggested that the phenotype was due to oxidation of the regulatory cysteine in subunit A rather than any mistranslation effects due to cysteine starvation (mutation of certain cysteine residues in subunit A also result in loss of activity (Taiz *et al.*, 1994)). This idea was reinforced by the finding that mutation of the regulatory cysteine in subunit A renders the enzyme insensitive to the effects of CYS4 mutation and glutathione (Oluwatosin and Kane, 1997).

Glutathione is produced by reduction of oxidised glutathione by reduced nictotinamide adenine dinucleotide phosphate (NADPH), which is also interconverted to reduced nictotinamide adenine dinucleotide (NADH), which is the substrate for ATP synthesis in mitochondria. So it might be that the oxidative inhibition of the V-ATPase is actually an NADH detection mechanism to avoid cell starvation. This cysteine-mediated REDOX regulation not only accounts for the

differences in the sensitivity of the two enzymes to NEM (section 1.3.5.2.1), but highlights one of the differences between the F and V type ATPases which fits well with their function. The F-ATPase is part of a system originally devised to allow prokaryotes to tolerate oxidative stress, so oxidative inhibition would be counterproductive. V-ATPase activity on the other hand appears to be controlled to ensure that the cell has sufficient ATP, by using disulphide bridge-mediated inhibition to detect NADH levels indirectly.

1.3.6.5 Nutrient availability

The V-ATPase is responsible for 10% of the total ATP consumption in *M. sexta* larva (Dow, 1984) so REDOX monitoring as a method of verifying the potential of the cell to generate ATP is not unexpected. However there is another mechanism of regulation that is more directly linked to verifying the potential of the cell to make ATP; regulation in response to glucose concentrations.

Starvation-induced dissociation of the V-ATPase was first demonstrated in *M. sexta*, (Sumner *et al.*, 1995), from discovering a 90% loss of V-ATPase activity and an absence of V_1 subunits in membrane preparations from moulting larvae. Consistent with these observations, the dissociated V_1 complex was purified from the cytoplasm and shown to have negligible Mg-ATPase activity (Graf *et al.*, 1996). This starvation response was also demonstrated in yeast (Kane, 1995), a more thorough investigation showing that this occurred independently of all known glucose-signalling pathways and was not dependent upon ATP levels (Parra and Kane, 1998). However, dissociation did require ATP hydrolysis since dissociation was inhibited by concanamycin A or by using ATPase-defective strains that were

still capable of assembly (Hirata *et al.*, 1997; Leng *et al.*, 1996). The suggested reason for this regulation was to conserve ATP during starvation conditions, whilst permitting a rapid restoration of activity upon addition of glucose to neutralise the drop in cytoplasmic pH seen when glycolysis is re-started (Beauvoit *et al.*, 1991; Parra and Kane, 1998).

When investigating the effects of alternative carbon sources, it was found that raffinose, despite its metabolism into glucose, did not induce reassembly to the same extent as glucose (Kane, 1995). Even cells grown in raffinose for prolonged periods show reduced levels of assembly (Kane, 1995), suggesting that the regulation is a graded rather than an absolute response.

1.3.6.6 Coupling efficiency

Despite the fact that the enzyme is designed to be as efficient as possible, there are cases when partial coupling is an advantage, for example in the early endosomal membranes where only a slight pH gradient is required. This was first observed in the F-ATPase (Groen *et al.*, 1990) and was later termed 'slip' in reference to the V-ATPase (Nelson, 1991). The advantage of using slip regulation might be that it can be a rapid and highly localised effect; ideal for use in the endocytic vesicle pathway.

1.3.6.7 Regulation of vacuolar pH

The chloride channel is thought to determine the balance between the electrochemical and pH gradient generated by the V-ATPase (Fuchs *et al.*, 1989a). The pH of endosomal compartments can also be regulated by the Na^+/K^+ ATPase, by way of alleviating the electrochemical gradient (Fuchs *et al.*, 1989b); consistent with this idea is the absence of the Na^+/K^+ ATPase in late endosomes and lysosomes.

1.3.7 Summary

The V-ATPase is a highly regulated enzyme responsible for driving a number of cellular activities, including the maintenance of the endocytic pathway for processing cell surface receptors. Because a number of viral oncoproteins bind to the proteolipid component of the enzyme (Conrad *et al.*, 1993; Faccini *et al.*, 1996; Franchini *et al.*, 1993; Goldstein and Schlegel, 1990) it is possible that its inhibition is implicated in their mechanism of cell transformation.

1.4 *Saccharomyces cerevisiae* as a model organism to investigate the effects of a family of ductin-binding E5 proteins on the vacuolar ATPase

Most, if not all papillomaviruses possess and open reading frame encoding a small hydrophobic protein which contributes towards cell transformation and binds to ductin (Conrad *et al.*, 1993; Faccini *et al.*, 1996; Franchini *et al.*, 1993; Goldstein *et al.*, 1991). Most of the E5 proteins also bind to growth factor receptors (Goldstein *et al.*, 1992a; Hwang *et al.*, 1995; Mulloy *et al.*, 1996), but this activity does always correlate with transforming potential (Nilson and DiMaio, 1993; Sparkowski *et al.*, 1996).

Ductin is a component of the gap junction, along with connexins. Not only are there a number of proteins involved in forming gap junctions, but they are also regulated by a number of pathways, and the role of gap junctional intercellular communication (GJIC) in cell transformation is unclear (see section 1.2.1). Ductin also forms the proton-conducting component of the vacuolar ATPase (V-ATPase), which acidifies the endosomal compartments that process and degrade growth factor receptors (section 1.2.2). Mutational analysis of BPV-1 E5 has shown that its ability to cause alkalinisation of the Golgi apparatus correlates with its ability to transform cells (Schapiro *et al.*, 2000). Direct inhibition of the V-ATPase was not shown, but it did suggest that the E5 proteins transform cells through binding to ductin and inhibiting the enzyme. The work in this thesis investigates the effects of a number of E5 proteins on the V-ATPase using a system well suited to the study of the enzyme.

1.4.1 Experimental rationale

V-ATPase inhibition by bafilomycin A_1 is cytotoxic in mammalian cells *in vitro* (Ohta *et al.*, 1998), with lower concentrations causing growth inhibition (Ohkuma *et al.*, 1993). Genetic inactivation of the V-ATPase is also lethal *in vivo* (Davies *et al.*, 1996), so an organism tolerant to V-ATPase inhibition was chosen to study the effects of the E5 proteins on the enzyme, *Saccharomyces cerevisiae.* The yeast V-ATPase contains at least as many subunits as the mammalian enzyme, suggesting similar architectures, and yeast ductin has 71% protein sequence identity to the bovine and human forms of ductin (92% identity in helices 3 and 4). In line with this conservation is the finding that BPV-1 E5 has been shown to bind to another form of yeast ductin; that of *Schizosaccharomyces pombe* (Goldstein *et al.*, 1992c). Vacuolar inactivation can be tolerated by yeast if the pH of the growth medium is lowered to 5.5, with neutral-buffered medium providing a simple screening technique to assay vacuolar function. Standard protocols exist for genetic modification and for isolation of milligram quantities of vacuoles. In addition, because of the popularity of using yeast to study the V-ATPase, techniques have also been devised to purify the enzyme from vacuolar preparations, and monoclonal antibodies are available against some of the subunits. Another advantage is that yeast do not naturally express the growth factor receptors that the E5 proteins interact with, reducing the ambiguity of the results.

In addition to wild type E5 protein constructs, BPV-4 E8 mutant forms were also expressed, which all bind to ductin (Ashrafi *et al.*, 2000) but exhibit different transforming abilities (O'Brien *et al.*, 1999). These activities did not correlate with

the disruption of one function of ductin, in gap junctional intercellular communication (Ashrafi *et al.*, 2000), so their activities may be due to disruption of the other function of ductin, in the V-ATPase. A dominant negative form of ductin, previously shown to cause V-ATPase inhibition (Andresson *et al.*, 1995) was included to verify the use of the yeast strain and plasmid combination to investigate the E5 proteins. A similar dominant negative form of ductin has also been shown to cause cell transformation (Andresson *et al.*, 1995), so by comparing its effects to those of the E5 proteins it will be possible to see if they act by similar mechanisms.

To allow detection of the E5 proteins, polymerase chain reaction (PCR) was used to add an HA epitope tag, a short peptide derived from the HA1 haemagglutinin gene of the influenza virus (Field *et al.*, 1988), which does not affect the transformation potential of either BPV-1 E5 or BPV-4 E8 (Andresson *et al.*, 1995; O'Brien *et al.*, 1999). In chapter 3 the use of *S. cerevisiae* is verified by assessing the binding efficacy of these epitope-tagged proteins to *S. cerevisiae* ductin by *in vitro* translation on microsomes followed by immunoprecipitation.

The E5 constructs were expressed using the pYPMA vector, (M. Harrison, Leeds University, UK), which was originally used to drive the overexpression of ductin mutants that required such high levels for detection (Jones *et al.*, 1995). In this case, overexpression was used to amplify any effects of the proteins, since the cytotoxic effects associated with V-ATPase inhibition are not seen when the E5 proteins are expressed in mammalian cells, suggesting that they exert subtle effects, if any, on the enzyme. The constructs were sequenced before

transformation into yeast, where their correct expression *in vivo* was verified by a number of methods, the results being shown in chapter 3.

The effects of the E5 proteins were first investigated in the YPH 500 strain of yeast grown in glucose medium. Its auxotrophy for uracil allows pYPMA maintenance, whilst the *ade2* mutation causes accumulation of a red pigment in the vacuole (Fisher, 1969), so provides an additional visual screen to that of growth. Chapter 4 shows the effects of the E5 proteins on growth rate, vacuolar acidification, V-ATPase activity and E5 binding *in vivo*, using the dominant negative form of yeast ductin as a control.

Chapter 5 shows the effects of the E5 proteins when expressed in yeast grown in galactose/raffinose medium. This has the effect of partially compromising the enzyme and possibly sensitising it to any further disruption by the E5 proteins. The effects in a doubly compromised system are shown in chapter 6, with the E5 proteins being expressed in a strain of yeast transgenic for *Nephrops norvegicus* ductin, grown in galactose/raffinose medium. *Nephrops* ductin has been shown to complement the yeast ductin knockout, albeit to produce a V-ATPase with an elevated Michaelis constant (Harrison *et al.*, 1994). This compromisation makes the transgenic system a useful model to detect any subtle effects on the V-ATPase. The original pTY-*Nephrops* ductin construct was used to minimise experimental variation, and because its tryptophan selection cassette is compatible with the leucine cassette replacing the wild type ductin, and the uracil selection used to maintain pYPMA.

The data presented in this thesis show that all of the E5 proteins bind to *S. cerevisiae* and *Nephrops* ductin *in vitro*, but do not remain associated with the active V-ATPase *in vivo*, nor affect its activity. This suggests that they do not directly inhibit the V-ATPase, but bind to ductin for other reasons.

2. Materials and methods

2.1 Materials

2.1.1 Equipment

Beckman (RIIC) Ltd. Oakley Court, Kingsmead business park, London road, High Wicombe, Buckinghamshire, HP11 1JU.

Polycarbonate centrifuge tubes

Bibby Sterlin Ltd. Tilling drive, Stone, Staffordshire, ST15 0SA

Bacteriological dishes (90mm)

Elkay Laboratory products PO BOX 6004, Basingstoke, Hampshire, RG24 8HL

Plastic pipette tips

Plastic tubes

Plastic cuvettes

Invitrogen BV, PO BOX 2321, 9704 CH Groningen, The Netherlands.

NOVEX gel apparatus

Mcquilkin & Co. 21 Polmadie avenue, Glasgow, G5 0BB

12ml glass homogeniser

2.1.2 Chemicals

Unless specified below, all chemicals were supplied by:

Fisher Scientific Ltd. Bishop Meadow Rd, Loughborough, Leics. LE11 5RG.

or by Sigma-Aldrich Co Ltd. Fancy Road, Poole, Dorset, BH12 4QH.

3M UK, 3M House, PO BOX 1, Marketplace, Bracknell, Berkshire, RG12 1JU

Fluorinert

Anachem Ltd. Anachem House, Charles St, Luton, Bedfordshire, LU2 OBE.

Galactose

Raffinose

Becton Dickinson, Northfield Road, Botherham, South Yorkshire, S60 1RR.

BACTO peptone

BACTO yeast extract

BACTO agar

James Burrough (F.A.D.) Ltd. 70 Eastways Industrial Park, Witham, Essex, CM8 3YE.

Ethanol 99.7%

2.1.3 Molecular biology reagents

Amersham Pharmacia . Amersham Place, Little Chalfont, Bucks, HP7 9NA.

Full and low range Rainbow molecular weight markers

ECL Western blotting system

Hybond ECL nitrocellulose membrane

Promix *in vitro* cell labelling mix

Cambridge Bioscience Ltd. 24-25 Signet Court, Newmarket Road, Cambridge, CB5 8LA.

Anti AU1 monoclonal antibody

Anti HA1 monoclonal antibody

Anti Vma1p monoclonal antibody

Anti Vph1p monoclonal antibody

ICN Biochemicals Ltd. Unit 18 Thame Park business centre, Whenham Road, Thame, Oxfordshire, OX9 3XA.

N-dodecyl maltoside

Yeast lytic enzyme, 70 000 units/g

Life Technologies. 3 Fountain Drive, Inchinnan Business Park, Paisley.

DH5α competent cells

DNA Restriction and modification enzymes

Low DNA Mass ladder

SOC medium

Promega UK Ltd. Chilworth Research Centre, Southampton, SO16 7NS.

Canine microsomal membranes

Nuclease treated rabbit reticulocyte lysate *in vitro* protein translation system

Stratagene Europe. Gebouw California, Hogehilweg 15, 1101 CB Amsterdam Zuidoost, Netherlands.

Dpn 1 restriction enzyme

Epicurial Coli XL10-gold ultracompetent cells

PfuTurbo DNA polymerase

2.1.4 Kits

AMS Biotechnology UK Ltd, 12 Thorney Leys Park, Witney, Oxon, OX8 7GE.

T7 mMESSAGE mMACHINE capped RNA synthesis kit

T7 MEGAshortscript short RNA synthesis kit

Cap analog ($m^7G(5')ppp(5')G$)

QIAGEN Ltd. Boundary Court, Gatwick Road, Crawley, West Sussex, RH10 2AX.

QIAprep spin miniprep kit

QIAquick spin PCR cleanup kit

Roche Diagnostics Ltd. Lewes, East Sussex, BN7 1LG.

Rapid ligation kit

2.1.5 Yeast strains

W303-1B Vatc	MATα *ura3 lys2 ade2 trp1 his3 leu2 vma3::*LEU2
YPH 500	MATα *ura3 lys2 ade2 trp1 his3 leu2*

2.1.6 Medium composition

Luria Broth (LB)	1%w/v bacto tryptone; 0.5%w/v bacto yeast extract; 1% NaCl, pH7.5. Heat sterilised
Complete glucose medium	1%w/v yeast extract; 2%w/v bacto peptone; 2%w/v glucose. Heat sterilised
Complete galactose/raffinose medium	As above, replacing glucose with 1%w/v galactose and 1%w/v raffinose.
Minimal glucose medium	0.67%w/v Bacto-yeast nitrogen base without amino acids; 2%w/v glucose; 50mM sodium succinate pH5.5; 20mg/l adenine; 20mg/l L-tryptophan; 20mg/l histidine; 30mg/l leucine; 30mg/l lysine. Filter sterilised.
Minimal galactose/ raffinose medium	As above, replacing glucose with 1%w/v galactose and 1%w/v raffinose.
Minimal galactose/ raffinose medium (for *Nephrops* ductin strains)	0.67%w/v Bacto-yeast nitrogen base without amino acids; 1%w/v galactose; 1%w/v raffinose; 50mM sodium succinate pH5.5; 20mg/l adenine; 20mg/l histidine. Filter sterilised.

2.2 Methods

2.2.1 Oligonucleotide design

BPV-1 E5, HTLV-1 p12^{I} and HPV-16 E5 were amplified by PCR using oligonucleotides incorporating *Bam*H 1 and *Kpn* 1 restriction sites and a HA epitope tag. These allowed ligation of the PCR product directly into the *Bam*H 1 site of the pYPMA vector, and rapid determination of orientation by *Kpn* 1 restriction digestion. The epitope tag allowed detection by commercially available antibodies (Field *et al.*, 1988). The sequences are shown below, with restriction sites in bold and the haemagglutinin epitope tag underlined. The BPV-1 E5 5' oligonucleotide was shorter because the template already contained the HA1 tag.

HA BPV-1 E5 5'

GCG**GGATCC**ATGCCAAATCTATACCCATACG

HA BPV-1 E5 3'

GCG**GGATCCGGTACC**TTAAAAGGGCAGACCTGTACAG

HA HTLV-1 p12^{I} 5'

G**GGATCC**GCCGCCACCATGTACCCATACGATGTTCCAGATTACGCTCTGTTTCGCCTTCTCAGCCC

HA HTLV-1 p12^{I} 3'

G**GGATCCCGGTACC**CTAGAAGAGGAAAGCCGCGG

HA HPV-16 E5 5'

GGC**GGATCC**TGCCGCCACCATGTACCCATACGATGTTCCAGATTACGCTACAAATCTTGATACTGCA

HA HPV-16 E5 3'

GGC**GGATCCGGTACC**TATTTATGTAATTAAAAAGCGTGC

The dominant negative ductin (Vma3p) construct was made in two stages. The first stage added the HA epitope tag and *Pst* 1 and *Xho* 1 restriction sites to allow directional cloning into pBluescript. The second stage mutated glutamate 137 to glycine, the final product being blunt-end ligated into pYPMA. The successive pairs of oligonucleotides are shown below, the mutated nucleotide being underlined and restriction sites in bold.

HA Vma3p 5'

GGG**CTGCAG**ATGACTGAATTGTGTCCTGTC

HA Vma3p 3'

GGG**CTCGAG**TTAAGCGTAGTCTGGAACATCGTATGGGTAACAGACAACATCTTGAGTAGCC

Vma3p E137G 5'

GATTTTGATTTTGATTTTTGCTGGAGTTTTGGGTCTATACGGTTT

Vma3p E137G 3'

AAACCGTATAGACCCAAAACTCCAGCAAAAATCAAAATCAAAATC

DNA templates for *in vitro* translation were synthesised by PCR, using oligonucleotides that included the T7 RNA polymerase promoter, to allow T7-mediated transcription, and a 'Kozak' sequence to ensure efficient (and consistent) translation of the RNA product. The T7 sites are underlined, and the Kozak sequences in bold. The HA T7 KOZAK oligonucleotide primed onto the HA-tag sequence so was also used for all of the E5 proteins except BPV-1 E5. All products were generated from pYPMA constructs so a universal 3' oligonucleotide, PMArev was designed for simplicity. This oligonucleotide was also used for sequencing the constructs.

Vma3p E137G T7 KOZAK 5'

AAATAATACGACTCACTATAGGGAGA**GCCACC**ATGACTGAATTGTGTCCTGTC

HA BPV-1 E5 T7 KOZAK 5'

AAATAATACGACTCACTATAGGGAGA**GCCACC**ATGCCAAATCTATACCCATACG

HA T7 KOZAK 5'

AAATAATACGACTCACTATAGGGAGA**GCCACC**ATGTACCCATACGATGTTCCAG

PMArev 3'

GGGAAGTAAGAGTTGAAGCC

2.2.2 PCR

PCR amplification of the E5 protein ORFs was performed using *PfuTurbo* (Stratagene), a high-fidelity polymerase, to minimise the chances of introducing errors into the PCR products. 50ng of template and 12.5pmols of each oligonucleotide were used in each 50μl reaction using the following cycles parameters.

Initial melt	94°C x 1min	
Melt	94°C x 30sec	
Anneal	55°C x 30sec	35 cycles
Extension	72°C x 1min	
Completion	72°C x 7min	

The PCR products were electrophoresed alongside low mass markers (Life Technologies) on a TAE (10mM Tris acetate pH8.0; 1mM EDTA) agarose gel at 100 volts for 30min. Bands corresponding to the correct product size were then excised and their DNA extracted using the silica-gel based 'QIAquick' gel

extraction kit (Qiagen), according to the protocol, including addition of isopropanol to aid binding to the silica matrix. 80μl of elution buffer was used in order to maximise the yield.

The 'Quickchange' kit (Stratagene) was used to generate the dominant negative form of Vma3p. A 50μl reaction was performed with 200ng of each oligonucleotide and 50ng of template using the following parameters.

Initial melt	94°C x 1min	
Melt	94°C x 30sec	
Anneal	55°C x 1min	12 cycles
Extension	68°C x 18min	

The 'Quickchange' PCR amplified the whole vector construct, so this was processed differently from the E5 ORF PCRs in that it was transformed directly into *E. coli* after removing the (unmutated) template by digestion with *Dpn* 1 (Stratagene) for 1 hour at 37°C .

2.2.3 Restriction digestion and ligation

The purified products were digested with appropriate restriction enzymes at 37°C for 2 hours, the reactions being terminated by purification using a 'QIAquick' PCR-purification kit. Only 30μl of elution buffer was used to maximise concentration of the samples and aid ligation. Vectors were prepared by restriction digestion

overnight, followed either by dephosphorylation with calf intestinal alkaline phosphatase (Life Technologies) for 30min at 37°C, or blunt end synthesis using polishing buffer and enzyme supplied with the pCRscript cloning kit (Stratagene). The PCR purification kit (Qiagen) was used to terminate the reactions and purify the DNA. The prepared PCR products were ligated to equimolar amounts of vector (as assayed by agarose gel electrophoresis), using a 'Rapid Ligation' kit (Roche).

2.2.4 *E. coli* transformation

DH5α competent cells (Life Technologies) were used for the majority of transformations. 1μl of fresh ligation product or 'Quickchange' digest was mixed with 20μl of competent cells in a 1.5ml tube at 4°C. The cells were incubated at 4°C for 30min then subjected to a 45 second heat shock at 42°C. The cells were incubated for 2min at 4°C and 100μl SOC medium (Life Technologies) was added. The cells were then spread directly onto LB agar plates (containing 0.7%w/v bacto agar; 50μg/ml ampicillin) and incubated overnight at 37°C. XL10-Gold ultracompetent cells (Stratagene) were used for transformation of constructs with low transformation efficiency, using the same protocol as above.

2.2.5 Plasmid DNA isolation and analysis

Plasmid DNA was extracted from 4ml cultures grown overnight in LB containing 200μg/ml ampicillin using the 'Qiaprep' kit (Qiagen), according to the manufacturer's protocol. This uses the alkaline lysis method to extract the

plasmid DNA, followed by purification on a silica matrix spin column. The purified DNA was digested and electrophoresed as for preparation of the PCR products. *Bam*H 1 digestion detected the presence of inserts and *Kpn* 1 was used to determine their orientation with respect to the promoter (*Kpn* 1 sites being 200bp upstream of the cloning site, and at the 3' end of the insert). Correctly assembled constructs were sequenced in both directions with external primers using the central automated sequencing facility.

2.2.6 Manipulation of *Saccharomyces cerevisiae*

2.2.6.1 Preparation of competent cells

200ml of cells were grown in complete glucose medium overnight at 30°C to an optical density of 0.6 at 600nm and harvested at 2000xg for 5min at room temperature. Cells were washed in 50ml sterile water, then in 40ml filter-sterilised SORB (1M Sorbitol; 100mM Lithium acetate; 10mM Tris-acetate pH8; 1mM EDTA.). The supernatant was removed completely, the cells resuspended in 1.5ml SORB, and 200μl carrier DNA added. (10mg/ml salmon sperm DNA, boiled for 1min, sonicated at 10μm for 1min.) The cells were stored in 20μl aliquots at -70°C.

2.2.6.2 Yeast transformation

100ng plasmid DNA was incubated with 20μl competent cells and 120μl filter-sterilised PEG (40%w/v polyethylene glycol 3350; 100mM lithium acetate; 10mM

Tris-acetate pH8; 1mM EDTA) for 30min at room temp, then 15μl DMSO was added and the cells placed at 42°C for 15min. The cells were harvested at 15,000xg for 1min, resuspended in 100μl minimal medium and spread onto minimal medium plates containing 2%w/v bacto agar.

2.2.6.3 Yeast DNA purification

The 'QIAprep' protocol was modified to allow yeast lysis by the buffers supplied in the kit. 15ml of yeast were grown to logarithmic phase in minimal medium, harvested at 3,000xg for 5min at room temperature then resuspended in 3ml digest buffer (1M sorbitol; 50mM Na_2HPO_4/NaH_2PO_4 pH7.5; 2%w/v glucose; 0.4%v/v β-mercaptoethanol; 0.5mg/ml yeast lytic enzyme). This was incubated for 30min before harvesting the spheroplasts at 2,000xg for 5min at room temperature. The plasmid DNA was isolated using the 'Qiaprep' kit as for bacterial cells, using only 30μl elution buffer to take into account the low yield obtained from yeast.

2.2.6.4 Vacuole isolation

This was performed using the method of (Uchida *et al.*, 1985). 200ml yeast cultures were grown in minimal medium overnight at 30°C to an optical density of between 0.5 and 1 at 600nm. Sugar (glucose or galactose/raffinose, see section 1.3.6.5) was added to restore the concentration to 2%(w/v) 2 hours before the cells were harvested at 5,000xg for 5min at room temperature. The cells were then washed in 20ml phosphate buffer (50mM Na_2HPO_4/NaH_2PO_4 pH7.5), using

50ml tubes to minimise loss of material during subsequent liquid transfers. Spheroplasts were prepared by resuspending in 20ml of digest buffer (1M sorbitol; 50mM Na_2HPO_4/NaH_2PO_4 pH 7.5; 2%w/v sugar source; 0.4%v/v β-mercaptoethanol; 0.5mg/ml yeast lytic enzyme) and incubating at room temperature for 1 hour. The spheroplasts were harvested at 2,000xg for 5min at room temperature and placed on ice, with all subsequent steps being carried out at 4°C. Of note is the presence of sugar for 3 hours before harvesting, which is necessary to prevent starvation-induced inactivation of the V-ATPase (Kane, 1995), section 1.3.6.5.

The spheroplasts were resuspended by vortex mixing in 6ml of floatation buffer (8%w/v Ficoll 400; 10mM MES-Tris pH6.9; 0.5mM $MgCl_2$; 0.1mM 4-(2-Aminoethyl)-benzenesulphonylfluoride, AEBSF; 1mM Benzamidine), then lysed by four strokes in a loose-fitting homogeniser. The suspension was poured into a Beckman SW40 thin-walled polyallomer tube, overlaid with floatation buffer and centrifuged at 51,900xg for 30min. The layer of vacuoles was harvested from the top of the tube with a spatula and resuspended in 1.5ml buffer C (10mM MES-Tris pH6.9; 5mM $MgCl_2$; 25mM KCl; 0.1mM AEBSF; 1mM benzamidine). The vacuoles were harvested by centrifugation at 20,000xg for 5min, resuspended to approximately 2mg/ml in buffer C and either used immediately or stored at -70°C in buffer C containing 10% glycerol (w/v)

Because of the slow growth in minimal medium, larger preparations were grown using complete medium. The 2μ-based plasmids are stable under non-selective conditions (Ludwig and Bruschi, 1991) and no effects of plasmid loss were seen (Figure 4.13).

2.2.7 Analysis of the vacuolar proton-translocating ATPase (V-ATPase)

2.2.7.1 Purification of the V-ATPase

This was performed using the method of (Uchida *et al.*, 1985), with adaptations for use on a benchtop ultracentrifuge. 1mg aliquots of vesicles were washed twice in TE (10mM Tris-Cl pH7.5; 1mM EDTA), harvesting by centrifugation at 400,000xg for 10min at 4°C. The final pellet was resuspended in the residual buffer, to which was added 50μl of 10%(w/v) N-dodecyl maltoside solution and 5μl yeast protease inhibitor cocktail (Sigma). The solution was mixed by slow pipetting, and then incubated at 4°C for 15min to solubilise the membrane proteins. Insoluble material was removed by centrifugation at 400,000xg for 10 min at 4°C and the supernatant carefully loaded onto 2ml of a 10 step density gradient (20%-50%w/v glycerol; 10mM Na-HEPES pH7.5; 50mM NaCl; 0.01%v/v yeast protease inhibitor cocktail.) Centrifugation was carried out in a TLS-55 swing-out rotor at 200,000xg for 5 hours at 4°C using the minimum acceleration and deceleration. 200μl fractions were collected into a 96-well plate by upward displacement using fluorinert (3M).

2.2.7.2 Protein estimation

This was performed using the bicinchoninic acid (BCA) method. BCA reagent (Sigma) was prepared fresh, by addition of 0.02 volumes of 5%(w/v) copper(II)sulphate to BCA solution. To this, an equal volume of 0.1%w/v SDS was added, and the mixture dispensed in 1ml aliquots. Approximately 5μg of

protein was added and the reaction mixed and incubated at 65°C for 30min. Absorbance at 562nm was measured and the protein concentration deduced from a standard curve of bovine serum albumin.

2.2.7.3 Measurement of V-ATPase activity

The rate of ATP hydrolysis was determined by measuring the amount of phosphate released in saturating concentrations of ATP. Specific activity was determined from the activity inhibited by N'N-dicyclohexylcarbodiimide (DCCD) in the presence of sodium azide (see section 4.3).

Duplicate reactions were set up, containing 200μl buffer C containing 0.02% w/v sodium azide and 5μl (≈10μg protein) of vacuoles. 5μl ethanol, with or without 5mg/ml DCCD was added to the respective sets of reactions which were briefly vortex mixed and then incubated at room temperature for 5 min. 30μl ATP (50mM in buffer C) was added to all reactions, which were then vortex mixed and incubated at 30°C for 30 min. The reaction was terminated, and the phosphate release determined by using a modification of the ammonium molybdate-based technique (Piper and Lovell, 1981). 750μl phosphate detection reagent (2%v/v sulphuric acid; 1%w/v SDS; 0.5%w/v ammonium molybdate; 0.1%w/v ascorbic acid.) was added to the reaction and the absorbance at 830nm measured after 10min. Phosphate concentrations were determine from a standard curve of Sodium phosphate.

For microplate assay of the purified V-ATPase, 50μl buffer C containing 5mM ATP and 0.02%w/v sodium azide was added to 5-30μl aliquots from the gradient fractions. The reactions were mixed, incubated at 30°C for 30min, developed using 100μl double-concentrate phosphate detection reagent, and the absorbance measured at 630nm against a standard curve of sodium phosphate. Kinetic analysis was performed in the same way, using 1-2μg vacuoles per well. For N-ethyl maleimide (NEM) sensitive ATPase activity determination, NEM was added to 100μM for 5min before addition of ATP.

2.2.8 *In vitro* binding assays

2.2.8.1 Capped RNA synthesis

The 'T7 mMessage mMachine' kit (Ambion) was used according to the manufacturer's protocol, doubling the reaction size to increase yield. The RNA was precipitated using the lithium chloride method, concentration estimated by agarose gel electrophoresis, and adjusted to 0.5mg/ml. For shorter transcripts (all of the E5 protein constructs) the T7 'MEGAshortscript' kit (Ambion) was used instead, following the manufacturer's protocol but including the cap analogue, (m^7G(5')ppp(5')G) (Ambion) and modifying the size of the reaction as above.

2.2.8.2 *In-vitro* translation

Rabbit reticulocyte lysate (Promega) was used to synthesise proteins in the presence of canine pancreatic microsomes (Promega), to allow signal-mediated

translation of the proteins (Dunlop *et al.*, 1995) and allow their primary purification by centrifugation. ^{35}S-Cysteine and ^{35}S-methionine (Amersham) were included to allow radiological detection. The reactions were carried out according to the manufacturer's protocol, using a total of 1μg RNA per reaction. Microsomes were collected by centrifugation at 400,000xg for 10min and resuspended in 400μl radoimmuniprecipitation (RIPA) buffer (20mM Na-MOPS pH7.5; 150mM NaCl; 10mM EDTA; 5mg/ml Triton X-100; 5mg/ml sodium deoxycholate; 1mg/ml SDS).

2.2.8.3 Immunoprecipitation

Five microlitres of anti-HA1 (E5 proteins) and anti-AU1 (ductin) antibodies were added to respective 200μl aliquots of the solubilised microsomes, to which a further 200μl radioimmunoprecipitation assay (RIPA) buffer was added. The mixture was then placed on a rotary mixer overnight at 4°C. 10μl protein D-sepharose was added and mixing continued for 1 hour at room temperature before harvesting the sepharose by centrifugation at 20,000xg for 5min. The samples were washed three times in 500μl RIPA, the final wash including incubation on a rotary mixer for 15min. The final pellet was resuspended in 10μl 2xSDS loading buffer (100mM Tris-Cl pH6.8; 10%w/v glycerol; 5%w/v SDS; 0.2%w/v bromophenol blue), which was electrophoresed on a 15% Tris-tricine polyacrylamide gel to optimise the resolution of the small proteins. The gel was fixed in 50%v/v methanol, 10%v/v acetic acid for 1 hour, dried onto Whatman paper and used to expose film (Fuji super RX) for approximately one week.

2.2.9 Protein analysis

2.2.9.1 Protein precipitation

Acetone:ethanol was chosen because it delipidated the sample and so improved the migration properties on SDS-PAGE. Four volumes of acetone:ethanol (1:1) were added to the protein sample, which was vortex-mixed and incubated at -20°C for 30min. The protein was collected by centrifugation at 20,000xg for 30min, and the supernatent removed completely. The sample was resuspended at 37°C in SDS loading buffer (50mM Tris-Cl pH 6.8; 5%w/v glycerol; 2.5%w/v SDS; 0.1%w/v bromophenol blue).

2.2.9.2 Tris-glycine SDS-PAGE

The larger V-ATPase subunits, Vma1p and Vph1p were separated using 10% Tris glycine polyacrylamide gels, prepared according to the method of (Laemmli, 1970). All protein electrophoresis was carried out using the NOVEX mini-gel system (Invitrogen), which allowed rapid processing without loss of resolution. Pre-stained markers (Amersham) were used to estimate the mass of the proteins in the gel.

2.2.9.3 Tris-tricine SDS-PAGE

All other protein separations by SDS-PAGE were carried out according to the method of (Schagger and von Jagow, 1987). Substituting glycine with tricine

improved the separation of smaller proteins from the SDS in the loading buffer, increasing their resolution during electrophoresis. 15% gels were prepared according to the protocol, including 10%(w/v) glycerol in the separating gel to allow direct overlaying of the 4% stacking gel. The gel contained high concentrations of Tris, which diffused into the wells and prevented samples from sinking completely, so the sample wells were thoroughly rinsed with cathode buffer (0.1M Tris; 0.1M tricine; 0.1%w/v SDS) before sample application.

2.2.9.4 Western blotting

Proteins separated by SDS-PAGE were transferred onto Hybond nitrocellulose membrane (Amersham) using a semi-dry blotting apparatus. The apparatus was assembled with the gel placed against the nitrocellulose membrane and sandwiched between stacks of six sheets of Whatman paper soaked in transfer buffer (100mM Tris; 100mM glycine; 0.1%w/v SDS), with 20%(v/v) methanol being included for Vma1p and Vph1p. A glass pipette was rolled over the surface to remove any air bubbles, and a current of 1mA/cm^2 was applied for 1 hour (2 hours for Vph1p). The efficacy of protein transfer was verified by staining in Ponceau S (Sigma) for 10min followed by 4x1min rinses in water.

The membrane was transferred to block solution (10mM Tris-Cl pH 7.5; 50mM NaCl; 0.01%w/v TWEEN; 10%w/v skimmed milk powder, Marvel) for 30min before rinsing in TBST (10mM Tris-Cl pH 7.5; 50mM NaCl; 0.01%w/v TWEEN) and incubating overnight at 4°C in primary antibody diluted 1000-fold in TBST containing 0.1% (w/v) Marvel. The membrane was rinsed four times in TBST and incubated for 1 hour in horseradish peroxidase-linked secondary antibody

(Amersham), diluted 5000-fold in TBST containing 0.1% (w/v) Marvel. To remove non-specifically bound antibody the membrane was rinsed twice in TBST, then four times for 10min. The membrane was then incubated in chemiluminescent detection solution (Amersham) for 1min, drained, and exposed to film (Kodak super RX) for 5 seconds to 1 hour, depending upon the intensity of the signal.

2.2.9.5 Dot blotting

To aid binding to the membrane, 10μg aliquots of vacuolar protein samples were precipitated in acetone:ethanol as described previously, then taken up in SDS loading buffer and spotted directly onto nitrocellulose membrane. The samples were allowed to dry completely before blocking and processing as for Western blots.

3. Results. Generation of epitope-tagged E5 protein pYPMA constructs: binding to yeast ductin and correct expression in *S. cerevisiae*

3.1 Introduction

As explained in chapter 1, *Saccharomyces cerevisiae* is a convenient model organism to use for studying the effects of the E5 proteins. The homology between *S. cerevisiae* and mammalian forms of ductin, and the demonstration of BPV-1 E5 binding to the *S. pombe* form (Goldstein *et al.*, 1992c) suggest that the E5 proteins will bind to the *S. cerevisiae* form. The similarities between mammalian and yeast protein expression and targeting mechanisms would also suggest the correct expression of the E5 proteins in transformed yeast strains.

To confirm these assumptions, HA-tagged E5 constructs were generated by PCR and cloned into the yeast expression vector, pYPMA. The binding of the products from these constructs to *S. cerevisiae* ductin was verified by co-translation on microsomes *in vitro*, followed by immunoprecipitation. The stability and integrity of the constructs *in vivo* was confirmed by PCR, and correct protein expression by western blotting and dot blotting.

3.2 PCR generation of epitope-tagged E5 protein constructs and demonstration of binding to *Saccharomyces cerevisiae* ductin *in vitro*

The expression vector for the E5 proteins was pYPMA (Figure 3.1 A); a derivative of the pYES2 vector (Invitrogen), which was constructed by replacing the GAL1 promoter construct of pYES2 with that of the yeast plasma membrane ATPase; *PMA1* (Villalba *et al.*, 1992). Other features of the vector are a URA3 gene, which allows for transformant selection by uracil omission from minimal medium, and a 2μ replication origin to maintain the plasmid at a high copy number and maximise expression levels. A pMB1 replication origin and a β-lactamase ampicillin resistance gene are present to allow propagation in *E. coli.* This vector was chosen because it had previously been shown to drive particularly high levels of integral membrane proteins in yeast (Jones *et al.*, 1995), and so would maximise any effects of the E5 proteins.

In addition to investigating the wild type E5 proteins, three mutant forms of BPV-4 E8 were included, which all bind to ductin but differ in their transforming capacities and their ability to disrupt one activity associated with ductin: gap junctional intercellular communication (GJIC) (Ashrafi *et al.*, 2000; O'Brien *et al.*, 1999). Wild type BPV-4 E8 induces anchorage-independent growth and reduces contact inhibition and GJIC in primary bovine fibroblasts. The N17S mutant confers anchorage independent growth, as seen for wild type E8, but does not inhibit communication or cause cells to overcome contact inhibition. The N17A mutant is a hypertransforming form that has all the properties of wild type E8, but induces higher levels of anchorage independent growth. The truncated E8 mutant (BPV-4

E8T) lacks the hydrophilic carboxy terminus of the protein and does not cause transformation or inhibit communication.

A positive control for V-ATPase inhibition was also included, a dominant negative form of ductin with the active glutamic acid mutated to glycine. Expression of this type of mutant has been shown to disrupt the V-ATPase in yeast, (Hughes *et al.*, 1996) and also to cause cell transformation (Andresson *et al.*, 1995).

The ORFs for the E5 proteins were available in mammalian expression vectors, and the HA-tagged BPV-4 E8 constructs were cloned from laboratory stocks into pYPMA by Joan Grindlay (Beatson Institute for Cancer research, Glasgow, UK). In order to add the HA epitope tag, and the *Bam*H 1 and *Kpn* 1 restriction sites for cloning and analysis, PCR was performed upon the ORFs using oligonucleotides containing these motifs. A high fidelity DNA polymerase (Pfu, Stratagene) was used to minimise the introduction of errors during synthesis. The *Bam*H 1 sites allowed direct ligation into pYPMA, and the *Kpn* 1 site made it possible to exploit the upstream *Kpn* 1 site in the vector and screen for correct orientation by restriction digestion; the site being placed at the 3' oligonucleotide, so digests yielding excised fragments of higher mass indicating the correct orientation. This screen was also useful for detecting insertion of concatamers which gave more than one species of excised DNA. Oligonucleotides specific for pYPMA, directed against sequences adjacent to the cloning site were used to sequence the constructs, the results being shown in Figure 3.1 B.

The sequencing data confirm the sequence integrity of the ORF PCR products, and their correct ligation into pYPMA. Despite use of a high fidelity polymerase,

errors did occur in HTLV-1 $p12^{I}$ and in BPV-4 E8 N17A. However, the $p12^{I}$ protein sequence was unaffected, and the difference caused by mutation of a leucine to a valine in the E8 construct was decided to be too small to be of any consequence; both residues have very similar size, shape and hydrophobicity, differing only by one methyl group.

The dominant negative ductin construct (Vma3p E137G) was constructed such that it and the unmutated form could also be ligated into other yeast expression vectors based on pBLUEscript (Stratagene). To this end, the HA tag, and *Pst* 1 and *Xho* 1 restriction sites were added to *S. cerevisiae* ductin (VMA3) by PCR, with the product being cloned into pYPMA by blunt end ligation and sequenced as before. The correctly oriented construct was then used as a template for site directed mutagenesis by PCR, using the Quickchange kit (Stratagene). A final round of sequencing was performed to confirm the mutation and check sequence integrity, shown in Figure 3.1.

To verify binding between the E5 proteins and *S. cerevisiae* ductin, both were translated in the presence of microsomes *in vitro* and analysed by radioimmunoprecipitation. DNA templates were generated by PCR using oligonucleotides containing identical T7 bacteriophage RNA polymerase recognition sites, and 'Kozak' mammalian ribosome docking sequences to encourage similar levels of transcription and translation. RNA transcripts were generated by the T7 mMessage mMachine kit (Ambion) and translated in the presence of microsomes. These endoplasmic reticulum (ER) derived vesicles are required for signal-recognition particle (SRP) dependent translation of ductin and the E5 proteins (Dunlop *et al.*, 1995; Faccini *et al.*, 1996). They also permit

interactions to occur in a membranous environment, and allow primary purification of membrane proteins from the reaction by centrifugation. ^{35}S-labelled methionine and cysteine were included to allow the translation products to be tracked by autoradiography. The proteins contained different proportions of sulphydryl residues so the RNA levels were adjusted to minimise the differences in the amount of labelling and so aid visual analysis of autoradiographs.

Before testing the binding capacities of the proteins, it was necessary to check the ability of the system to correctly translate and detect them. To this end, single translations were carried out and the proteins precipitated with both types of antibodies. The results of SDS-PAGE and autoradiography of the samples are shown in Figure 3.2 A. The precipitation of the proteins by antibodies to their epitope tags confirms the specificity of the antibodies and also confirms the correct translation of the epitope tags on the proteins. The correct size of the proteins and their presence in the microsomal membranes suggests that all of the proteins were correctly translated *in vitro*. HTLV-1 p12^{I} seems to have been affected by a gel artefact, but this is resolved in subsequent experiments (Figure 3.2 B).

Co-translations were carried out to test for interactions between the E5 proteins and Vma3p, with RNA levels adjusted to give similar levels of ^{35}S incorporation for the different proteins. Translation of the E5 proteins in the presence of Vma3p followed by immunoprecipitation and SDS-PAGE (Figure 3.2 B) demonstrates interaction between the two proteins. A control was attempted, using PPA1 which has previously been shown not to bind to the E5 proteins (Faccini *et al.*, 1996) but correct translation could not be achieved so the validity of the results was inferred

from the fact that the experimental conditions were identical to those previously used to show specific interactions between *Nephrops* ductin and E5 proteins (Faccini *et al.*, 1996), (I. Rodriguez, manuscript in preparation).

The E5 proteins of BPV-1 and HPV-16 co-precipitate with Vma3p irrespective of which protein the antibody is directed against. The same also occurs for HTLV-1 p12^{I}, suggesting that these three proteins bind to *S. cerevisiae* ductin. BPV-4 E8 is only detected when precipitated via anti-AU1 in co-translations, consistent with published results (Faccini *et al.*, 1996). This could represent a weaker association, or could be due to poor translation when having to compete with the Vma3p template in the co-translation reaction. However, the mutant forms of BPV-4 E8 all bind to *S. cerevisiae* ductin to a similar level. They have also been shown to bind to *Nephrops* ductin in a similar manner (Ashrafi *et al.*, 2000). Because the truncated mutant has almost no hydrophilic domains, it demonstrates its interaction with ductin is most likely mediated by its transmembrane domain, consistent with results published for BPV-4 E8 and BPV-1 E5 (sections 1.1.5.1 and 1.1.5.3) as well as unpublished data on HPV-16 E5 (I. Rodriguez, manuscript in preparation).

The significance of the varied intensities of the proteins is not known. It may be due to differences in the levels of translation, possibly as a result of competition from the Vma3p template. However it is unlikely to explain the differences between the E8 constructs, which are almost identical in sequence so their varied intensities may represent differences in binding affinity to ductin.

In summary, all of the E5 proteins co-immunoprecipitated with Vma3p, showing for the first time that they are capable of binding to *S. cerevisiae* ductin and verifying its use as a model to study the effects of these proteins on the V-ATPase.

3.3 Viral E5 proteins bearing amino terminal epitope tags are expressed in *S. cerevisiae* and are found in the vacuolar membrane

The E5 proteins have been shown to bind to *S. cerevisiae* ductin, so the effects of their expression *in vivo* can now be examined in the light of early indications of V-ATPase inhibition, found by Meagher and Finbow (Figure 6.2). To this end, the YPH 500 strain was used since it has been used in other studies on the V-ATPase (Umemoto *et al.*, 1991) and it is auxotrophic for uracil, allowing pYPMA maintenance by nutrient selection. The *ade2* genotype also causes accumulation of a red pigment in the vacuole (Ugolini and Bruschi, 1996) which is dependent upon V-ATPase activity (Figure 4.3), providing another method of screening for disruption of the enzyme. The haploid strain was transformed with pYPMA constructs containing the HA-tagged E5 protein ORFs, the HA-tagged dominant negative ductin construct, as a positive control, and the empty pYPMA vector as a negative control. Transformants were selected by growth on medium lacking uracil, then tested for their ability to maintain the pYPMA constructs, and correctly express and target the proteins.

To check the integrity of the ORFs in pYPMA, PCR analysis was used to determine if the plasmids were maintained in the transformants. Plasmid DNA was isolated from equal amounts of yeast grown in minimal medium, and PCR performed using oligonucleotides specific for the insert and the vector; the 5'

oligonucleotides being directed against the PMA1 promoter sequence, and the 3' oligonucleotide against the 3' end of each of the inserts. The 'no template' control reaction contained all of the oligonucleotides to check for contamination.

Figure 3.3 shows the result of agarose gel electrophoresis of the products from the reactions. The products were of the expected size, confirming the presence of correctly oriented E5 protein ORFs in pYPMA in the transformed yeast strains. The BPV-4 E8 constructs should have migrated to the same position as BPV-1 E5, but because they were ligated into pYPMA as fragments excised from another vector it is possible that upstream sequence from their original vector was incorporated, increasing their apparent size. The small product seen in the empty vector lane was expected since the oligonucleotides in this reaction were 20 nucleotides in length and directed against sequences 40bp apart.

Because codon preferences vary between different organisms, this may effect the translation of the E5 proteins oustide their natural hosts. None of the E5 proteins are naturally expressed in yeast and although the presence of the desired plasmid constructs suggests expression of the protein, the only way to be certain is to detect the proteins directly. BPV-1 E5 and HPV-16 E5 have been detected previously by western blotting (Burkhardt *et al.*, 1989; Hwang *et al.*, 1995), but $p12^{I}$ and E8 have not.

Because the V-ATPase is predominantly found in the yeast vacuole, immunoblotting was carried out on purified vacuolar membranes, the results being shown in Figure 3.4 A. The dominant negative form of ductin and HPV-16 E5 were detected by western blotting, their molecular masses corresponding with

those expected, and those seen in *in vitro* translation. This evidence for correct expression and vacuolar targeting of the proteins *in vivo* is reinforced by the observation of higher order species, which have been seen in other preparations of these proteins (Burkhardt *et al*., 1989; Hwang *et al*., 1995).

The reasons why the other E5 proteins were not detected are not known. It may be that their small size may have caused them to pass through nitrocellulose during electroblotting, but molecular markers were visible at the expected size interval, and reducing the membrane pore size and the transfer voltage made no difference, as did increasing the methanol concentrations. Using different buffer systems at all stages of the protocol also had no effect (positive or negative) so the problems could be pleiotropic, perhaps due to the combination of small size and high hydrophobicity. It could also be due to inaccessibility of the epitope tag. The N-termini of the proteins only remain hydrophilic for a few residues, and the hydrophobic region may repel antibody molecules. Possible solutions might be to attach multiple tags or to use a larger tag, or a hydrophilic 'linker' peptide to distance the epitope from the protein. Detection of BPV-4 E8 by western blotting and immunofluorescence has been performed using Green Fluorescent Protein (GFP) as an epitope tag (M. Zago, personal communication), but it is not known whether this affects the transforming potential of the protein.

An alternative strategy was tried to circumvent this problem. 10μg aliquots of vacuolar protein were precipitated in acetone:ethanol, solubilised in SDS loading buffer and spotted directly onto the nitrocellulose membrane, the results of immunodetection being shown in Figure 3.4 B. With this approach, the dominant negative ductin and HPV-16 E5 gave the strongest signal, corresponding to their

being detectable by western blotting, however, the relative intensities were not the same as those seen in western blotting, so it seems that either electrophoresis and blotting changes the affinity of the proteins for the antibody (possibly by altering the accessibility of the epitope tags), or that some of the protein is lost during concentration. The latter possibility is supported by the variations in reactivity seen for the E8 proteins, which would have been expected to react equally given their sequence similarity.

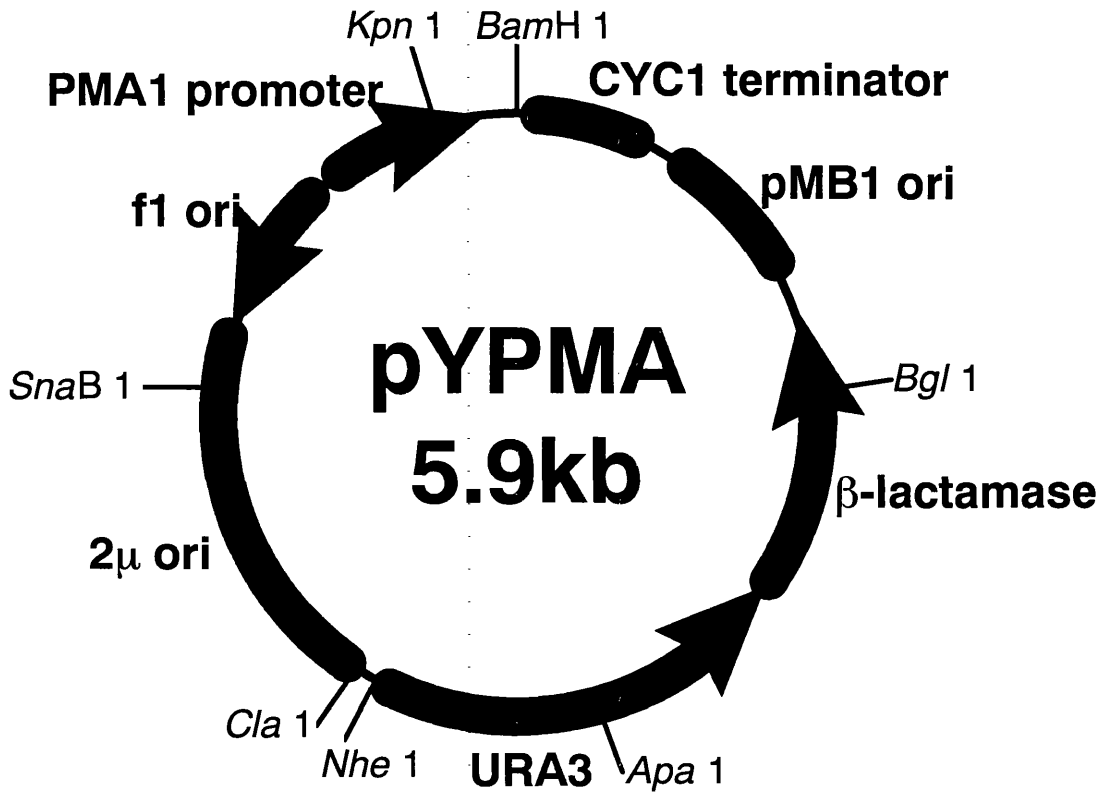
The *in vitro* translation experiments have shown that the E5 proteins bind to the *S. cerevisiae* form of ductin, and western blotting confirmed the correct expression of the dominant negative form of ductin and HPV-16 E5 *in vivo*. Although that of the other E5 proteins could not be verified directly, their correct expression was inferred from sequencing data and PCR analysis showing the integrity of the sequences and plasmid maintenance *in vivo*, and dot blot analysis of vacuolar protein showing expression of HA-tagged proteins in the vacuole.

Figure 3.1 pYPMA vector map and sequences of the E5 protein constructs

A: pYPMA contains a pMB1 replication origin and ampicillin resistance gene to allow propagation in bacteria, the 2μ origin and URA3 gene to allow maintenance of high copy number in yeast using uracil deficient medium, a PMA1 promoter to drive high levels of transcription, a BamH *1 cloning site and a* Kpn *1 site to enable directional analysis by restriction digestion.*

B: The E5 protein and dominant negative ductin constructs were sequenced in both directions to verify their integrity and orientation. Vector sequences are underlined, mutations and the haemagglutinin epitope tag being shown in red. HTLV-1 p12^{I} contained a point mutation which did not affect the protein sequence. The BPV-4 E8 N17A mutant contained a point mutation which converted a leucine to a valine.

A


Kpn 1
BamH 1
PMA1 promoter
CYC1 terminator
pMB1 ori
f1 ori
pYPMA
5.9kb
SnaB 1
Bgl 1
β-lactamase
2μ ori
Cla 1
Nhe 1
URA3
Apa 1

HA-tagged VMA3 E137G

```
Forward GACGGCATCGATCCCCCGGGCTGCAGATGACTGAATTGTGTCCTGTCTACGCCCCTTTCTTTGGTGCCATTGGTTGTGCCTCTGCAATTATCTTCACCTCATTAGGTGCTGCTTACGGTACTGCTAAGTCTGGTGTT
                                  M  T  E  L  C  P  V  Y  A  P  F  F  G  A  I  G  C  A  S  A  I  I  F  T  S  L  G  A  A  Y  G  T  A  K  S  G  V

GGTATCTGTGCCACTTGTGTGTTGAGACCAGACCTATTATTCAAGAACATTGTTCCTGTTATTATGGCTGGTATCATTGCCATTTACGGTTTAGTTGTTTCCGCTTTGGTTTGTTATTCGTTGGGTCAAAAGCAAGCTTTGTACA
G  I  C  A  T  C  V  L  R  P  D  L  L  F  K  N  I  V  P  V  I  M  A  G  I  I  A  I  Y  G  L  V  V  S  V  L  V  C  Y  S  L  G  Q  K  Q  A  L  Y  T

CCGGTTTCATCCAATTGGGTGCCGGTCTATCAGTCGGTTTGAGTGGTCTAGCTGCTGGTTTCGCTATTGGTATTGTCGGTGATGCAGGTGTTAGAGGTTCCTCTCAACAA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CCGGTTTCATCCAATTGGGTGCCGGTCTATCAGTCGGTTTGAGTGGTCTAGCTGCTGGTTTCGCTATTGGTATTGTCGGTGATGCAGGTGTTAGAGGTTCCTCTCAACAACCAAGATTATTCGTCGGTATGATTTTGATTTTGAT
  G  F  I  Q  L  G  A  G  L  S  V  G  L  S  G  L  A  A  G  F  A  I  G  I  V  G  D  A  G  V  R  G  S  S  Q  Q  P  R  L  F  V  G  M  I  L  I  L  I

                                                                                                                                                ACCCATACGATGTTCCAGATTACGCTTAACTCGAGATTCCCTCNAGGGAAATGGCATTCTAATA

TTTTGCTGGAGTTTTGGGTCTATACGGTTTGATTGTTGCTTTGTTGTTGAACTCCAGGGCTACTCAAGATGTTGTCTGTT
 F  A  G  V  L  G  L  Y  G  L  I  V  A  L  L  L  N  S  R  A  T  Q  D  V  V  C  Y  P  Y  D  V  Y  D  Y  A  *
```

HA-tagged BPV-1 E5

```
Forward       CTCGACGGGATCCATGCCAAATCTATACCCATACGATGTTCCAGATTACGCTAGCTTGAATCTATGGTTTCTATTGTTCTTGGGACTAGTTGCTGCAATGCAACTGCTGCTATTACTGTTCTTACTCTTGTT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AATATACCCCAGCCTCGACGGGATCCATGCCAAATCTATACCCATACGATGTTCCAGATTACGCTAGCTTGAATCTATGGTTTCTATTGTTCTTGGGACTAGTTGCTGCAATGCAACTGCTGCTATTACTGTTCTTACTCTTGTT
                        M  P  N  L  Y  P  Y  D  V  P  D  Y  A  S  L  N  L  W  F  L  L  F  L  G  L  V  A  A  M  Q  V  L  L  L  L  F  L  L  L  F

TTTTCTTGTATACTGGGATCATTTTGAGTGCTCCTGTACAGGTCTGCCCTTTTAAGGTACCGGATCCCGTCGAGGGAA
||||||||||||||||||||||||||||||||||||||||
TTTTCTTGTATACTGGGATCATTTTGAGTGCTCCTGTAC
 F  L  V  Y  W  D  H  F  E  C  S  C  T  G  L  P  F  *
```

HA-tagged HTLV-1 p12[I]

```
Forward                                                                                                                                                GCTTTCTCCGGGCGAAGTCAGCGGCCTTCTTCTCCGCCCGCTTCCTGCGCCGTG
                                                                                                                                                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CCATGTACCCATACGATGTTCCAGATTACGCTCTGTTTCGCCTTCTCAGCCCCTTGTCTCCACTTGCGCTCACGGCGCTCCTGCTCTTCCTGCTTTCTCCGGGCGAAGTCAGCGGCCTTCTTCTCCGCCCGCTTCCTGCGCCGTG
  M  Y  P  Y  D  V  P  D  Y  A  L  F  R  L  L  S  P  L  S  P  L  A  L  T  A  L  L  L  F  L  L  S  P  G  E  V  S  G  L  L  L  R  P  L  P  A  P  C

CCTTCTCCTCTTCCTTCCTTTTCAAATACTCAGCAATCTGCTTTTCCTCCTCTTTCTCCCGCTCTTTTTTTCGCTTCCTCTTCTCCTCAGCCCGTCGCTGCCGATCACGATGCGTTTCCCCGCGAGGTGGCGCTTTCCCCCCTGG
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CCTTCTCCTCTTCCTTCCTTTTCAAATACTCAGCAATCTGCTTTTCCTCCTCTTTCTCCCGCTCTTTTTTTCGCTTCCTCTTCTCCTCAGCCCGTCGCTGCCGATCACGATGCGTTTCCCCGCGAGGTGGCGCTTTCCCCCCTGG
 L  L  L  F  L  P  F  Q  I  L  S  N  L  L  F  L  L  F  L  P  L  F  F  S  L  P  L  L  L  S  P  S  L  P  I  T  M  R  F  P  A  R  W  R  F  P  P  W

AGGGCCCCGTCGCAGCCGGCCGCGGCTGACACCTATCGCTATATAGCCGCGGCTTTTCTCTTCTAGGGTAC
|||||||||||||||||||||||||||||||||||||||||||
AGGGCCCCGTCGCAGCCGGCCGCGGCTGACACCTATCGCTATAT
R  A  P  S  Q  P  A  A  A  D  T  Y  R  Y  I  A  A  A  F  L  F  *
```

B

HA-tagged HPV-16 E5

```
Forward CCCCAGCCTCGACGGATCCGCCGCCACCATGTACCCATACGATGTTCCAGATTACGCTACAAATCTTGATACTGCATCCACAACATTACTGGCGTGCTTTTTGCTTTGCTTTTGTGTGCTTTTGTGTGTCTGCCTAT
                                M  Y  P  Y  D  V  P  D  Y  A  T  N  L  D  T  A  S  T  T  L  L  A  C  F  L  L  C  F  C  V  L  L  C  V  C  L  L

TAATACGTCCGCTGCTTTTGTCTGTGTCTACATACACATCATTAATAATATTGGTATTACTATTGTGGATAACAGCAGCCTCTGCGTTTA
      TGCTTTTGTCTGTGTCTACATACACATCATTAATAATATTGGTATTACTATTGTGGATAACAGCAGCCTCTGCGTTTAGGTGTTTTATTGTATATATTATATTTGTTTATATACCATTATTTTTAATACATAC
 I  R  P  L  L  L  S  V  S  T  Y  T  S  L  I  I  L  V  L  L  L  W  I  T  A  A  S  A  F  R  C  F  I  V  Y  I  V  F  V  Y  I  P  L  F  L  I  H  T

ACATGCACGCTTTTTAATTACATAAATAGGTACCGGATCCCGTCGAGGGAATTGTCATTCTAATATTTTATCCA
 H  A  R  F  L  I  T  *
```

HA-tagged BPV-4 E8

```
Reverse ATACCCCAGCCTCGACGGATCCGAGACATGTACCCATACGATGTTCCAGATTACGCTAGCTTGATCATGTCTTTGTGGCTTATCTATGTTTTGTTGCTTTTCTGGTGTGCTTTTAATTTTCTTGCACTGTTATTTGC
                               M  Y  P  Y  D  V  P  D  Y  A  S  L  I  M  S  L  W  L  I  Y  V  L  L  L  F  W  C  A  F  N  F  L  A  L  L  F  A

                                                                                                        TTTTGCTATATATAGTGGAGATTATGCGTTTGGTGTTATTGCT
AATTATTGTGTACCTTCTGTTAATTTCTACTATTACTCGGTTAGATGGATGGGATTGAATTTTTATTTATTGTGTTTATTATTTGCTTGATTTCTGATTTGCTTTTGCTATATATAGTGGAGATTATGCGTTTGGTGTTATTGCT
I  I  V  Y  L  L  L  I  S  T  I  T  R  L  D  G  W  D  *

GTCTTTGCTAGATGAATTACAACAGGAGGAACAGTAAATAGAATCGGATCCCGTCGAGGGAATTGTCATTCTAATAT
```

HA-tagged BPV-4 E8 N17S

```
Reverse ACCCCAGCCTCGACGGATCCGAGACATGTACCCATACGATGTTCCAGATTACGCTAGCTTGATCATGTCTTTGTGGCTTATCTATGTTTTGTTGCTTTTCTGGTGTGCTTTTAGTTTTCTTGCACTGTTATTTGCAA
                             M  Y  P  Y  D  V  P  D  Y  A  S  L  I  M  S  L  W  L  I  Y  V  L  L  L  F  W  C  A  F  S  F  L  A  L  L  F  A  I

  ATTGTGTACCTTCTGTTAATTTCTACTATTACTCGGTTAGATGGATGGGATTGAATTTTTATTTATTGTGTTTATTATTTGCTTGATTTCTGATTTGCTTTTGCTATATATAGTGGAGATTATGCGTTTGGTGTTATTGCTGT
TTATTGTGTACCTTCTGTTAATTTCTACTATTACTCGGTTAGATGGATGGGATTGAATTTTTATTTATTGTGTTTATTATTTGCTTGATTTCTGATTTGCTTTTGCTATATATAGTGGAGATTATGCGTTTGGTGTTATTGCTGT
  I  V  Y  L  L  L  I  S  T  I  T  R  L  D  G  W  D  *

CTTTGCTAGATGAATTACAACAGGAGGAACAGTAAATAGAATCGGATCCCGTCGAGGGAATTGTCATTCTAATATTTT
```

B

HA-tagged BPV-4 E8 N17A

```
Forward                          GCAGATTACGCTAGCTTGATCATGTCTGTGTGGCTTATCTATGTTTTGTTGCTTTTCTGGTGTGCTTTTGCTTTTCTTGCACTGTTATTTGCAATTATTGTGTACCTTCTGTTAAT
                                 ||||||||||||||||||||||||||||||||||                                                                ||||||||||||||||||||||||||||||||
GGATNNGAGACATGTACCCATNCGATGTTCCAGATTACGCTAGCTTGATCATGTCTGTGT                                                                        ATTTGCAATTATTGTGTACCTTCTGTTAAT
          M  Y  P  Y  D  V  P  D  Y  A  S  L  I  M  S  V  W  L  I  Y  V  L  L  L  F  W  C  A  F  A  F  L  A  L  L  F  A  I  I  V  Y  L  L  L  I

TTCTACTATTACTCGGTTAGATGGATGGGATTGAATTTTTATTTATTGTGTTTATTATTTGCTTGATTTCTGGTGTGCTTTTGCTATATATAGTGGAGATTATGCGTTTGGTGTTATTGCTGTCTTTGCTAGATGAATTAGAACA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TTCTACTATTACTCGGTTAGATGGATGGGATTGAATTTTTATTTATTGTGTTTATTATTTGCTTGATTTCTGGTGTGCTTTTGCTATATATAGTGGAGATTATGCGTTTGGTGTTATTGCTGTCTTTGCTAGATGAATTAG
 S  T  I  T  R  L  D  G  W  D  *

GGAGGAACAGTAAATAGAATCGGATCCCGTCGAGGGAATTG
```

HA-tagged BPV-4 E8T

```
Reverse AGCCTCGACGGATCCGAGACATGTACCCATACGATGTTCCAGATTACGCTAGCTTGATCATGTCTTTGTGGCTTATCTATGTTTTGTTGCTTTTCTGGTGTGCTTTTAATTTTCTTGCACTGTTATTTGCAATTATT
                                 M  Y  P  Y  D  V  P  D  Y  A  S  L  I  M  S  L  W  L  I  Y  V  L  L  L  F  W  C  A  F  N  F  L  A  L  L  F  A  I  I

GTGTACCTTCTGTTAATTTGAACTATTACTCGGTTAGATGGATGGGATTGAATTTTTATTTATTGTGTTTATTATTTGCTTGATTTCTGATTTGCTTTTGCTATATATAGTGGAGATTATGCGTTTGGTGTTATTGCTGTCTTTG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
              TAATTTGAACTATTACTCGGTTAGATGGATGGGATTGAATTTTTATTTATTGTG
V  Y  L  L  L  I  *

CTAGATGAATTAGAACAGAAGGAACAGTANNATAGAATCGGATCCCGTCGAGGGAATTGTCATT
```

B

Figure 3.2 *In vitro* E5 protein binding assays

To assess if the E5 proteins bound to the S. cerevisiae *form of ductin (Vma3p), the proteins were translated* in vitro *in the presence of microsomes and* ^{35}*S-labelled methionine and cysteine. The microsomes were collected by centrifugation, solubilised in RIPA buffer, split into two equal volumes and incubated overnight at 4°C with antibodies to the AU1 and HA1 epitopes, which bind to the epitope-tagged Vma3p and E5 proteins respectively. The bound proteins were precipitated by incubation with sepharose-linked protein G for one hour, washed three times in RIPA buffer, taken up in sample loading buffer and run on 15% Tris-tricine gels. These were fixed in 50% methanol 10% acetic acid for one hour, vacuum dried onto Whatman paper and exposed to autoradiographic film for 2-20 days. The single translation gels (A) verify the correct translation of the proteins and specificity of the antibodies to their respective epitopes. In co-translations (B), BPV-4 E8 and its truncated mutant are harder to detect by HA1 precipitation than in single translations, which may be due to competition by the Vma3p template. All of the E5 proteins co-immunoprecipitated with Vma3p, demonstrating binding* in vitro.

A
Single translations
Molecular mass (kDa)
23
17
13
7
4
AU1 (ductin)
HA1 (E5 proteins)
Vma3p
BPV-1 E5
HLTV-1 p12I
HPV-16 E5
BPV-4 E8
BPV-4 E8 N17S
BPV-4 E8 N17A
BPV-4 E8T
B
Vma3p co-translations

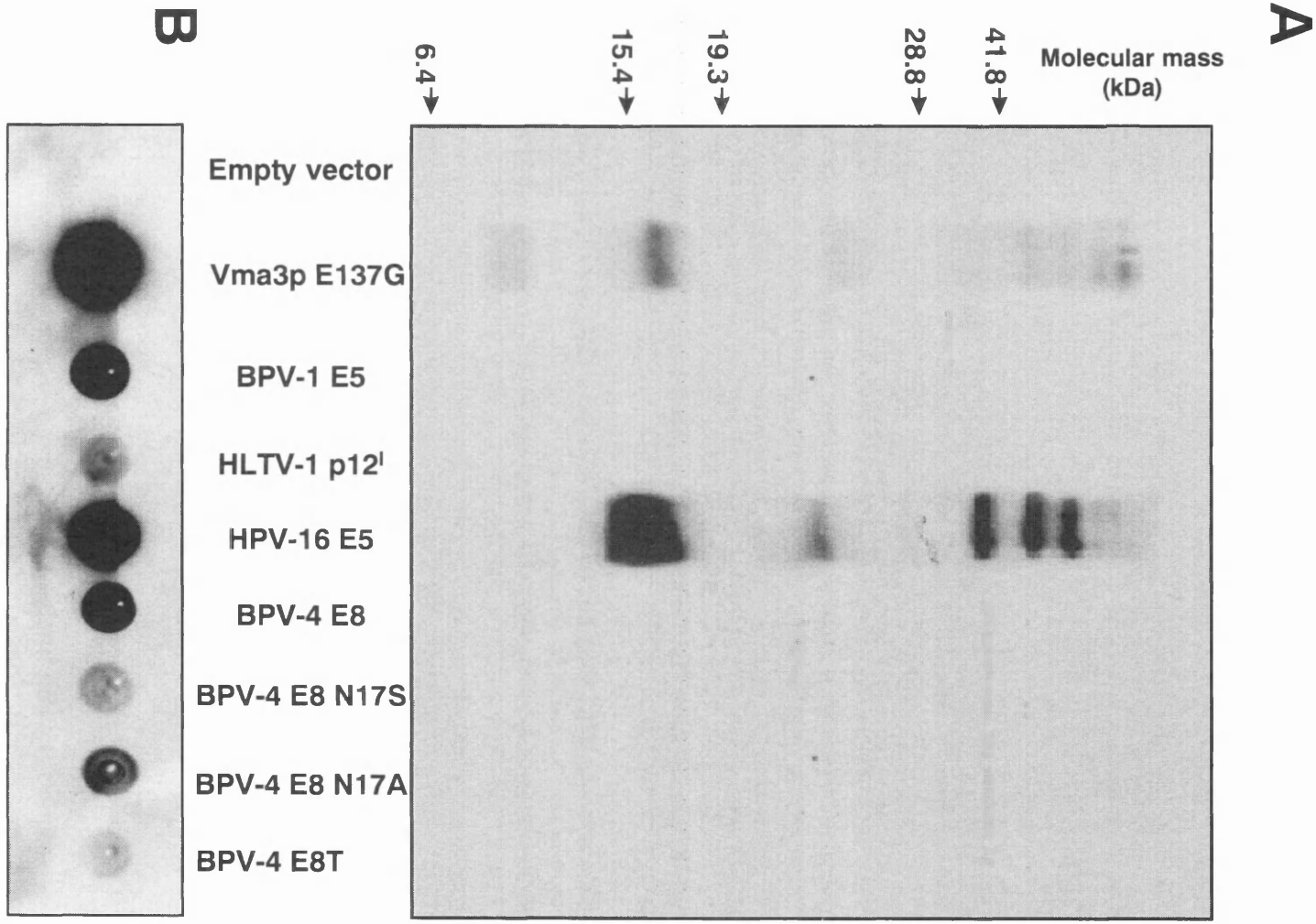
Figure 3.3 PCR analysis of plasmid DNA isolated from transformants

To confirm the presence and integrity of the pYPMA constructs in the yeast transformants, PCR was performed using one oligonucleotide directed against the PMA1 promoter and one against the 3' terminus of the constructs, (or a site upstream of the cloning site in the case of the empty vector control). The 'no template' control contained a mixture of all of the oligonucleotides. The products were run on a 2% TBE agarose gel stained with ethidium bromide and visualised under ultra-violet light. The presence of bands at the expected size intervals confirmed that the plasmids were correctly maintained in vivo.

Size (base pairs)
2000
1200
800
400
200
100
No template
Empty vector
VMA3p E137G
BPV-1 E5
HLTV-1 p12I
HPV-16 E5
BPV-4 E8
BPV-4 E8
N17S
BPV-4 E8
N17A
BPV-4 E8T

Figure 3.4 Immunodetection of tagged proteins in yeast vacuoles

Aliquots of vacuoles containing 10μg of protein were either run on a 15% Tris-glycine polyacrylamide gel and electroblotted onto nitrocellulose membrane (A), or precipitated in acetone:ethanol, solubilised in SDS loading buffer and spotted directly onto the membrane (B). Chemiluminescent immunodetection was performed using 5μg/ml of 12CA5 monoclonal anti-HA antibody (Roche) in TBST. The dominant negative ductin mutant and the HPV-16 E5 proteins were detected at the correct size intervals on the western blot (A), confirming correct translation and vacuolar targeting in vivo. *Dot blotting detected the other E5 proteins, with variations in their levels of detection.*


B
A
6.4
15.4
19.3
28.8
41.8
Molecular mass (kDa)
Empty vector
Vma3p E137G
BPV-1 E5
HLTV-1 p12I
HPV-16 E5
BPV-4 E8
BPV-4 E8 N17S
BPV-4 E8 N17A
BPV-4 E8T

4. Results: Analysis of the E5 proteins using *S. cerevisiae* supported on glucose

4.1 Introduction

The binding of BPV-1 E5 to ductin involves the glutamic acid at the active site of ductin (Andresson *et al.*, 1995), which has also been shown to lie on the outside face of the Vo complex with partial exposure to lipid (Harrison *et al.*, 1999). If the mechanism of rotational catalysis is the same as that of F-ATPase (Stock *et al.*, 1999), this binding would cause steric hindrance, and may inhibit the rate of catalysis, consistent with observations by Finbow and Meagher (Figure 6.2). Both the BPV-4 and HPV-16 proteins have been shown to disrupt another activity associated with ductin, gap junctional intercellular communication (Faccini *et al.*, 1996; Oelze *et al.*, 1995). Again, in this form of ductin complex, the critical glutamic acid is exposed to lipid (Finbow *et al.*, 1992), however, inhibition of GJIC does not fully correlate with the transforming ability of E8 (Ashrafi *et al.*, 2000), so if ductin-binding does account for a conserved mode of transformation it is likely to be via effects on the V-ATPase.

Efficient translation and ductin-binding has been demonstrated for all of the proteins in the previous chapter, so the first step towards investigating their effects upon the V-ATPase is to express them in yeast grown in optimal conditions, thereby maintaining the V-ATPase in its fully assembled state.

The effects of the E5 proteins on the ATP hydrolysis and proton translocation by the V-ATPase were probed by a number of means; the amenability of yeast towards vacuole isolation making it possible to study the enzyme in more detail, both in terms of kinetics and of assembly of the enzyme. To maintain optimal glucose levels, and prevent starvation-induced enzyme inactivation, (Parra and Kane, 1998), glucose concentrations were restored two hours before harvesting, and maintained at all stages prior to cell lysis.

4.2 Overexpression of ductin-binding E5 proteins does not affect cell viability

One of the difficulties of expressing foreign proteins at high levels is that they may exert toxic effects not seen when expressed at their normal levels in their usual host cell. A well characterised instance of this is in bacterial overexpression systems, where cell death is a major obstacle (Studier *et al.*, 1990). BPV-4 E8 overexpression with *ras* is lethal in primary bovine fibroblasts (Pennie *et al.*, 1993), so to rule out any similar effects in yeast, growth rates were determined for the yeast transformants, using minimal medium buffered to pH5.5 to preserve the plasmid levels whilst removing the direct influence of the V-ATPase on cell viability. The optical densities of exponential phase cultures were monitored over eight hours and plotted on a logarithmic scale graph, shown in Figure 4.1. The average growth rates were calculated from the gradients of the curves, and converted into doubling times (Figure 4.2).

YPH 500 cells containing the empty vector had a doubling time of approximately three hours. Cells expressing any of the E5 proteins had similar doubling times

confirming that overexpression of these proteins is not deleterious to growth. However, both the ductin knockout (W303-1B Vatc) and dominant negative ductin strains grew at around half the rate of that of cells bearing the empty vector, suggesting that V-ATPase disruption even affects growth at low pH. This is probably due to mitochondrial dysfunction as a side-effect of V-ATPase inhibition (Eide *et al.*, 1993). A consequence of mitochondrial dysfunction is the 'pet' phenotype; an abbreviation for 'petite', describing the small colonies seen when grown in medium where the principal carbon source is non-fermentable (Whittaker, 1979). Because strains expressing the E5 proteins grew at a different rate to the strain expressing the dominant negative form of ductin, it suggests that they may have different effects upon the V-ATPase.

4.3 The E5 proteins do not affect vacuolar acidification or the rate of ATP hydrolysis by the V-ATPase

To test for vacuolar disruption by the E5 proteins, the transformed yeast strains were inoculated onto solid medium containing either 100mM calcium chloride, or buffered to pH 7.5, which select for V-ATPase function in yeast. Non-selective plates, buffered to pH 5.5 were included as controls, with representative results from experiments repeated at least three times being shown in Figure 4.3. Both the ductin knockout and the dominant negative transformant, Vma3p E137G, failed to grow at pH 7.5 but the dominant negative form grew slowly in complete medium containing calcium, suggesting that the stringency varies with the type of screening and the nutrient composition of the medium. The loss of red colouration of the V-ATPase-defective strains when grown on complete glucose medium at pH 5.5 confirms the use of this visual test to detect inhibition of the V-ATPase.

However all of the strains expressing the E5 proteins grew to the same extent as the wild type, with growth on minimal medium confirming that this was not due to plasmid loss. Taken together, these data suggest that the E5 proteins do not inhibit the V-ATPase to the same extent as that seen by expression of the dominant negative form of ductin. However, published data show that only a low level of V-ATPase activity is required to permit growth at high pH (Finbow *et al.*, 1994) consistent with results presented in chapter 5 showing that growth at high pH (Figure 5.4) still occurs despite a 65% loss of V-ATPase activity due to substitution of glucose for galactose/raffinose (Figure 5.1). So it is possible that the E5 proteins cause only partial inhibition, which is not detected by this assay. To address this possibility, the V-ATPase activity of the transformed strains was quantified. Proton pumping is tightly coupled to Magnesium ATP hydrolysis in the V-ATPase (Puopolo *et al.*, 1992b; Zhang *et al.*, 1992), so the effects of the E5 proteins on the enzyme could be determined by measuring the rates of ATP hydrolysis of the purified V-ATPase.

Standard isolation procedures are available to isolate milligram quantities of vacuoles and a number of V-ATPase inhibitors are available for determination of enzyme activity; bafilomycin A_1, concanamycin A, dicyclohexylcarbodiimide (DCCD) and N-ethylmaleimide (NEM) (Arai *et al.*, 1987a; Drose and Altendorf, 1997; Flatmark *et al.*, 1982). Concanamycin A and bafilomycin A_1 are the most specific in activity (Drose and Altendorf, 1997) but are expensive and difficult to store. NEM has none of these drawbacks, but is less specific, inhibiting other membrane-associated ATPases such as the NEM-sensitive factor required for vesicle fusion (Block *et al.*, 1988). DCCD reacts with acidic residues in hydrophobic environments, and so also inhibits the F-type ATPase (Altendorf,

1977), however this activity can be eliminated by inclusion of 0.02% sodium azide in reactions, effectively making DCCD specific for the V-ATPase. In addition, both DCCD and bafilomycin A_1 cause the same level of ATPase inhibition in purified vacuoles (Harrison *et al.*, 1994; Kane *et al.*, 1989), supporting the use of DCCD as a way of overcoming the practical limitations of bafilomycin A_1 whilst maintaining the specificity of inhibition.

Figure 4.4 shows the specific activities of vacuoles prepared for the various strains of yeast, assayed under saturating concentrations of ATP, i.e. at concentrations at least ten times greater than the published Michaelis constant (Km) for the enzyme, such that the reaction proceeds at near maximal rate and is not affected by substrate depletion over its course. In this case, the published Km of the *S. cerevisiae* V-ATPase is 0.2mM (Jones *et al.*, 1995), so 5mM ATP was used. The activities are similar to published results (Jones *et al.*, 1995), with both the ductin knockout and dominant negative strains showing a loss of activity. However, no inhibition of the V-ATPase was seen in any of the E5 protein transformants.

The fact that the E5 proteins do not inhibit the V-ATPase is a little unexpected, given that BPV-1 E5 binds at the active site of ductin, the same binding site as DCCD (Finbow *et al.*, 1992). One possibility is that the proteins affect the affinity of the enzyme for ATP rather that the maximal rate of hydrolysis. To address this possibility, the Km for the V-ATPase preparations was determined by repeating the enzyme assays using a number of ATP concentrations around the published Km. The volume of the reaction was increased to minimise the effects of substrate depletion over time, and the ATP concentrations chosen so as to be equally spaced when plotted on a double reciprocal Lineweaver-Burke graph. The

action of inhibitors can interfere with kinetic studies, so DCCD was not used to determine the specific activity, however the V-ATPase is the principal ATPase in the vacuole so the data obtained from this analysis are representative of those for the enzyme.

Representative graphs are shown in Figure 4.5 which shows that this form of analysis is capable of distinguishing between the effects of the ductin knockout and dominant negative expression. A comparison of the maximal enzyme activities (Vmax) is shown in Figure 4.6. The results are similar to published results (Jones *et al.*, 1995), and those shown in Figure 4.4, being slightly higher because they represent the total, rather than the specific V-ATPase activity. This effect is most pronounced in the ductin knockout and dominant negative activities since the majority of their total activity is attributable to other ATPases. In any case, the only samples showing consistent inhibition are the ductin knockout and dominant negative, confirming that none of the E5 proteins inhibit the maximal rate of hydrolysis by the V-ATPase significantly. However, the possibility still remains that they alter the affinity of the enzyme for ATP

The Michaelis constant gives an indication of the affinity of the enzyme for a substrate. It can be used to detect competitive and uncompetitive inhibition, which often have little or no effect upon the Vmax, but reduce the rate of catalysis at non-saturating concentrations of substrate. The E5 proteins do not bind at the ATP-binding site, so competitive inhibition is not expected, however it is possible that their binding to ductin could alter the conformation of the ATP-binding subunits, as in the case of bafilomycin A_1, which binds to the Vo sector (Landoltmarticorena *et al.*, 1995; Zhang *et al.*, 1994). This irreversible

uncompetitive inhibition would be expected to alter the Vmax as well as the Km, which was not seen, but because the Km and Vmax can be altered to different degrees, it is possible that effects may only be revealed from comparisons of the Km. The Michaelis constants were calculated from the X intercepts of Lineweaver-Burke plots of vacuole activities, and are shown in Figure 4.7.

The dominant negative (Vma3p E137G) strain shows that disruption of the proteolipid core does decrease the affinity of the V-ATPase for ATP, interestingly an effect not seen in the ductin knockout strain. The reason for this is not known, but it could be explained if the dominant negative ductin became incorporated into the enzyme, rather than blocking its formation. Because of the tight coupling in the enzyme, this disruption of the Vo would impair the binding of ATP, elevating the Km. However, since only 40% of the ATPase activity is sensitive to DCCD in the strains with an inactivated V-ATPase, these observations could be due to other ATPases.

None of the E5 proteins significantly alter the affinity of the V-ATPase for ATP. The significance of the p value for BPV-4 E8 is unclear because a similar effect is not seen in any of the E8 mutants, including the hypertransforming N17A mutant. So it seems that in yeast grown under optimal conditions, the E5 proteins have no discernible effect upon proton translocation or ATP hydrolysis by the V-ATPase, despite binding *in vitro* and correct expression in the vacuole *in vivo*. One possibility is that the proteins dissociate from the enzyme when delivered to the vacuole, so the *in vivo* binding of the E5 proteins to the V-ATPase was investigated.

4.4 HPV-16 E5 does not appear to bind to the active V-ATPase *in vivo*.

HPV-16 E5 was chosen to represent the E5 proteins in this analysis since it is capable of transforming a number of cell types (Straight *et al.*, 1993) and can be detected by western blotting.

The vacuoles were solubilised in N-dodecyl maltoside, a non-ionic detergent which does not denature the V-ATPase (Harrison *et al.*, 1999). In addition, this detergent would not be expected to disrupt the E5-ductin interaction in the light of it being milder than the detergents in RIPA buffer used during *in vitro* binding assays of the E5-ductin complexes (section 2.2.8.3). The solubilised protein was overlaid onto a buffered, continuous gradient of 20%-50% glycerol (w/v), containing 0.1% detergent to retain the proteins in solution. The samples were centrifuged on a benchtop ultracentrifuge, which allowed faster separation and so minimised mixing by diffusion and the inactivation of the enzyme over time. A swing out rotor using low rates of acceleration and deceleration was used to minimise turbulent mixing. Separation was achieved on the basis of size and shape, with large protein complexes (such as the V-ATPase) migrating furthest down the gradient. Fractions were collected by upward displacement, again to minimise mixing. Whole fractions were precipitated in acetone:ethanol to delipidate and concentrate the samples before electrophoresis on a polyacrylamide gel. The type of gel used was dependent upon the proteins being observed; 10% acrylamide in tris-glycine buffer being the best for resolving the large Vph1p subunit, and 15% acrylamide in tris-tricine buffer, according to (Schagger and von Jagow, 1987) being used for all other proteins due to its

resolving power at low molecular weights. The electrophoresed proteins were then blotted onto nitrocellulose membrane for immunodetection.

To analyse the separation of proteins in the gradient, and provide a direct reference for subsequent immunodetection data, the nitrocellulose membranes were stained with Ponceau-S, the results being shown in Figure 4.8. The separation of proteins is demonstrated by the banding patterns along the gradient, and reveals differences between preparations that contain an active V-ATPase and those that do not. This change of protein profile was induceable by expression of the dominant negative form of ductin, but not by HPV-16 E5, which reinforces earlier results showing that it and the other E5 proteins do not disrupt the V-ATPase, or at least not in the same manner as the dominant negative ductin.

To compare the activities of the V-ATPase purifications, and provide a second point of reference against immunological analysis, the ATPase activities of the fractions were determined in the presence of sodium azide (Figure 4.9). The restriction of activity to a small number of fractions, compared to the distribution of protein throughout the gradient seen in Figure 4.8 demonstrates the purification of the enzyme from other vacuolar protein components, and the activity for HPV-16 E5 supports previous findings that the E5 proteins do not inhibit the V-ATPase.

DCCD and bafilomycin A_1 were not used to determine the specific activity since they do not inhibit the solubilised form of the enzyme (M. Finbow, unpublished results). However, 80% of the total ATPase activity was attributable to the V-ATPase before fractionation, and the loss of activity in purifications from both

ductin defective strains shows that the activity is linked to that of the V-ATPase. Nevertheless, solubilisation causes a 2-fold increase in ATPase activity (Finbow and Harrison, personal communication), which may be due to activation of other ATPases. These might interfere with the ATPase profile of the gradient, so NEM was used to determine the V-ATPase activity more specifically. NEM reacts with the cysteine residue at the active site of subunit A (section 1.3.5.2.1), inhibiting the V-ATPase. The NEM-sensitive activity is shown in Figure 4.10, revealing activity due to other ATPases and identifying the V-ATPase as being present in the denser fractions of the gradient.

Antibodies are commercially available against some subunits in the Vo and V_1 components of the yeast V-ATPase, so it was possible to analyse the assembly of the enzyme, and the binding of HPV-16 E5 *in vivo*. Antibodies against the catalytic V_1 subunit (Vma1p) and the ductin-associated Vo glycoprotein (Vph1p) were used to track the position of the enzyme components, and the 12CA5 anti HA antibody was used to detect the dominant negative ductin and HPV-16 E5 proteins. The locations of the three proteins were compared against each other and against the location of fractions containing the peak of V-ATPase activity (Figure 4.11-Figure 4.14).

Analysis of the wild-type V-ATPase purification in Figure 4.11 shows that the Vo and V_1 subunits co-localise around the fractions containing the peak of NEM-inhibitable ATPase activity, supporting the assertion that this activity is due to the V-ATPase. The reactivity in the anti-HA blot may represent a poorly-reacting endogenous protein that has been highly concentrated by the purification (potentially representing its total occurrence in 1mg of vacuoles.)

Figure 4.12 shows the immunoblotting profiles for the ductin knockout strain, the lack of V-ATPase subunits agreeing with work showing that ductin is required for assembly of both V_1 and Vo subunits onto the vacuolar membrane (Kane *et al.*, 1992). Figure 4.13 shows a similar picture for the dominant negative transformant, but the occurrence dominant negative ductin peak in the same fractions as those seen for the holoenzyme in Figure 4.11, suggests that the dominant negative form of ductin is incorporated into a large protein complex. Because none of the other V-ATPase subunits are present, it is unlikely that the migration of ductin is due to its incorporation into the V-ATPase, however it is possible that it forms aggregates with itself, supported by the occurrence of larger immunoreactive species on the blot.

If HPV-16 E5 was integrated into the enzyme similar to the dominant negative form of ductin, a similar anti-HA profile would be seen, however, Figure 4.14 shows that this is not the case. Some HPV-16 E5 is present in the same fractions as the peak of ATPase activity, but the majority is found in lighter fractions. These fractions would appear to contain only scant amounts of ATPase activity, which may be attributable to other ATPases.

4.5 Summary

All of the E5 proteins bound to *S. cerevisiae* ductin *in vitro* and were correctly expressed in the vacuole, but subsequent analysis showed that this interaction did not affect:

- The growth of yeast in liquid culture
- The growth of yeast in conditions requiring an active V-ATPase
- The maximal catalytic rate of the V-ATPase
- The affinity of the V-ATPase for ATP
- The assembly of the V-ATPase

Western blot analysis suggested that HPV-16 E5 did not remain associated with the enzyme *in vivo*, which would be consistent with these observations, however studies in mammalian cells suggest that the E5 proteins do inhibit the V-ATPase (Schapiro *et al.*, 2000; Straight *et al.*, 1995). One possibility is that the E5 proteins only exert subtle effects on the enzyme, and that a more sensitive system is required to detect their effects in yeast. This can be achieved by changing the carbon source upon which the yeast strains are grown, which causes partial dissociation of the V-ATPase (Kane, 1995) and so may sensitise the enzyme to the effects of the E5 proteins.

Figure 4.1 Representative growth curves of *S. cerevisiae* strains expressing the E5 proteins

To check for V-ATPase independent toxic effects of the E5 proteins, optical densities of exponential-phase cells were monitored over 8 hours of growth in minimal glucose medium buffered to pH 5.5. The ductin knockout strain, W303-1B Vatc, and the ductin dominant negative, Vma3p E137G are included as vacuole-defective controls and show reduced growth rates.

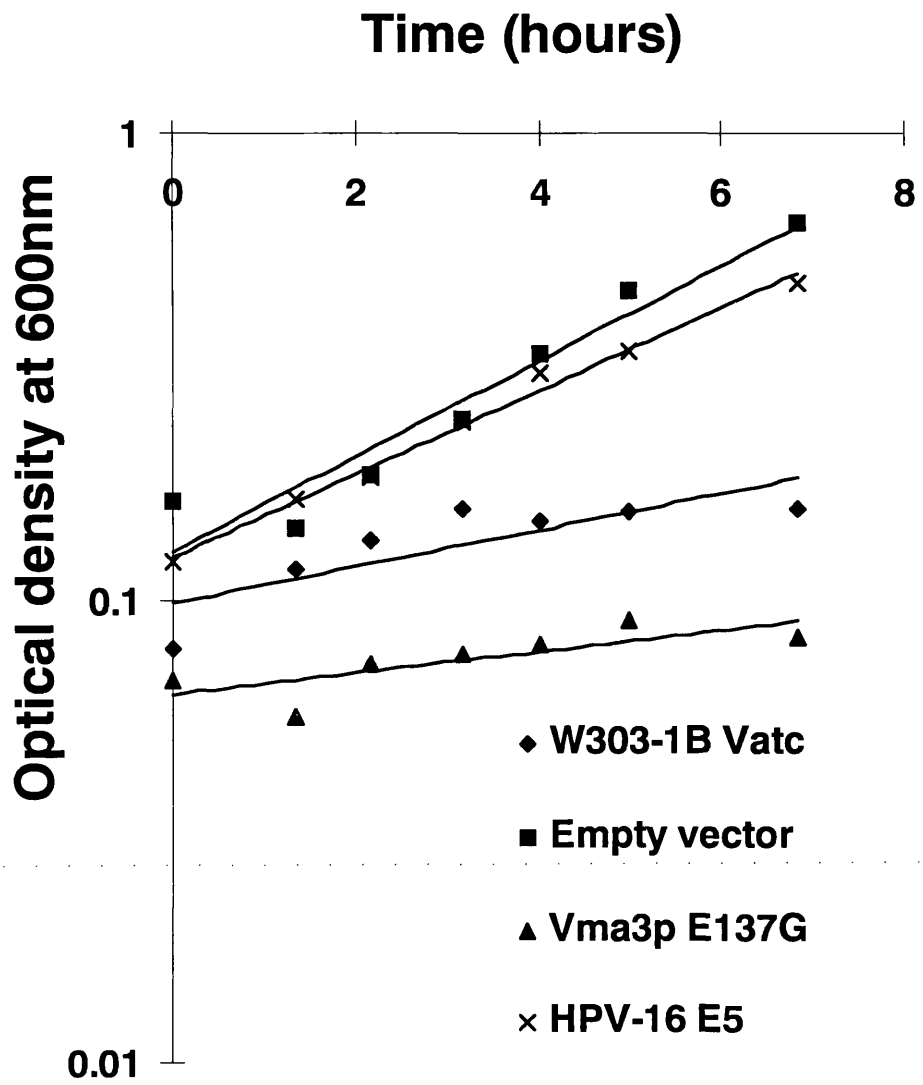

Time (hours)
Optical density at 600nm
1
0.1
0.01
0
2
4
6
8
W303-1B Vatc
Empty vector
Vma3p E137G
HPV-16 E5

Figure 4.2 Growth rates of *S. cerevisiae* strains grown in minimal glucose medium

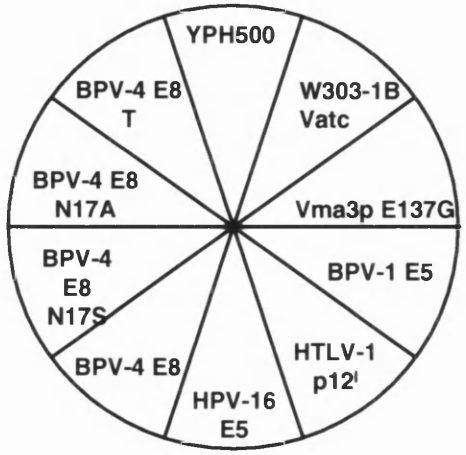
Doubling times were calculated from logarithms of the average growth rates, converted to base 2. This allowed statistical analysis of the growth rates, and also the calculation of inoculation densities for exponential phase culture preparation. The ductin knockout strain (W303-1B Vatc), and the ductin dominant negative (Vma3p E137G) show that vacuolar inactivation retards growth, but this effect is not seen for any of the E5 proteins, suggesting that they do not affect vacuolar function.

Doubling time (hours)
0
1
2
3
4
5
6
7
8
9
10
W303-1B Vatc
Empty vector
Vma3p E137G
BPV-1 E5
HTLV-1 p12
HPV-16 E5
BPV-4 E8
BPV-4 E8 N17S
BPV-4 E8 N17A
BPV-4 E8T
n=3 p=0.03
n=3
n=3 p=0.02
n=3 p=0.88
n=3 p=0.71
n=3 p=0.19
n=3 p=0.80
n=3 p=0.09
n=3 p=0.13
n=3 0.25

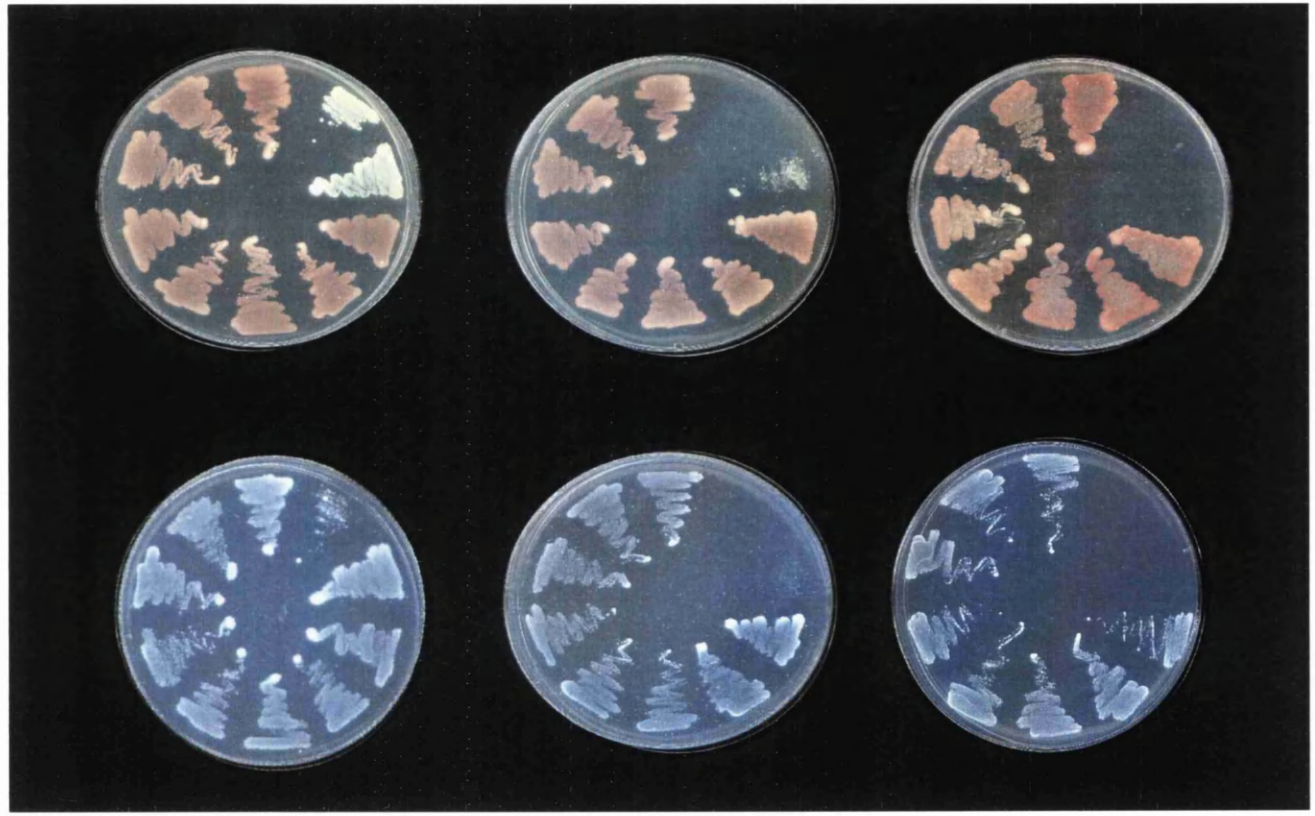
Figure 4.3 Screening for V-ATPase defects in wild-type yeast in glucose medium

Transformed strains of YPH 500 were streaked onto plates of complete and minimal glucose medium. 100mM calcium chloride and 100mM MOPS-Cl pH 7.5 were used to screen for vacuolar defects. The ductin knockout strain, W303-1B Vatc and the dominant negative ductin transformant, Vma3p E137G were used as positive controls. Medium buffered to pH 5.5 confirms that the phenotypes are pH or calcium dependent. The plates represent results from experiments repeated three times, the growth of the E5 protein expressing strains showing that they do not inactivate the V-ATPase.

Complete glucose medium



Minimal glucose medium



pH 5.5 100mM calcium pH 7.5

Figure 4.4 V-ATPase assay of YPH 500 strains expressing the E5 proteins

To quantify the effects of the E5 proteins on the V-ATPase, the vacuoles were purified by floatation on 8% Ficoll and the amount of V-ATPase activity determined by DCCD inhibition in the presence of 0.02% sodium azide. Glucose concentrations were maintained at 2% for 3 hours before lysis of the spheroplasts to minimise starvation-induced dissociation of the enzyme. The dominant negative form of ductin inactivated the V-ATPase but this effect was not seen for any of the E5 proteins.

V-ATPase activity (μmols/mg protein/min)
0.0
0.1
0.2
0.3
0.4
0.5
Empty vector
Vma3p E137G
BPV-1 E5
HTLV-1 P12
HPV-16 E5
BPV-4 E8
BPV-4 E8 N17S
BPV-4 E8 N17A
BPV-4 E8T
n=10
n=5 p=0.0001
n=10 p=0.31
n=10 p=0.48
n=10 p=0.65
n=10 p=0.16
n=10 p=0.17
n=10 p=0.23
n=10 p=0.20

Figure 4.5 Lineweaver - Burke plot of representative enzyme activities

To allow more detailed kinetic analysis, activities were obtained for a range of ATP concentrations around the published Km of the V-ATPase and plotted on a double reciprocal graph. The Y and X intercepts were used to calculate the Vmax and Km respectively. This plot distinguishes between the ductin knockout and dominant negative strains, but does not reveal any effects of HPV-16 E5.

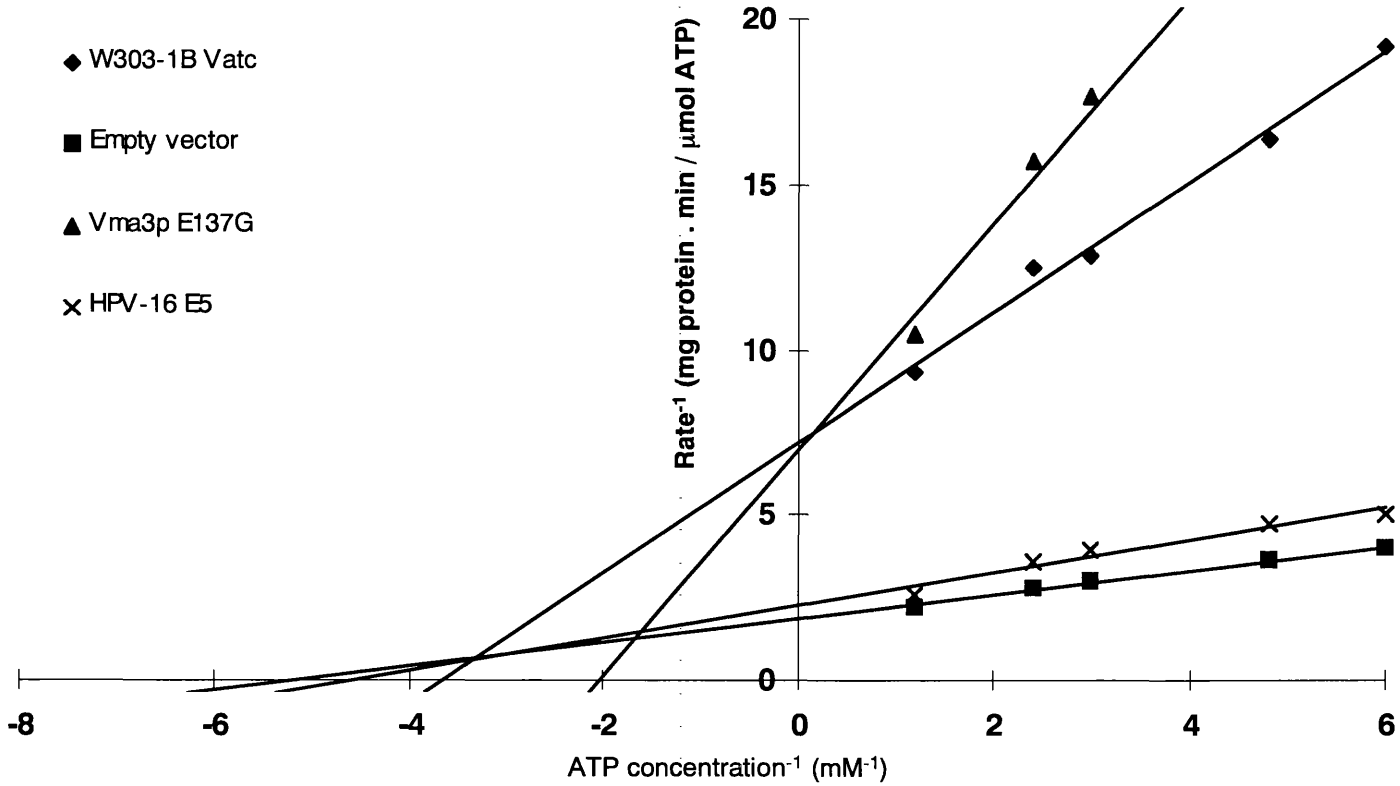

W303-1B Vatc
Empty vector
Vma3p E137G
HPV-16 E5
Rate-1 (mg protein . min / μmol ATP)
20
15
10
5
0
-8
-6
-4
-2
0
2
4
6
ATP concentration-1 (mM-1)

Figure 4.6 Maximal V-ATPase activity as determined by Lineweaver-Burke kinetic analysis

The Y intercepts of Lineweaver-Burke plots of enzyme activities were used to calculate the maximal rate of catalysis. None of the E5 proteins inhibited the V-ATPasae by the same amount seen in strains with a defective V-ATPase.

Vmax (μmols/mg protein/min)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
W303-1B Vatc
Empty vector
Vma3p E137G
BPV-1 E5
HTLV-1 p12
HPV-16 E5
BPV-4 E8
BPV-4 E8 N17A
BPV-4 E8 N17S
BPV-4 E8T
n=6 p=0.001
n=5
n=6 p=0.001
n=6 p=0.29
n=6 p=0.52
n=7 p=0.84
n=7 p=0.54
n=8 p=0.04
n=7 p=0.67
n=8 p=0.84

Figure 4.7 ATP affinity determined by Lineweaver-Burke analysis

To check for uncompetitive inhibition by the E5 proteins, the Km was determined from the X intercept of Lineweaver-Burke plots of enzyme activity. The dominant negative form of ductin appeared to increase the Km. BPV-4 E8 seemed to produce a similar effect, but this was not seen for any other of the E5 proteins.

Km (μmol)
0
0.1
0.2
0.3
0.4
0.5
0.6
W303-1B Vatc
Empty vector
Vma3p E137G
BPV-1 E5
HTLV-1 p12
HPV-16 E5
BPV-4 E8
BPV-4 E8 N17A
BPV-4 E8 N17S
BPV-4 E8T
n=6 p=0.31
n=5
n=6 p=0.02
n=6 p=0.91
n=6 p=0.98
n=7 p=0.22
n=7 p=0.04
n=8 p=0.51
n=7 p=0.41
n=8 p=0.33

Figure 4.8 V-ATPase assembly in as revealed by Ponceau-stained blots

Vacuolar membranes were prepared from wild-type yeast transformants grown in complete glucose medium. Total fractions from 20%-50% glycerol-gradient purifications of 1mg aliquots of vacuolar membranes were precipitated by acetone:ethanol and run on 15% tris-tricine polyacrylamide gels. These were electroblotted onto nitrocellulose and stained with Ponceau S. V-ATPase inactivation produces differences in protein profile, but this was not seen for HPV-16 E5.

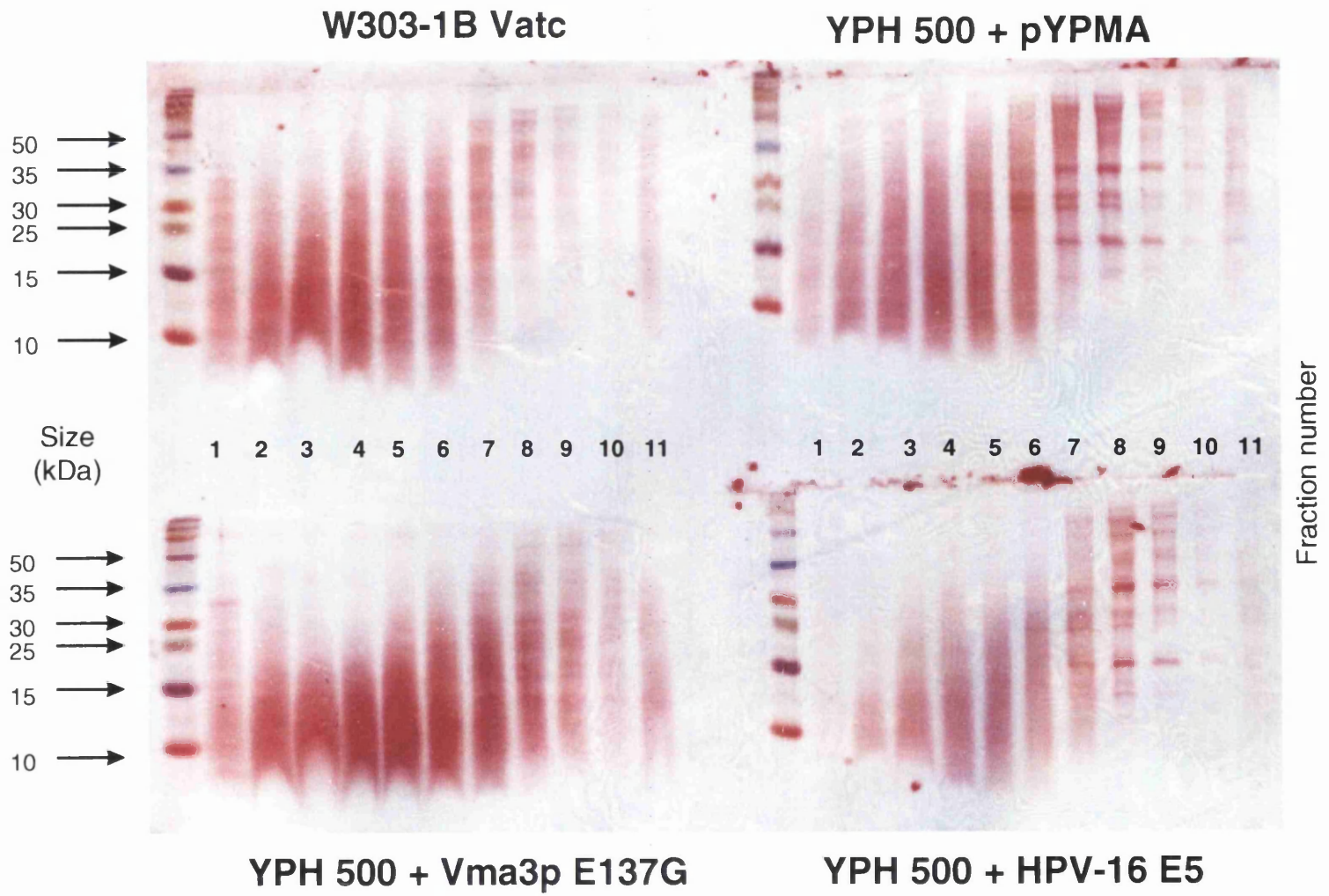

W303-1B Vatc
YPH 500 + pYPMA
Size (kDa)
50
35
30
25
15
10
1 2 3 4 5 6 7 8 9 10 11
YPH 500 + Vma3p E137G
YPH 500 + HPV-16 E5
Fraction number

Figure 4.9 ATPase activity of fractions obtained by glycerol gradient purification of vacuolar membranes

To locate the fractions containing the V-ATPase in the glycerol gradient purifications, the ATPase activity was determined for 10μl aliquots of the fractions collected from 20%-50% glycerol gradient purifications of 1mg aliquots of vacuoles solubilised in N-dodecyl maltoside. All reactions contained 0.02% sodium azide to annul any contaminating F-ATPase activity.

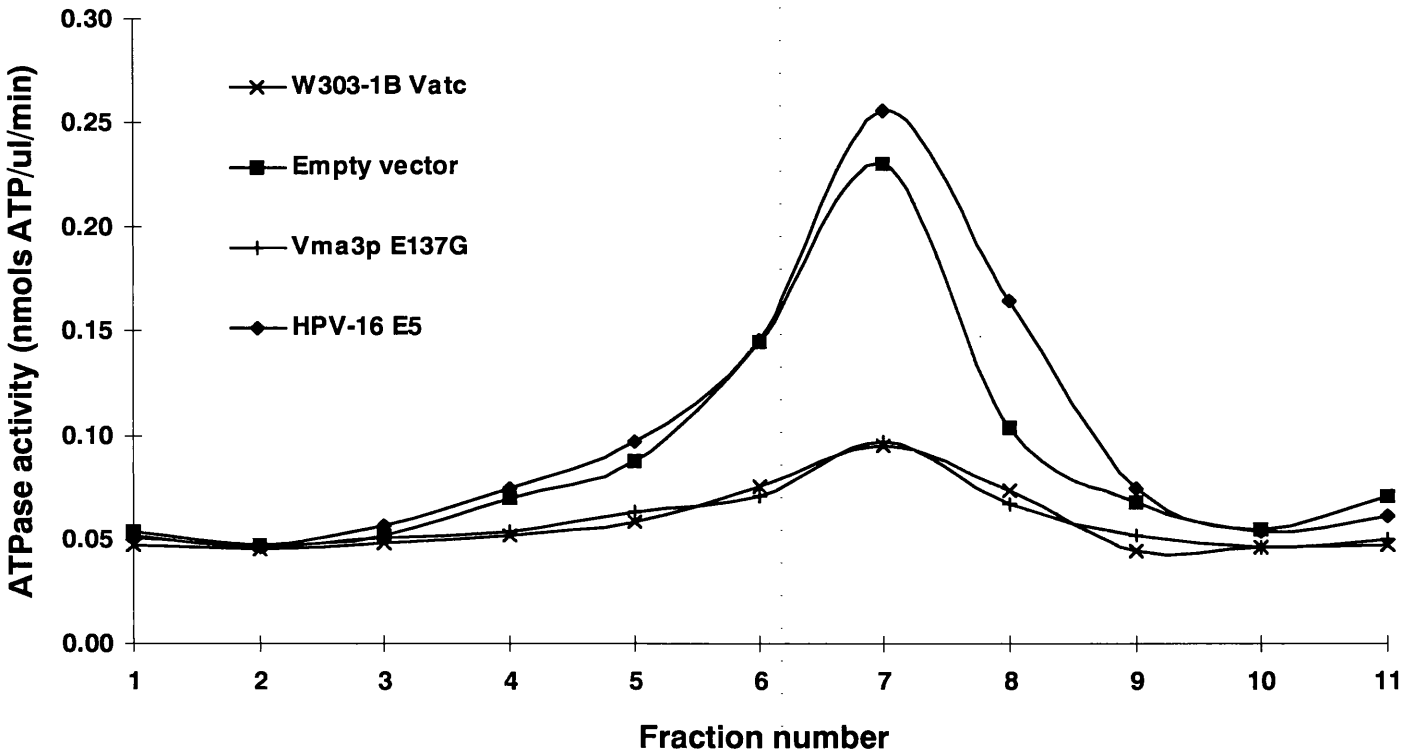

0.30
0.25
0.20
0.15
0.10
0.05
0.00
ATPase activity (nmols ATP/ul/min)
W303-1B Vatc
Empty vector
Vma3p E137G
HPV-16 E5
1
2
3
4
5
6
7
8
9
10
11
Fraction number

Figure 4.10 NEM inhibition of the purified V-ATPase

100μM NEM was used to confirm that the activity peak is due to the V-ATPase. The majority of the activity was due to the V-ATPase, but the peak of activity seemed to be centred between fractions 7 and 8.

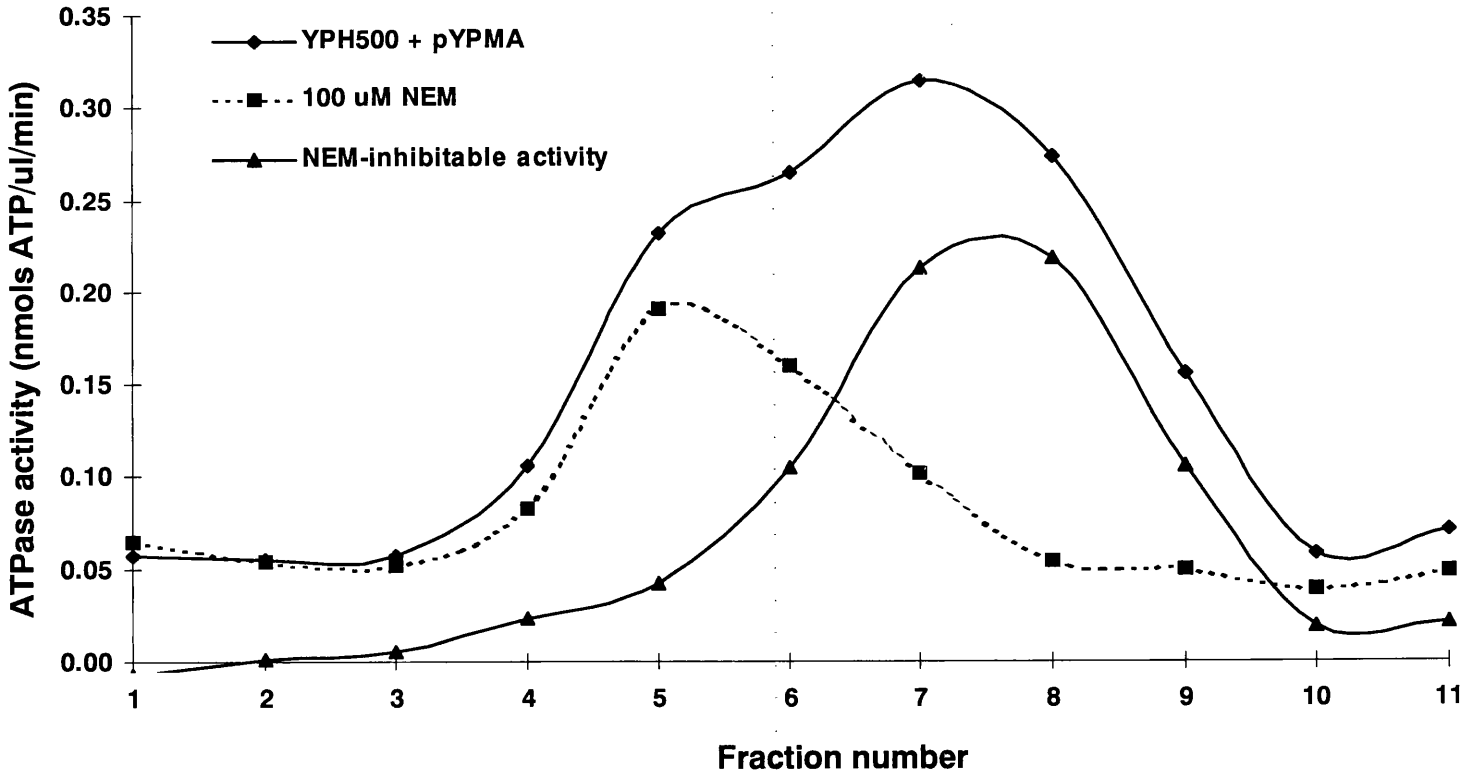

YPH500 + pYPMA
100 uM NEM
NEM-inhibitable activity
ATPase activity (nmols ATP/ul/min)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
1
2
3
4
5
6
7
8
9
10
11
Fraction number

Figure 4.11 Western blot analysis of purified V-ATPase fractions from YPH 500 bearing the empty pYPMA expression vector

Whole fractions from glycerol gradient purifications were precipitated with acetone:ethanol and run on a 10% tris-glycine polyacrylamide gel, (15% tris-tricine for anti-HA blots to enhance resolution). Electroblotting was carried out for 2 hours, including 20% methanol in the transfer buffer only for anti-HA and anti-Vma1p blots. The fractions containing peak V-ATPase activities are denoted by asterisks. The HA immunoreactivity is most likely to be an artefact produced during blotting, since it is not seen in any of the other enzyme purifications.

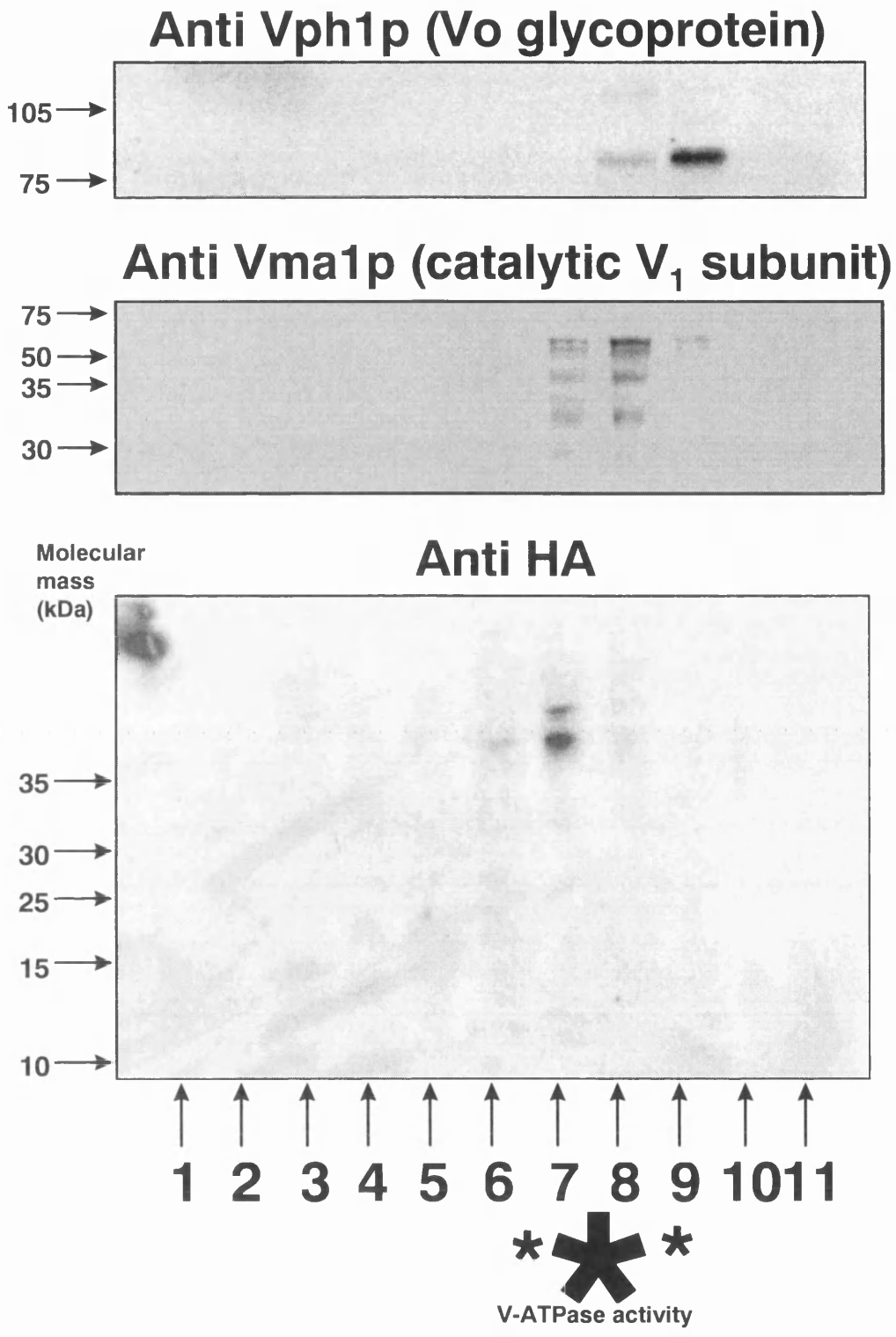

Anti Vph1p (Vo glycoprotein)
105
75
Anti Vma1p (catalytic V1 subunit)
75
50
35
30
Molecular mass (kDa)
Anti HA
35
30
25
15
10
1 2 3 4 5 6 7 8 9 10 11
V-ATPase activity

Figure 4.12 Western blot analysis of purified V-ATPase fractions from the W303-1B Vatc yeast strain

Methodology was as for YPH 500 vacuoles. The absence of activity and enzyme subunits agrees with the expected lack of assembly.

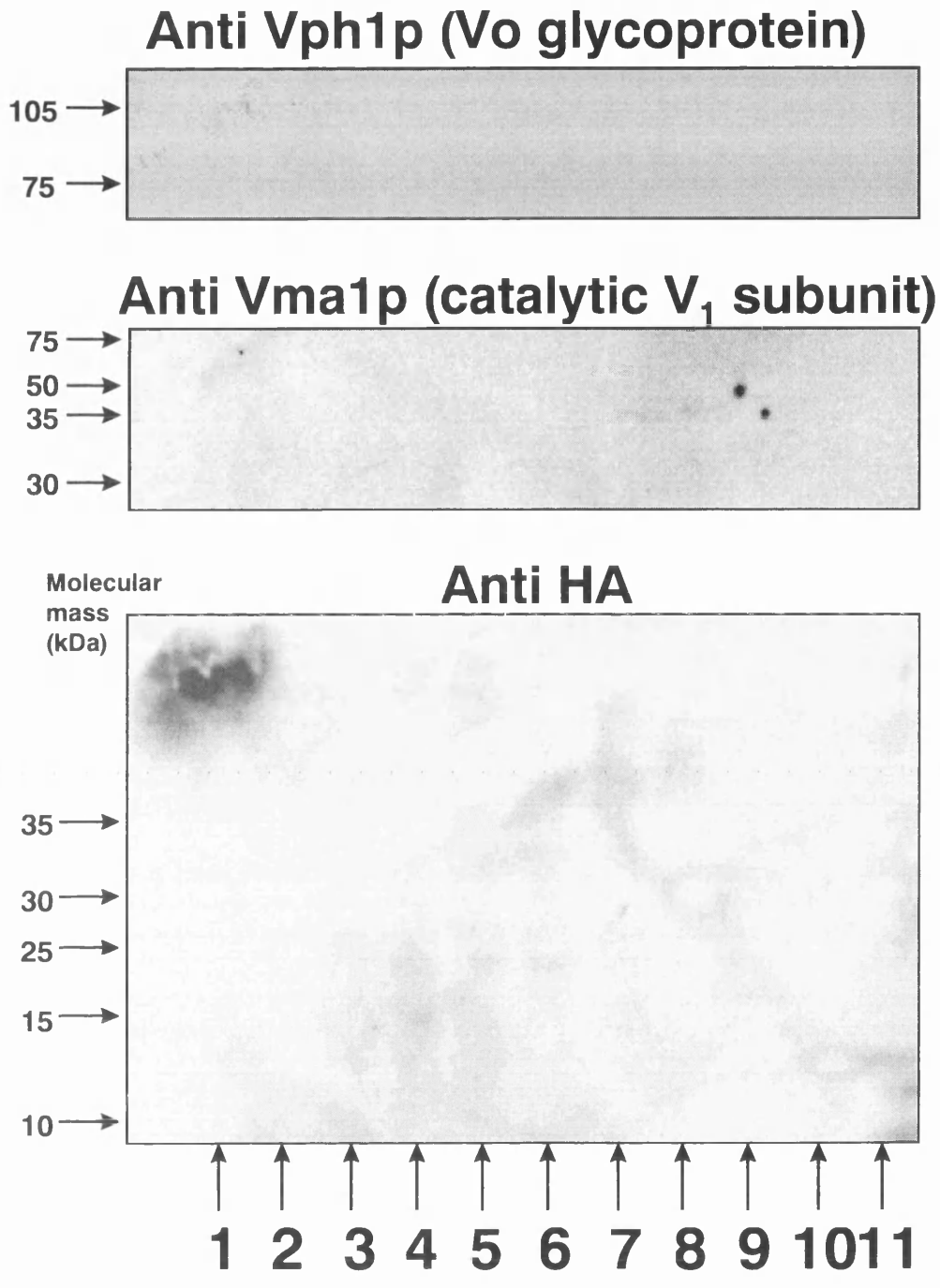

Anti Vph1p (Vo glycoprotein)
105
75
Anti Vma1p (catalytic V1 subunit)
75
50
35
30
Molecular mass (kDa)
Anti HA
35
30
25
15
10
1 2 3 4 5 6 7 8 9 10 11

Figure 4.13 Western blot analysis of purified V-ATPase fractions from wild-type yeast expressing the dominant negative form of ductin

Methodology was as for YPH 500 vacuoles. The reduction in the levels of the Vo glycoprotein suggest that the dominant negative ductin inhibits assembly of the Vo, the presence of larger species suggetsing that it forms aggregates, which may explain its migration into the gradient.

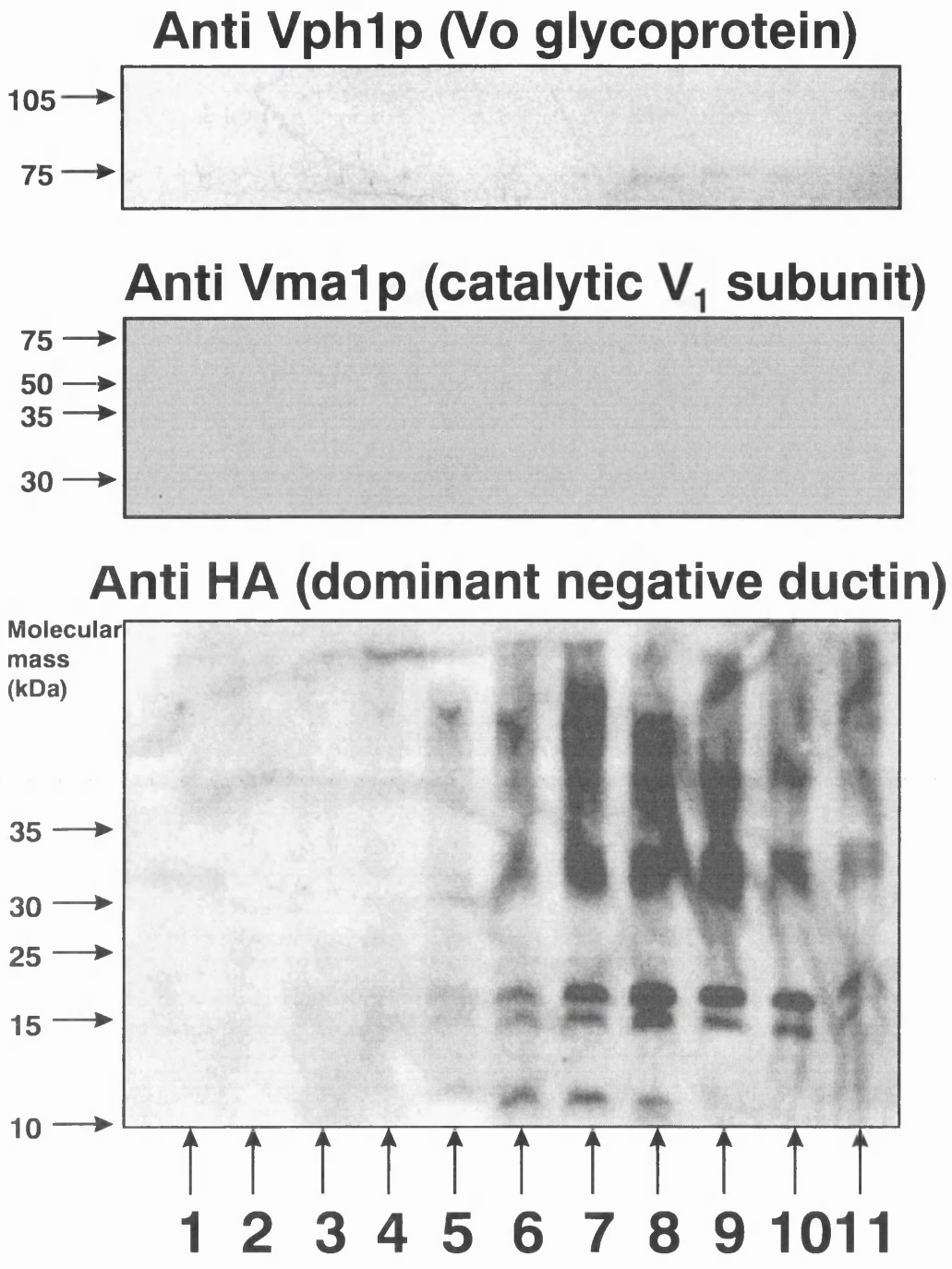
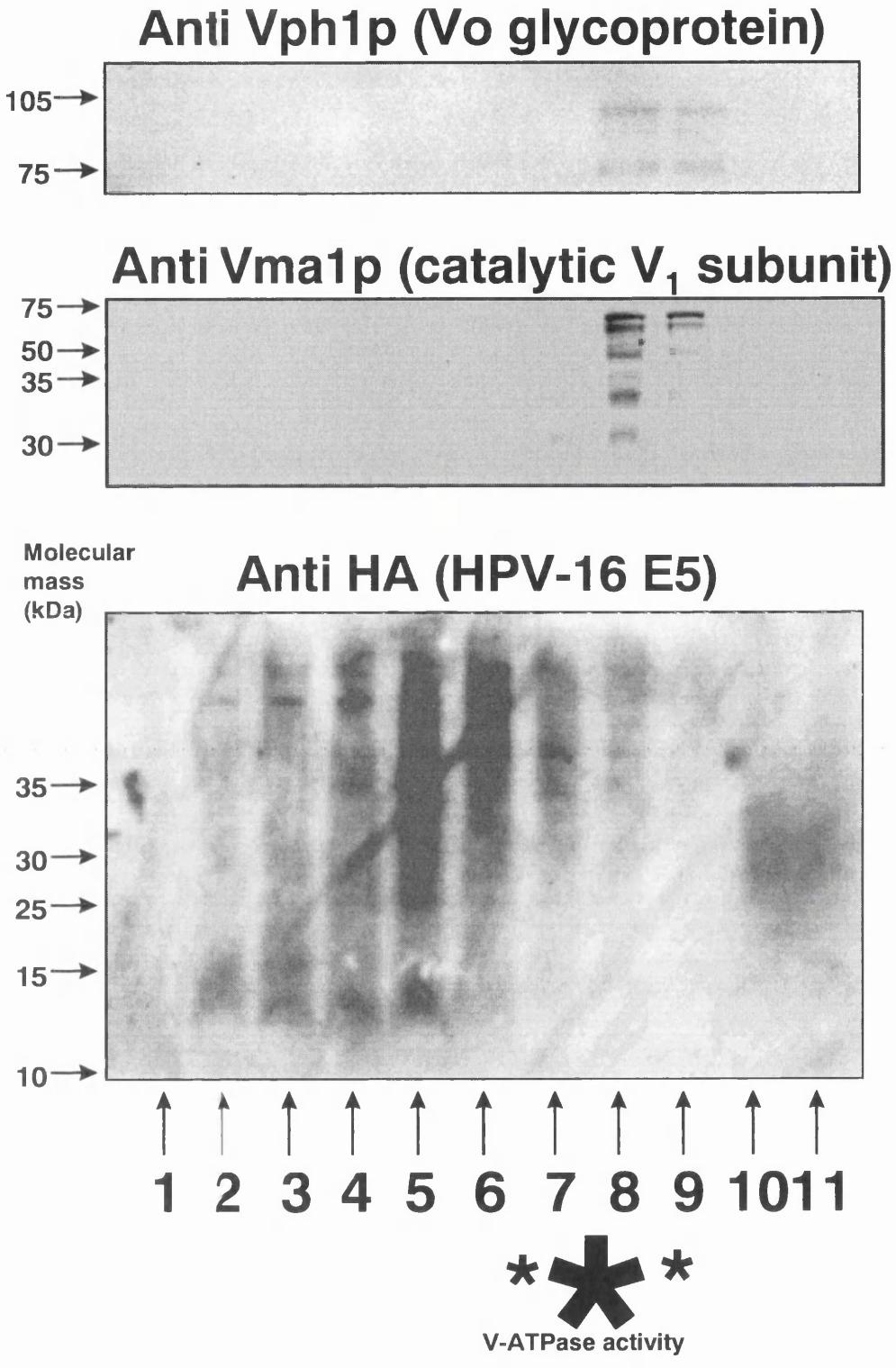

Anti Vph1p (Vo glycoprotein)
105
75
Anti Vma1p (catalytic V1 subunit)
75
50
35
30
Anti HA (dominant negative ductin)
Molecular mass (kDa)
35
30
25
15
10
1 2 3 4 5 6 7 8 9 10 11

Figure 4.14 Western blot analysis of purified V-ATPase fractions from wild-type yeast expressing the HA-tagged HPV-16 E5 protein

Methodology was as for YPH 500 vacuoles. HPV-16 E5 was located in different fractions to those containing V-ATPase activity and enzyme subunits, suggesting that the protein is not associated with the enzyme in vivo.


Anti Vph1p (Vo glycoprotein)
105
75
Anti Vma1p (catalytic V1 subunit)
75
50
35
30
Molecular mass (kDa)
Anti HA (HPV-16 E5)
35
30
25
15
10
1 2 3 4 5 6 7 8 9 10 11
V-ATPase activity

5. Results: Analysis of the effects of viral E5 proteins on the *S. cerevisiae* V-ATPase supported by galactose/ raffinose

5.1 Introduction

Another feature with using yeast is that the assembly and activity of the V-ATPase can be modulated by carbon source. The V_1 sector rapidly dissociates from the Vo sector in response to glucose removal from the medium (Kane, 1995). This also occurs when glucose is replaced by other carbon sources such as raffinose, and overnight growth in raffinose results in only partial reassembly of the enzyme (Kane, 1995). The carbon source also affects the activity of the enzyme (Figure 5.1), with transfer to galactose/raffinose initially causing the same level of inactivation as the ductin knockout. However, even after prolonged growth in galactose and raffinose the activity is only restored to a third of its level in glucose medium.

This compromisation of the V-ATPase by carbon source may sensitise it to the effects of the E5 proteins, so the activity of the enzyme was assessed under these restrictive growth conditions using the same techniques as those for glucose-based medium.

5.2 Galactose/raffinose supports growth of all yeast strains at a reduced rate, but does not reveal any effects of the E5 proteins

Firstly, to confirm that YPH 500 respond in the same manner described by (Kane, 1995), the V-ATPase activity was determined under a variety of nutrient conditions (Figure 5.1). Cells grown and spheroplasted in the presence of glucose had the expected, high levels of activity, however if the same culture was spheroplasted in the presence of galactose/raffinose, the activity fell to the levels comparable to that of the ductin knockout. Growth of cells in galactose/raffinose prior to digestion in galactose/raffinose resulted in partial restoration of activity, to approximately 30% of that when prepared using glucose. This is consistent with the enzyme disassembly seen by (Kane, 1995), so to maintain a constant level of enzyme compromisation all cultures were grown in the presence of galactose/raffinose for 7 days before being studied.

The growth of the dominant negative ductin strain at high calcium concentrations (Figure 4.3) despite its loss of V-ATPase activity (Figure 4.4) suggests that the enzyme can tolerate a certain degree of disruption before complete loss of function. It is also apparent that the E5 proteins do not disrupt the enzyme to the same extent as the dominant negative form of ductin, however growth in galactose/raffinose may provide a means of sensitising the V-ATPase to their effects.

Experiments in glucose medium have shown that the E5 proteins are neither toxic, nor able to induce the 'petite' phenotype (Figure 4.2). To assess whether this was the case when the activity V-ATPase is partially compromised and its activity

markedly reduced, the growth of exponential-phase cultures grown in pH 5.5-buffered galactose/raffinose growth medium was followed (Figure 5.2). The cultures were sensitive to dilution in the medium, so use of identical inoculation densities produced different starting densities for the experiment. However, all cultures were grown for 16 hours before analysing so the 'lag' phase of growth should have been completed.

Analysis of the doubling times, shown in Figure 5.3, reveals that all strains grew at approximately half of that in glucose (Figure 4.2), and that the effects of vacuolar inactivation were more pronounced, suggesting that galactose/raffinose sensitises the cells to the effects of V-ATPase disruption. However, the E5-expressing strains grew at the same rate as the wild type, supporting previous observations that they are neither toxic or able to induce the petite phenotype through effects on the V-ATPase.

5.3 Growth on galactose/raffinose increases the stringency of vacuolar screening but does not reveal any effects of the E5 proteins

A more stringent assessment of vacuolar function under conditions of reduced V-ATPase assembly was carried out by screening on galactose/raffinose-based solid medium containing 100mM calcium chloride or MOPS chloride buffered to pH 7.5. All strains were acclimatised to galactose/raffinose for one week before inoculation to prevent screening effects due to loss of V-ATPase activity upon change of carbon source.

Just as the growth rate in liquid medium is lower in galactose/raffinose, so was the growth rate on solid medium; colonies taking up to one week to appear compared to two days when grown on glucose. Figure 5.4 shows that unlike on glucose medium, the dominant negative transformant fails to grow on complete medium containing calcium when maintained on galactose/raffinose, suggesting that the overall level of stringency has been increased by the change of carbon source.

As seen in experiments carried out in glucose (Figure 4.3), the E5 protein-expressing strains grew as efficiently as the wild type, suggesting that the proteins do not inhibit the V-ATPase. There did appear to be a slight reduction in growth rate when screened at pH 7.5 in minimal medium but this was not consistently observed, and the nature of this type of experiment meant that this observation could not be quantified. However, because yeast vacuoles can be isolated in sufficient quantity to permit kinetic analysis of the V-ATPase, it was possible to analyse the effects of the E5 proteins on the enzyme in more detail.

5.4 The viral E5 proteins do not inhibit the V-ATPase of cells supported on galactose/raffinose

Growth on galactose/raffinose reduces the population of assembled V-ATPase by an unknown mechanism (Parra and Kane, 1998). This disassembly only occurs in catalytically competent enzymes, so may work in synergy with enzyme inhibitors to deactivate the V-ATPase population. The growth patterns in galactose/raffinose medium show that this restrictive carbon source does sensitise the vacuole to disruption, but that the E5 proteins do not perturb proton translocation sufficiently to compromise the function of the vacuole. By determining the activity of V-

ATPase isolates, the effects of these proteins could be measured more accurately.

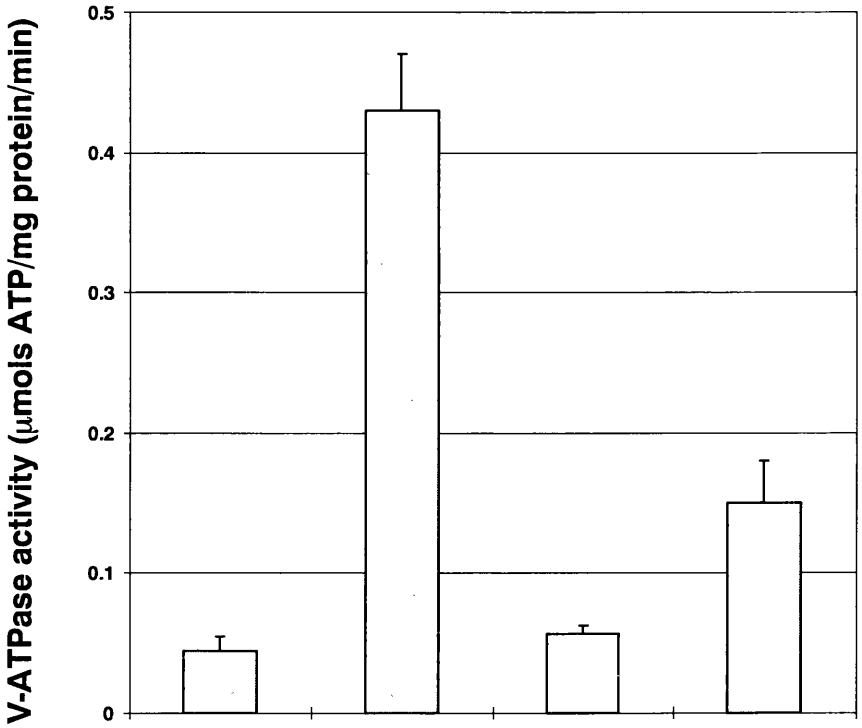
As for growth rate determination, all yeast cultures were grown in galactose/raffinose medium for one week prior to inoculation and overnight culture. Galactose/raffinose concentrations were maintained at 2% (w/v) at all stages three hours prior to lysis, to avoid starvation-induced dissociation of the enzyme. Vacuoles were isolated and the V-ATPase activity assayed, the results being shown in Figure 5.5. The activities were between a third and a half of those obtained in glucose medium (Figure 4.4), with the dominant negative ductin inactivating the enzyme. However, no inhibition was seen in any strains expressing the E5 proteins, supporting the emerging picture that these proteins do not contribute towards cell transformation by inhibiting the V-ATPase.

5.5 Summary

Replacement of glucose for galactose/raffinose in the growth medium caused a transient loss of V-ATPase activity, which is partially restored over time. Yeast strains grown on galactose/raffinose grew at a slower rate than on glucose, and also exhibited lower levels of V-ATPase activity in their vacuoles; the latter effect sensitising the vacuole to inactivation by expression of a dominant negative form of ductin. Despite this sensitisation, none of the E5 proteins had any discernible effect on the V-ATPase, so it seems that if these proteins do perturb activity of the enzyme, then a further level of compromisation is needed to allow detection.

Figure 5.1 V-ATPase modulation by carbon source

Overnight cultures of S. cerevisiae *grown in glucose or galactose/raffinose minimal medium were harvested and transferred to minimal medium containing either glucose or galactose/raffinose for two hours. Cells were then harvested and converted into spheroplasts in the presence of the same carbon source. Vacuoles were isolated and assayed for V-ATPase activity revealing that galactose/raffinose initially causes inactivation of the V-ATPase, but activity is partially restored after prolonged growth.*



Strain	W303-1B Vatc	YPH 500	YPH 500	YPH 500
Growth medium	Glucose	Glucose	Glucose	Galactose raffinose
Digest conditions	Glucose	Glucose	Galactose raffinose	Galactose raffinose
n	4	10	3	7
p		$5x10^{-5}$	0.24	0.01

Figure 5.2 Growth curves of *S. cerevisiae* strains supported on medium containing galactose/raffinose

To check for V-ATPase independent toxic effects of the E5 proteins in yeast grown under restrictive conditions, optical densities of exponential-phase cells were monitored over 8 hours of growth in minimal galactose/raffinose medium buffered to pH 5.5. The ductin knockout strain, W303-1B Vatc, and the ductin dominant negative, Vma3p E137G are included as vacuole-defective controls and show reduced growth rates.

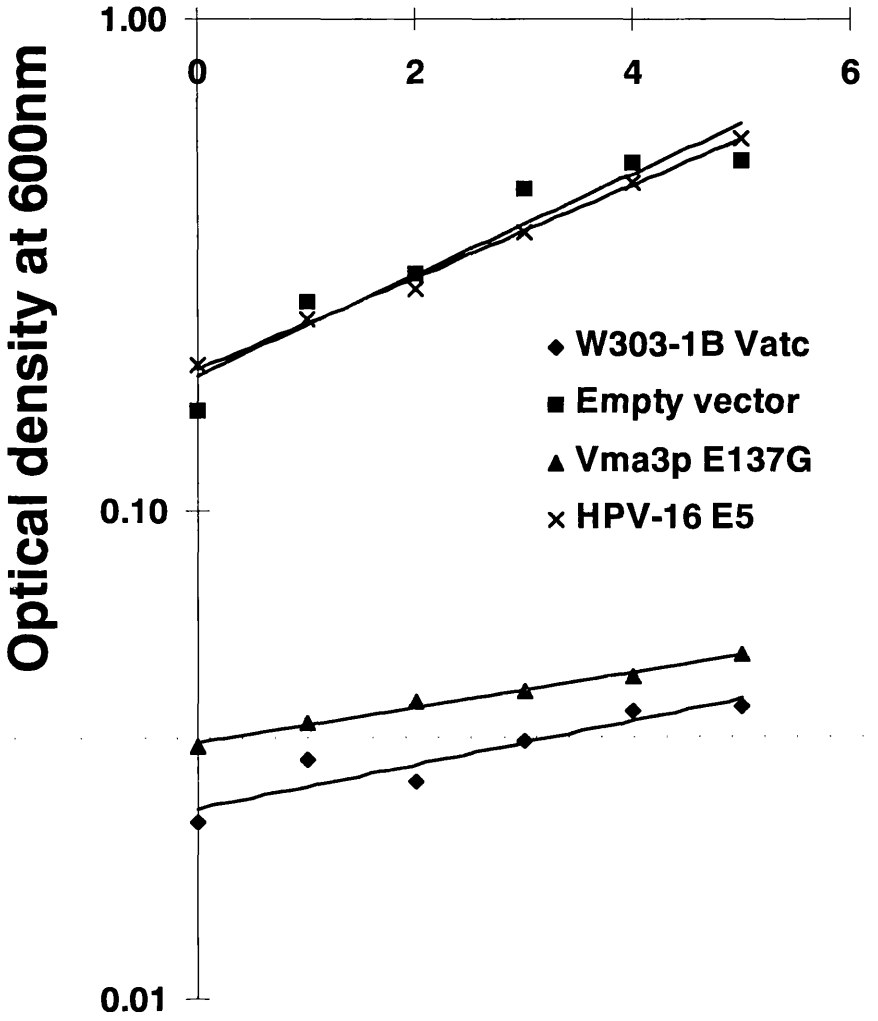

Time hours)
Optical density at 600nm
1.00
0.10
0.01
0
2
4
6
W303-1B Vatc
Empty vector
Vma3p E137G
HPV-16 E5

Figure 5.3 Growth rates of *S. cerevisiae* strains grown in minimal galactose/raffinose medium

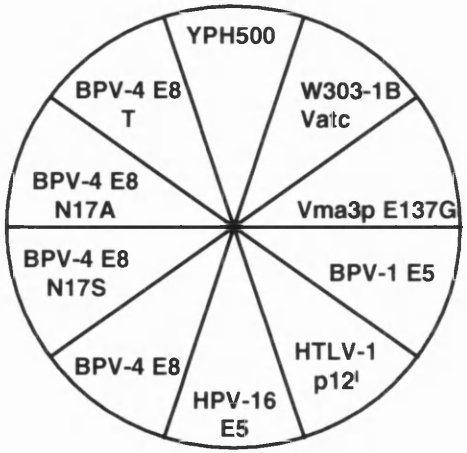
Doubling times were calculated from logarithms of the average growth rates, converted to base 2. This allowed statistical analysis of the growth rates, and also the calculation of inoculation densities for exponential phase culture preparation. The ductin knockout strain (W303-1B Vatc), and the ductin dominant negative (Vma3p E137G) show that vacuolar inactivation retards growth, but this effect is not seen for any of the E5 proteins, suggesting that they do not affect vacuolar function in yeast with a partially compromised V-ATPase.

Doubling time (hours)
0
2
4
6
8
10
12
14
16
18
W303-1B Vatc
Empty vector
Vma3p E137G
BPV-1 E5
HTLV-1 p12
HPV-16 E5
BPV-4 E8
BPV-4 E8 N17S
BPV-4 E8 N17A
BPV-4 E8T
n=3 p=0.01
n=3
n=3 p=0.03
n=3 p=0.90
n=3 p=0.77
n=3 p=0.52
n=3 p=0.82
n=3 p=0.74
n=3 p=0.52
n=3 0.83

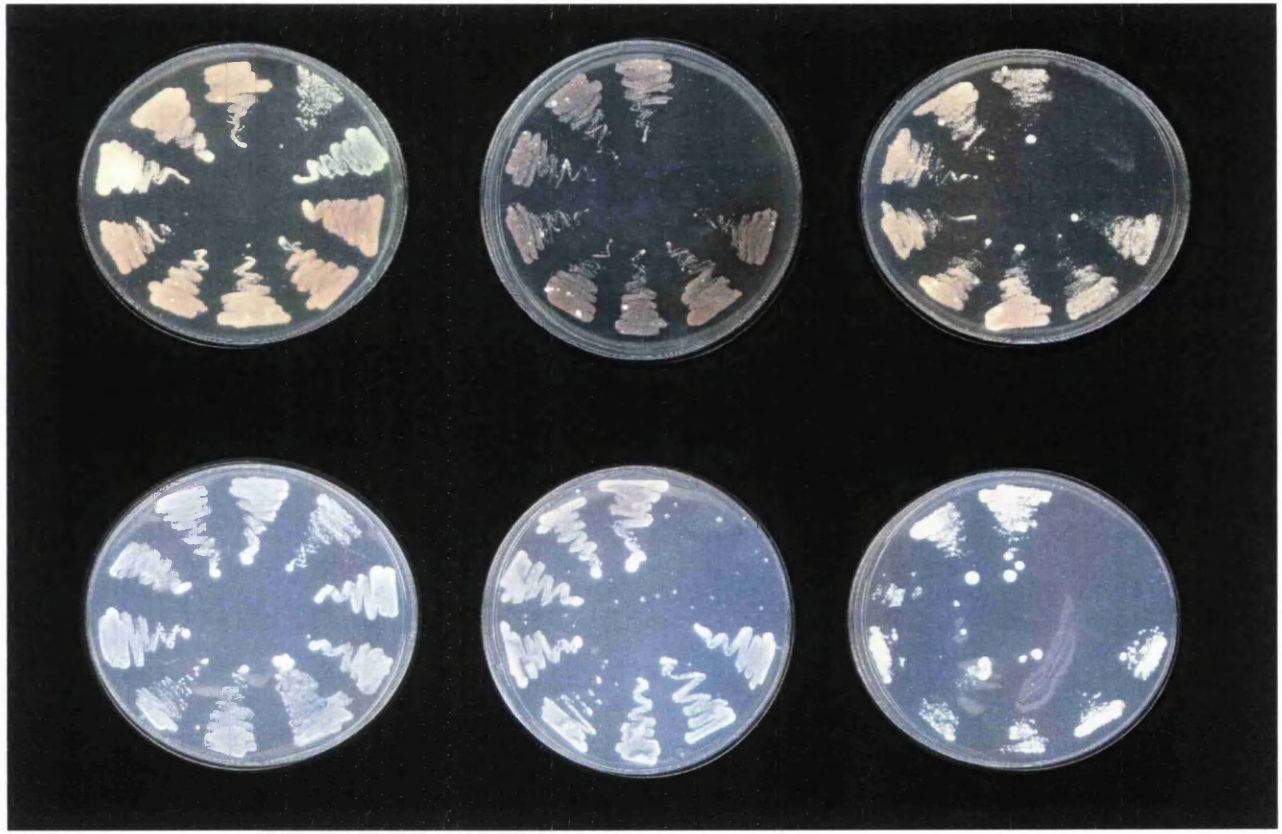
Figure 5.4 Screening for V-ATPase defects in wild-type yeast in galactose/raffinose medium

Representative results of three experiments where transformed strains of YPH 500 were streaked onto selective plates of complete and minimal galactose/raffinose medium. The ductin knockout strain, W303-1B and the dominant negative ductin transformant, Vma3p E137G were used as positive controls. Non-selective plates buffered to pH 5.5 confirm that the phenotypes are pH and calcium dependent. All of the strains expressing the E5 proteins grew under selection showing that they do not cause inactivation of the partially compromised V-ATPase.

Complete galactose/ raffinose medium



Minimal galactose/ raffinose medium



pH 5.5 100mM calcium pH 7.5

Figure 5.5 V-ATPase assay of wild type yeast transformants grown in minimal galactose/raffinose medium

Vacuoles were isolated by floatation on 8% Ficoll and assayed for V-ATPase activity by measuring the rate of DCCD-inhibitable ATP hydrolysis in the presence of 0.02% sodium azide. Galactose/raffinose concentrations were maintained at 2% for 3 hours before lysis of the spheroplasts to minimise starvation-induced dissociation of the enzyme. The enzyme activity was lower than that when glucose was used as a carbon source, illustrating the compromisation of the enzyme, but none of the E5 proteins affected the activity in the same way as that of dominant negative ductin.

V-ATPase activity (μmols/mg protein/min)
0.25
0.20
0.15
0.10
0.05
0.00
n=7
Empty vector
n=5
p=0.01
Vma3p E137G
n=6
p=0.82
BPV-1 E5
n=6
p=0.17
HTLV-1 P12
n=5
p=0.96
HPV-16 E5
n=5
p=0.54
BPV-4 E8
n=5
p=0.19
BPV-4 E8 N17S
n=5
p=0.81
BPV-4 E8 N17A
n=5
p=0.29
BPV-4 E8T

6. Results: Investigation using a highly compromised V-ATPase transgenic for *Nephrops* ductin and grown on galactose/raffinose.

6.1 Introduction

So far, it has been shown that the E5 proteins do not inhibit the V-ATPase under optimal or restrictive growth conditions. The possibility still remains that their effects are too subtle to be seen by analysis of the native form of the enzyme, and that a less stable form may exaggerate their effects, it being more liable to dissociate or lose activity in some way upon E5 binding.

To this end, a strain of yeast expressing a histidine-tagged form of the Norway lobster (*Nephrops norvegicus)* ductin was used to investigate the E5 proteins. *Nephrops* ductin complements for yeast ductin but doubles the Km of the V-ATPase, the inclusion of a hexahistidine tag causing a further 5 fold increase (Harrison *et al.*, 1994), qualitatively compromising the enzyme in addition to the quantitative effects from growth in galactose/raffinose. As with Vma3p, it has been well established that all four E5 proteins bind to *Nephrops* ductin *in vitro* (Figure 6.1), (Ashrafi *et al.*, 2000; Faccini *et al.*, 1996), (I. Rodriguez, manuscript in preparation). In addition, preliminary results from work carried out by L. Meagher and M. Finbow suggested that the BPV-1 E5 and HTLV-1 $p12^{I}$ are capable of inhibiting this *Nephrops* transgenic V-ATPase (Figure 6.2).

This study extends upon those results, using the original pYES2-based plasmid construct to conserve the properties of the system, but with a thorough investigation into the effects of all of the E5 proteins, exploiting the availability of antibodies to *Nephrops* ductin to determine the nature of *in vivo* associations.

6.2 *Nephrops* ductin partially restores vacuolar function, but does not reveal any effects of the E5 proteins.

Despite the published ability of *Nephrops* ductin to restore the activity of the V-ATPase, this could not be achieved consistently with the yeast stocks held in our laboratory (data not shown). The reason for this is not known, but the loss of activity appeared to be a consequence of the age of the stocks and might be explained by mitochondrial loss over time due to the 'pet' phenotype described in section 4.2. Both the ductin knockout and the *Nephrops* transgenic strain exhibited this phenotype (M. Finbow, personal communication) but although this is only a partial phenotype in V-ATPase-defective strains (Supek *et al.*, 1994), perhaps the length of storage caused total mitochondrial loss, restricting ATP production further and thus the ability of the cells to support a large population of active V-ATPases.

Nonetheless, because only partial restoration of V-ATPase activity is sufficient to restore the function of the vacuole, *Nephrops* ductin may be able to restore the growth of yeast under selective conditions. So to test this, and also to screen for effects of the E5 proteins, galactose/raffinose cultures were inoculated onto solid galactose/raffinose medium buffered to pH 5.5 and 7.5. Figure 6.3 shows the results of these selective plates spread with yeast strains bearing combinations of

Nephrops ductin and viral E5 proteins. All colonies grew very slowly, taking up to two weeks to appear under selection, and despite its inability to restore growth rates in liquid medium (data not shown), *Nephrops* ductin restored the ability to grow at high pH in complete medium, confirming its reconstitution of the yeast V-ATPase. (Calcium plates were not as effective in distinguishing the effects of the *Nephrops* protein, perhaps due to the prolonged growth periods required, so were omitted).

No growth occurred on minimal medium buffered to pH 7.5, marking the functional threshold of the transgenic V-ATPase and demonstrating the additional level of compromisation in this system. As expected, the dominant negative form of yeast ductin prevented growth under selection, but none of the E5 proteins had any effect, despite the increased sensitivity of the system.

To further investigate the reconstitution of the V-ATPase by *Nephrops* ductin, the enzyme was purified through a glycerol gradient as described in section 2.2.7.1 and the ATPase activities determined in each of the fractions (Figure 6.4). The results show that *Nephrops* ductin does restore a small amount of ATPase activity, but the different activity profile to the wild type enzyme (Figure 4.10) suggests that the activity is not due to the V-ATPase; the activity is found in less dense fractions than the V-ATPase. This ATPase activity could be due to the vesicle-associated NEM-sensitive factor (Block *et al.*, 1988). However, this ATPase activity seems to be dependent upon restoration of the V-ATPase since it is not seen in either the ductin knockout or dominant negative ductin strains. Because the activity is present in the strain expressing HPV-16 E5 it again suggests that the E5 proteins do not perturb the activity of the V-ATPase.

To test if *Nephrops* ductin restored the assembly of the V-ATPase, and to determine the nature of its interaction with dominant negative ductin and HPV-16 E5 *in vivo*, the purified fractions were probed by western blotting.

6.3 Western blot analysis of the purified *Nephrops* ductin reveals enzyme reconstitution and differences in the behaviour of HPV-16 E5 *in vivo*.

The fractions from glycerol gradient purifications were concentrated and subjected to SDS-PAGE and western blotting, as detailed in section 4.4. The locations of the catalytic V_1 subunit (Vma1p), *Nephrops* ductin and HA-reactive proteins are shown in Figure 6.5 - Figure 6.8.

Figure 6.5 shows the protein profiles for the ductin knockout strain, with no V-ATPase subunits or E5 proteins being detected in the vacuole, as seen when the same experiment was performed using glucose in the growth medium (Figure 4.12).

Figure 6.6 shows that the expression of *Nephrops* ductin increases the levels of the catalytic V_1 subunit in the same fractions as those of the wild type enzyme (Figure 4.11), but separate from those containing the ATPase activity (Figure 6.4), further suggesting that this activity does not represent that of the V-ATPase. The correct mass of *Nephrops* ductin shows that it is expressed correctly and targeted to the vacuole, and its appearance in the same fractions as the catalytic V_1 subunit, and of those fractions containing V-ATPase activity in the wild type enzyme (Figure 4.10) are consistent with data showing it being capable of reconstituting the V-ATPase (Figure 6.3), (Harrison *et al.*, 1994).

Figure 6.7 shows that both the *Nephrops* and dominant negative yeast ductin are located in the same fractions, so are likely to be incorporated into the same complexes; their co-migration with the catalytic V_1 subunit suggesting incorporation into the V-ATPase. The catalytic V_1 subunit did not appear in purifications of the wild type enzyme in the presence of dominant negative ductin (Figure 4.13), the most likely explanation being suppression of the effects of the dominant negative due to the elevated levels of *Nephrops* ductin expression on the pTY plasmid compared to those of wild type ductin.

From work with the wild type enzyme, HPV-16 E5 appeared not to bind to the V-ATPase *in vivo,* on the basis that it was not found in the same fractions as those containing other V-ATPase subunits (Figure 4.14). However, when this experiment was repeated for the *Nephrops* ductin V-ATPase, HPV-16 E5 was present in fractions containing *Nephrops* ductin and the catalytic V_1 subunit (Figure 6.8), and in fractions which contained V-ATPase activity in the wild type enzyme (Figure 4.10). In addition, HPV-16 E5 shows a lower tendency to form aggregates in the *Nephrops* ductin system.

This suggests that its migration into the gradient is by way of its association with another large protein complex (such as the V-ATPase), as opposed to forming large complexes of itself, so it is possible that HPV-16 E5 does bind to the *Nephrops* transgenic V-ATPase *in vivo*, however there are fractions where HPV-16 E5 and *Nephrops* ductin are found separately, suggesting that the two proteins do not form a stable association. In either case, the activity of the enzyme is unaffected, dissociating the binding activity of HPV-16 E5 from direct inhibition of the enzyme.

6.4 Summary

Nephrops ductin restores the function of the V-ATPase as assessed by growth at high pH and assembly of the purified enzyme. The failure to restore growth in minimal medium at high pH demonstrates the slim margin by which function has been restored, and thus the sensitivity of the system to V-ATPase perturbation. Despite this sensitivity, none of the E5 proteins affected the activity of the enzyme.

These results agree with those obtained from experiments with a fully active and partially compromised V-ATPase, suggesting that E5 proteins do not directly inhibit the V-ATPase in the vacuole.

Figure 6.1 *In vitro* binding of the E5 proteins to *Nephrops* ductin

Representative figures from data published by (Ashrafi et al., 2000; Faccini et al., 1996) and I. Rodriguez (Manuscript in preparation) demonstrating the ability of the E5 proteins to bind to hexahistidine-tagged Nephrops *ductin. The E5 proteins were co-translated with* Nephrops *ductin in the presence of microsomes and radiolabelled cysteine, then immunoprecipitated with antibodies to their respective epitope tags, the co-precipitation of the proteins demonstrating their association.*

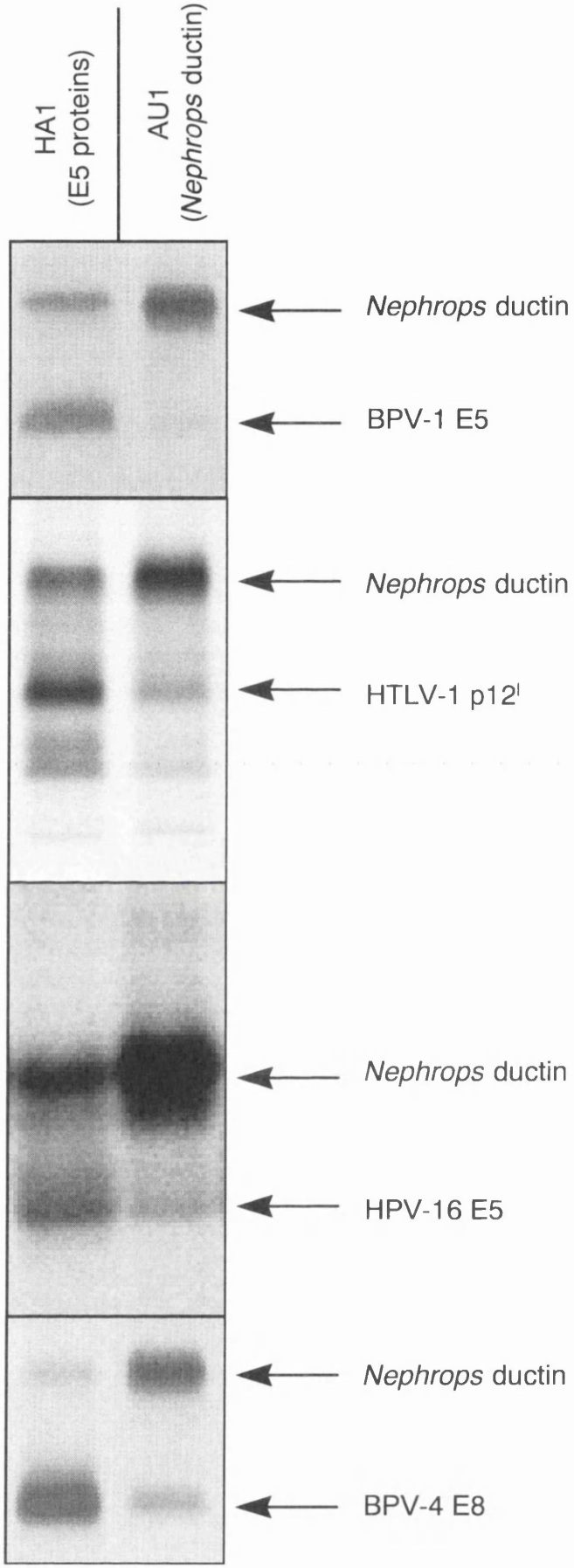

HA1
(E5 proteins)
AU1
(Nephrops ductin)
Nephrops ductin
BPV-1 E5
Nephrops ductin
HTLV-1 p12I
Nephrops ductin
HPV-16 E5
Nephrops ductin
BPV-4 E8

Figure 6.2 Inhibition of the *Nephrops* transgenic V-ATPase in strains expressing the E5 proteins

Vacuoles were isolated from S. cerevisiae *strains transgenic for* Nephrops *ductin by M. Finbow and L. Meagher, the inactivation seen in the E5 and p12^{I}-expressing strains suggesting that these proteins were capable of inhibiting the V-ATPase.*

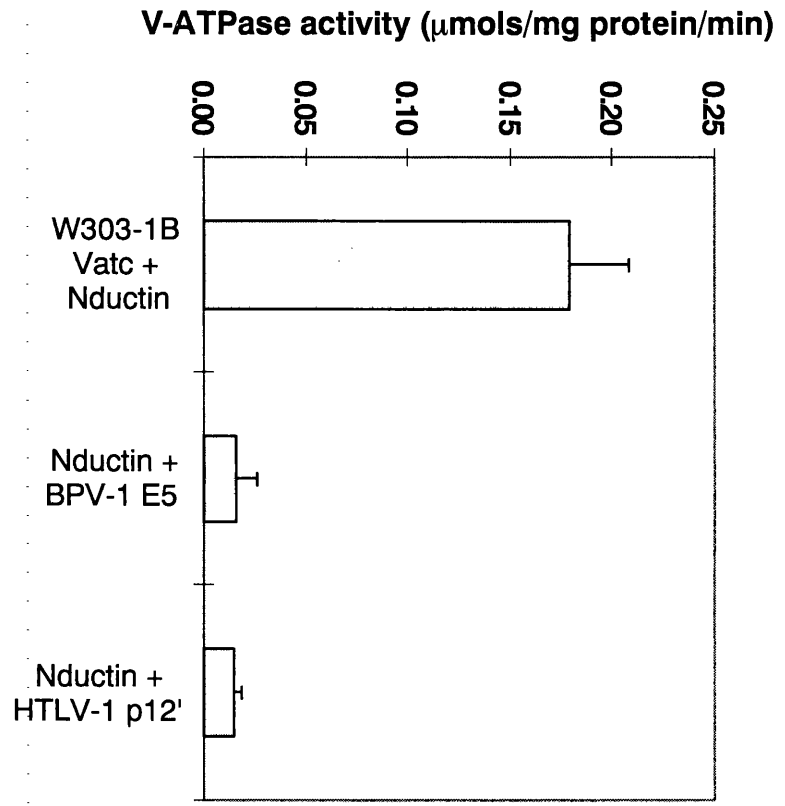
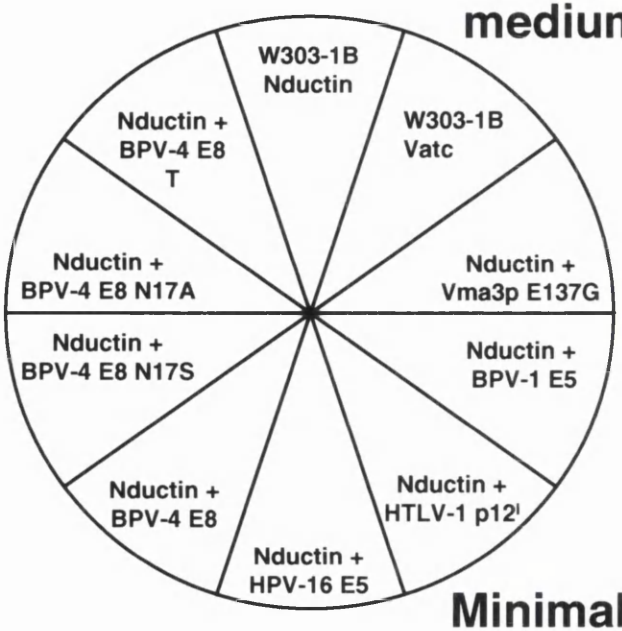

V-ATPase activity (μmols/mg protein/min)
0.00
0.05
0.10
0.15
0.20
0.25
W303-1B
Vatc +
Nductin
Nductin +
BPV-1 E5
Nductin +
HTLV-1 p12'

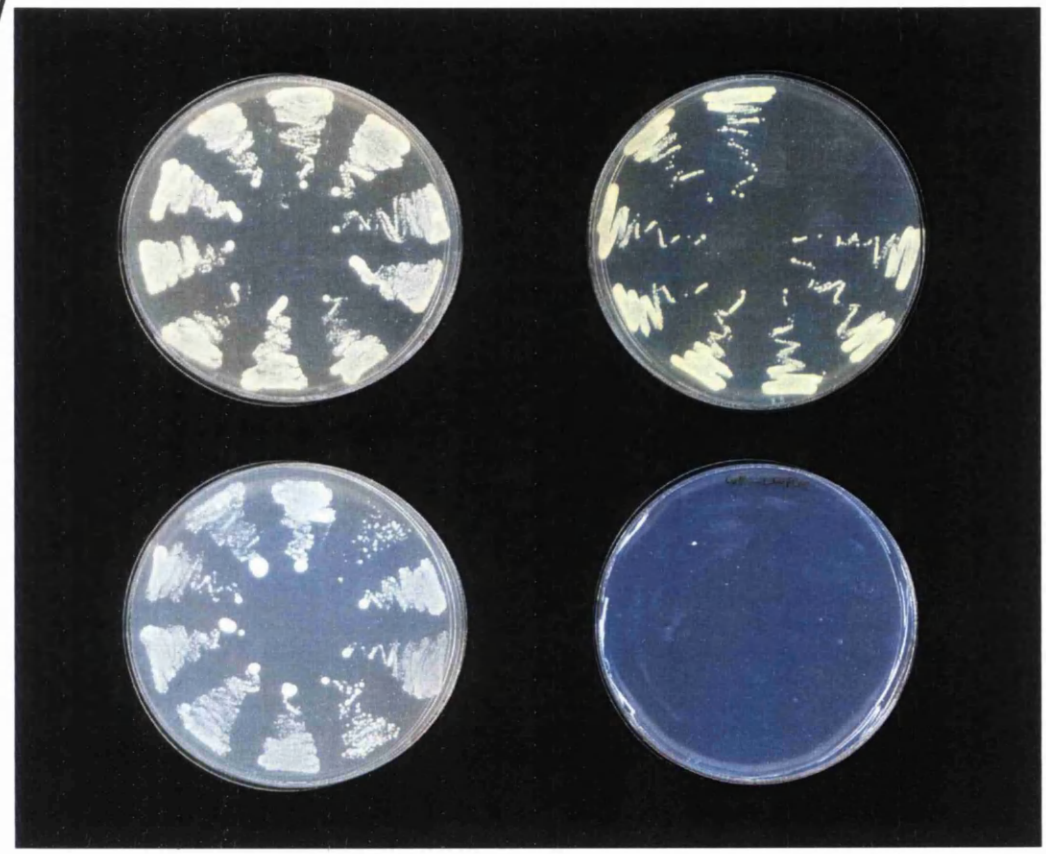
Figure 6.3 Screening for V-ATPase defects *Nephrops* ductin transgenic yeast strains expressing viral E5 proteins

Representative results of three experiments where transformed W303-1B Vatc strains were streaked onto selective plates of complete and minimal galactose/raffinose medium. The ductin knockout strain, W303-1B Vatc contains no form of ductin, and the dominant negative ductin transformant, Vma3p E137G was used as a positive control for inhibition. Non-selective plates buffered to pH 5.5 confirm viability of the yeast strains, plates buffered to pH 7.5 screen for vacuolar defects. Calcium plates were omitted because their stringency was below that of pH plates and gave no additional data. Nephrops *ductin restored vacuolar function, but not sufficiently to permit growth in minimal medium buffered to pH 7.5. Despite this level of compromisation, no effects of the E5 proteins were seen.*



Complete galactose/ raffinose medium

Minimal galactose/ raffinose medium



pH 5.5 **pH 7.5**

Figure 6.4 ATPase activity of fractions obtained by glycerol gradient purification of vacuolar membranes

1mg aliquots of TE-washed vacuoles were separated on a 20%-50% glycerol gradient into 11 fractions. ATPase activity was calculated from the rate of ATP hydrolysis in the presence of 0.02% sodium azide. ATPase activity was restored by Nephrops *ductin expression, but in a different location to that of the wild type V-ATPase, suggesting that the activity is attributable to another enzyme.*

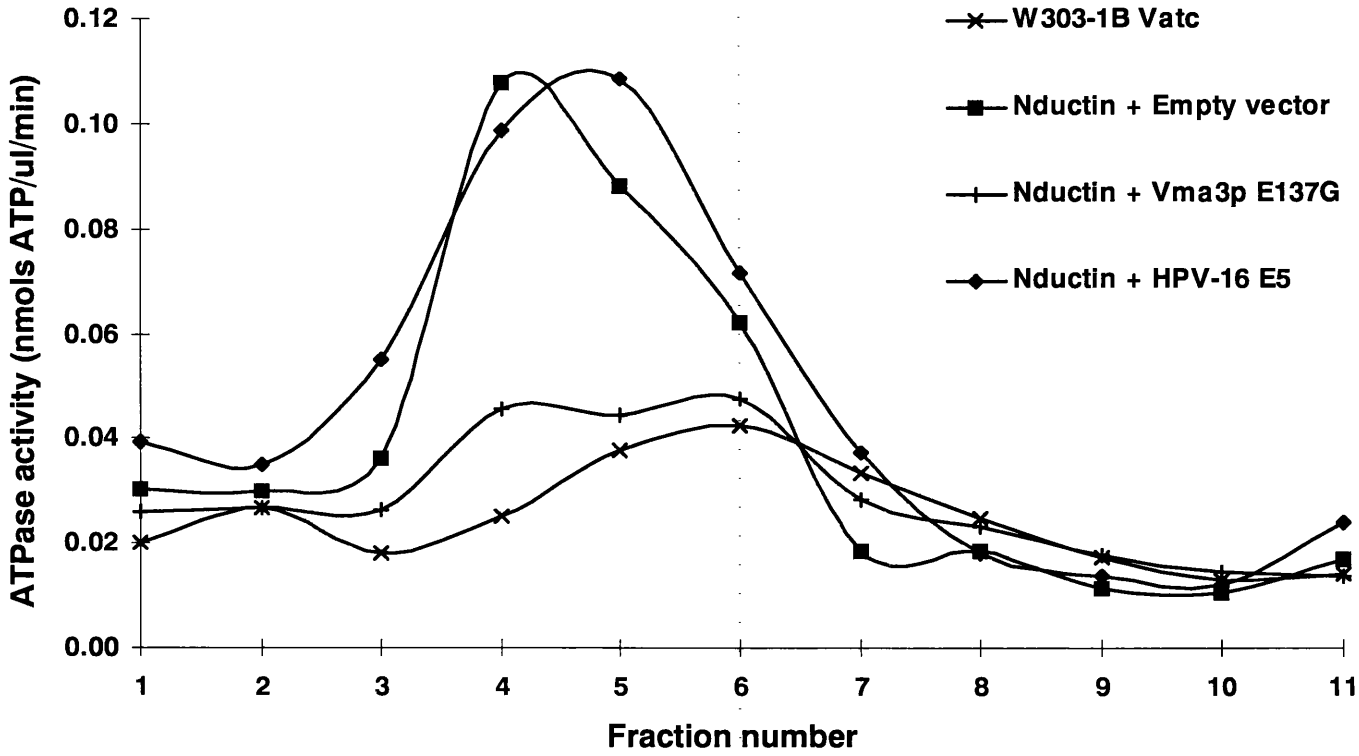

0.12
0.10
0.08
0.06
0.04
0.02
0.00
ATPase activity (nmols ATP/ul/min)
1
2
3
4
5
6
7
8
9
10
11
Fraction number
W303-1B Vatc
Nductin + Empty vector
Nductin + Vma3p E137G
Nductin + HPV-16 E5

Figure 6.5 Western blot analysis of purified V-ATPase fractions from the W303-1B ductin-knockout yeast strain bearing the empty pYPMA expression vector

Whole fractions were precipitated with acetone:ethanol and run on a 10% tris-glycine polyacrylamide gel, (15% tris-tricine for anti-HA blots to enhance resolution). Electroblotting was carried out for 2 hours including 20% methanol in the transfer buffer.

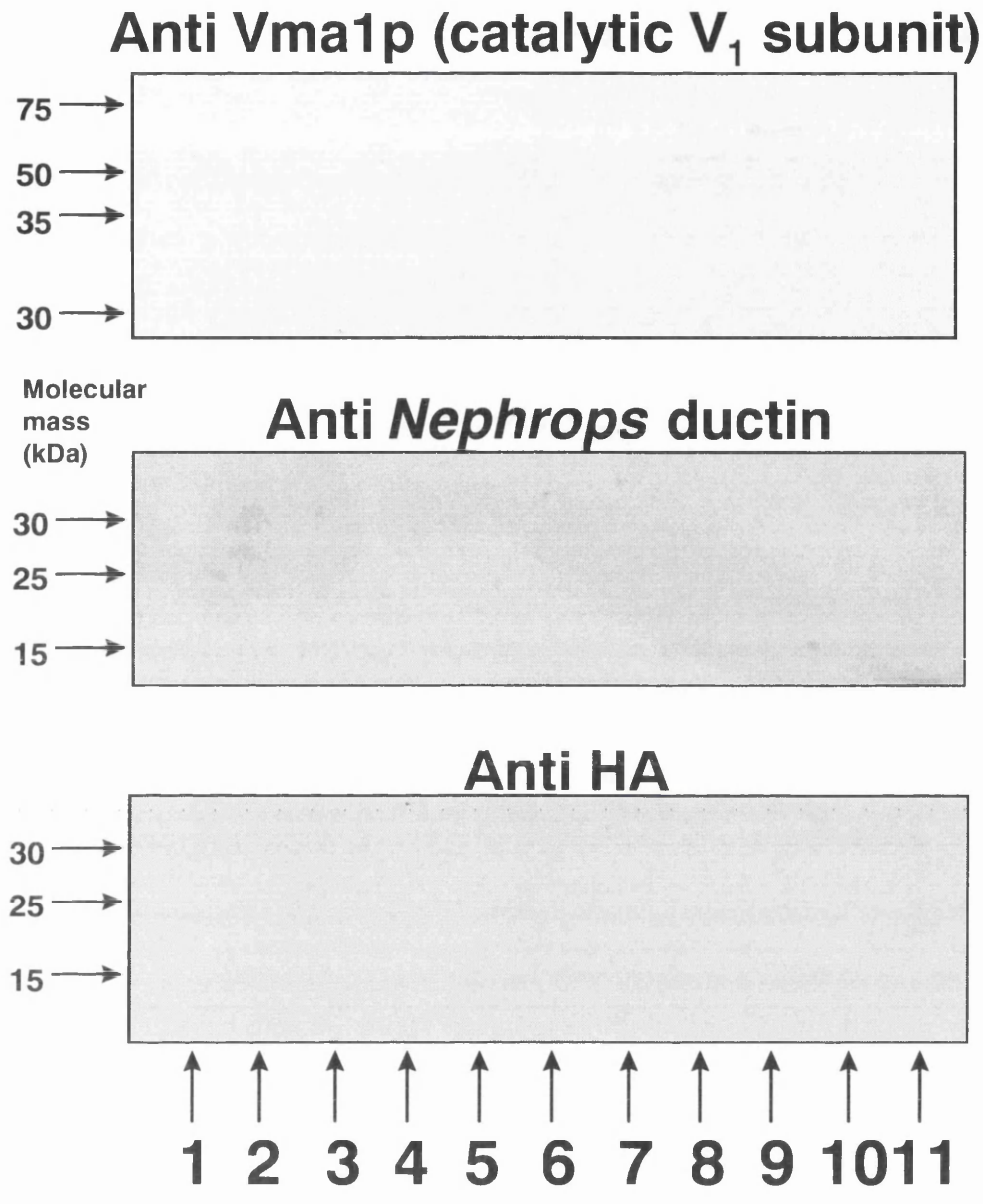

Anti Vma1p (catalytic V_1 subunit)
75
50
35
30
Molecular mass (kDa)
Anti *Nephrops* ductin
30
25
15
Anti HA
30
25
15
1 2 3 4 5 6 7 8 9 10 11

Figure 6.6 Western blot analysis of purified V-ATPase fractions from the W303-1B Vatc yeast strain expressing *Nephrops* ductin and bearing the empty pYPMA expression vector

Methodology was as for W303-1B Vatc vacuoles. Nephrops *ductin is detected by western blotting, and the presence of the catalytic V_1 subunit suggests that it restores assembly of the V-ATPase.*

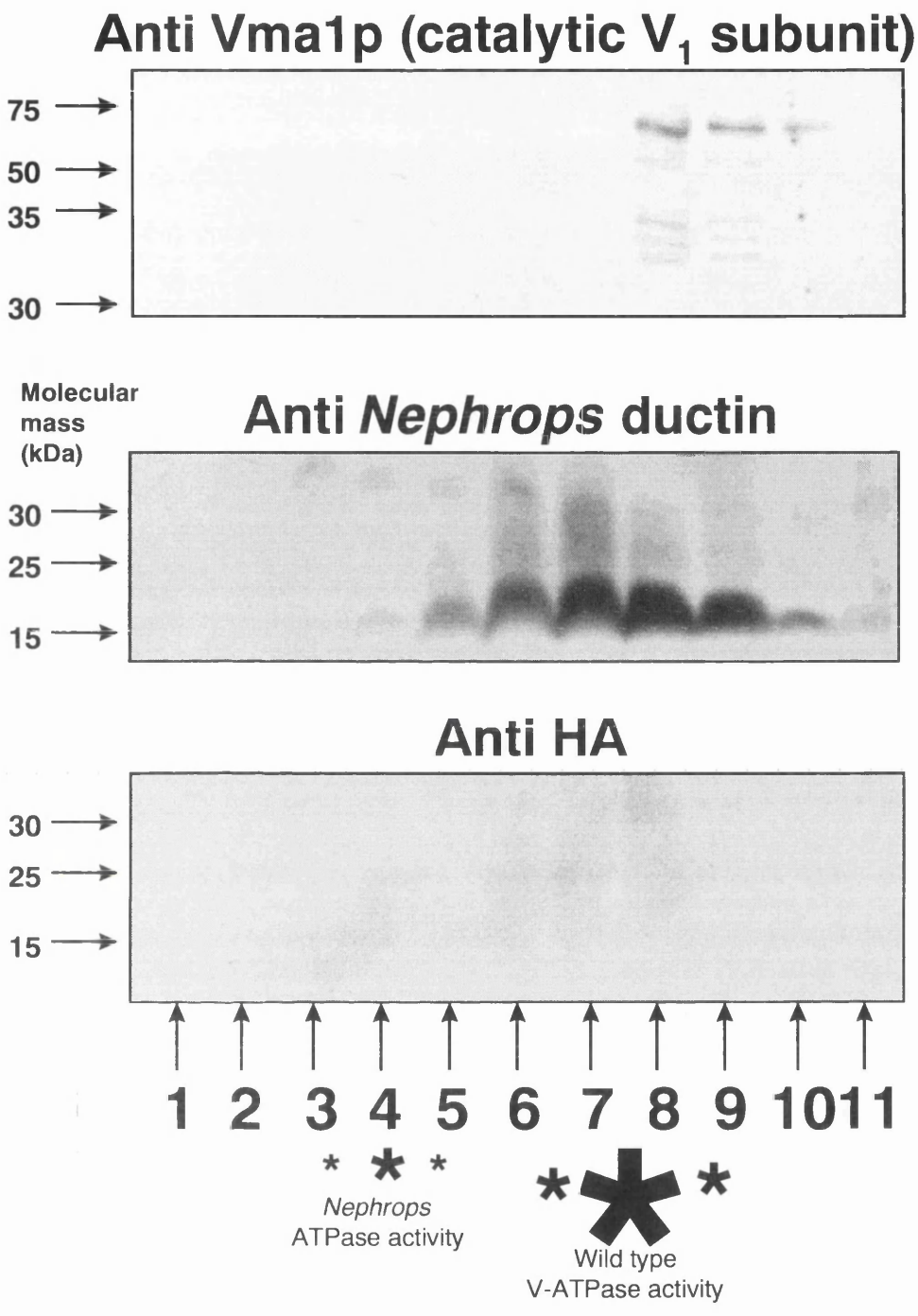

Anti Vma1p (catalytic V_1 subunit)
75
50
35
30
Molecular mass (kDa)
Anti *Nephrops* ductin
30
25
15
Anti HA
30
25
15
1 2 3 4 5 6 7 8 9 10 11
Nephrops ATPase activity
Wild type V-ATPase activity

Figure 6.7 Western blot analysis of purified V-ATPase fractions from the W303-1B Vatc yeast strain expressing *Nephrops* ductin and the dominant negative form of ductin

Methodology was as for W303-1B Vatc vacuoles. Contradictory to results obtained for the wild type enzyme, the appearance of catalytic V_1 subunit suggests that the dominant negative form of S. cerevisiae *ductin does not block assembly of the* Nephrops *ductin V-ATPase.*

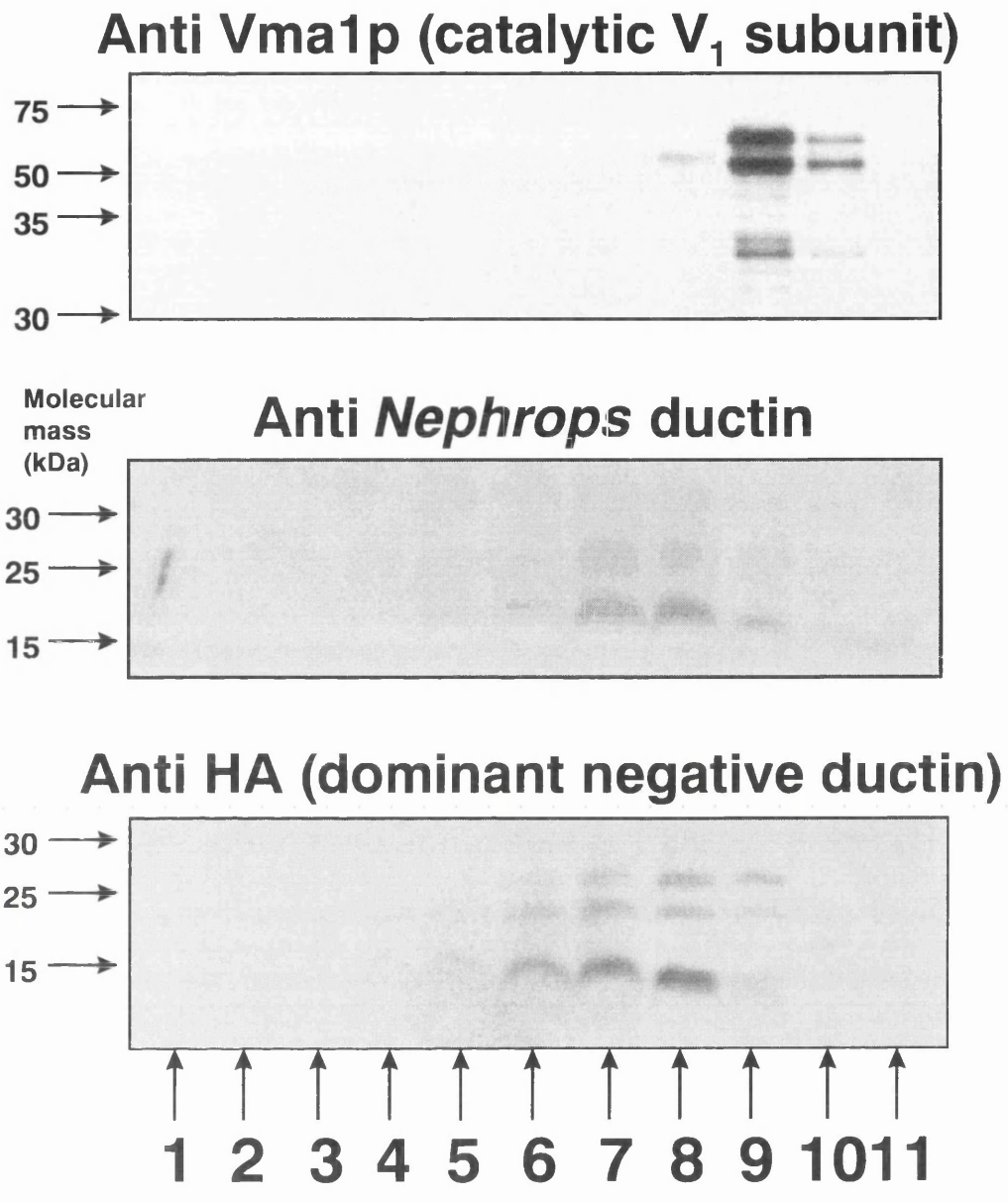
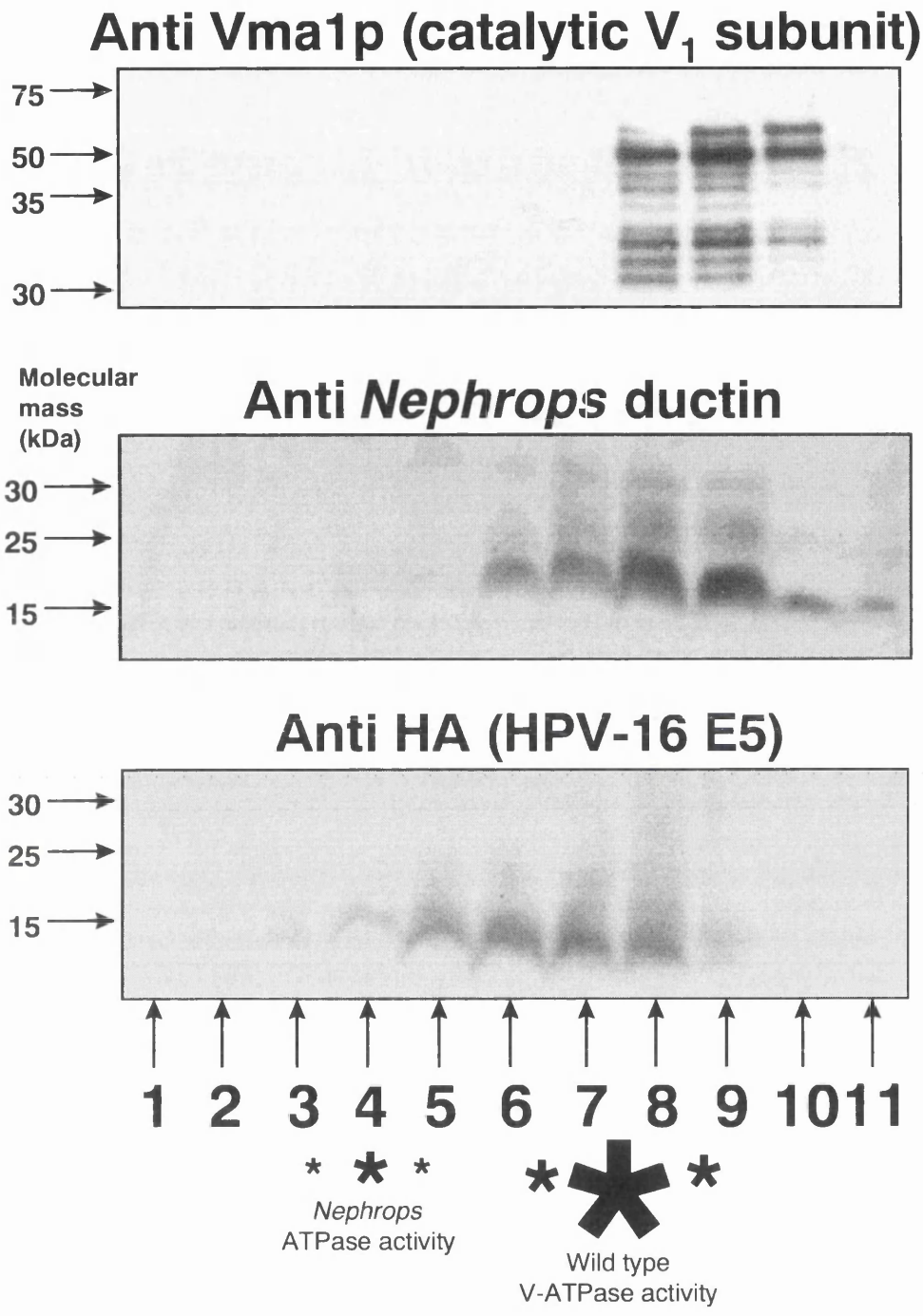

Anti Vma1p (catalytic V_1 subunit)
75
50
35
30
Molecular mass (kDa)
Anti *Nephrops* ductin
30
25
15
Anti HA (dominant negative ductin)
30
25
15
1 2 3 4 5 6 7 8 9 10 11

Figure 6.8 Western blot analysis of purified V-ATPase fractions from the W303 1B-Vatc yeast strain expressing *Nephrops* ductin and the HA-tagged HPV-16 E5 protein

Methodology was as for W303-1B Vatc vacuoles. HPV-16 E5 is found in fractions that contain Nephrops *ductin and catalytic V_1 subunit suggesting that it remains bound to the* Nephrops *ductin V-ATPase* in vivo.


Anti Vma1p (catalytic V_1 subunit)
75
50
35
30
Molecular mass (kDa)
Anti *Nephrops* ductin
30
25
15
Anti HA (HPV-16 E5)
30
25
15
1 2 3 4 5 6 7 8 9 10 11
Nephrops ATPase activity
Wild type V-ATPase activity

7. Discussion

The E5 proteins are a family of small, structurally-related hydrophobic transforming proteins. They all bind to ductin, a proteolipid component of the gap junction and the vacuolar ATPase (V-ATPase) but the effects of this binding are unknown. Both of these complexes are thought to be involved in growth control (Eghbali *et al.*, 1991; Mellman, 1992), but gap junctions are also composed of connexins and their precise role in cell transformation is unclear. Two of the E5 proteins have been shown to inhibit functions associated with the V-ATPase (Schapiro *et al.*, 2000; Straight *et al.*, 1995), so it was suggested that they directly inhibit the enzyme through their association with ductin.

Because of the practical limitations associated with using mammalian cells, it has not been possible to study the direct effect of these proteins on the V-ATPase. These problems are circumvented if yeast is used as the model organism since V-ATPase inhibition can be tolerated by lowering the pH of the growth medium, standard protocols exist for large scale vacuole purification, and subsequent study of the V-ATPase is aided by the availability of antibodies to many of the subunits. The yeast V-ATPase is also almost identical in composition to that in mammalian cells and yeast ductin shares 71% sequence identity with mammalian forms of ductin.

Although yeast is not the natural host of the viruses which encode the E5 proteins, the activities of the proteins in mammalian cells have been well characterised, and the intention of this study was to determine the effects of the E5 proteins on the V-

ATPase in isolation of any of their other effects upon cells. In fact, the differences between yeast and mammalian cells could be beneficial since yeast cells do not express the same growth factor receptors found in mammalian cells, eliminating any competitive effects due to these proteins.

This study addresses for the first time the question of whether the E5 proteins inhibit the V-ATPase through their binding to ductin. Epitope-tagged E5 proteins were analysed for their ability to bind *Saccharomyces cerevisiae* ductin *in vitro*, and to perturb the activity of the V-ATPase *in vivo*, using successive levels of V-ATPase compromisation to aid the detection of subtle effects. Western blot analysis of the purified V-ATPase was also performed to test for their ability to perturb assembly of the V-ATPase, and also for their ability to bind to the V-ATPase *in vivo*.

The growth characteristics of the strains expressing the dominant negative and *Nephrops* forms of ductin demonstrate the different levels of screening that can be achieved by varying the carbon source and enzyme composition. Although no effects of the E5 proteins on the vacuolar function were revealed, this is the first time that these successive levels of stringency have been demonstrated, which may prove useful in future studies of the V-ATPase.

All of the E5 proteins were correctly expressed, and bound to *S. cerevisiae* ductin *in vitro*. The correct expression of the dominant negative ductin and HPV-16 E5 in the vacuole was shown by western blotting, and that of the other E5 proteins was inferred from the specific detection of HA-tagged protein by dot blotting, their correct expression *in vitro*, and detection of the correct plasmid constructs *in vivo*.

The reason why the proteins were not detected by western blotting, and possible solutions were discussed in section 3.3.

7.1 The E5 proteins appear to dissociate from the V-ATPase *in vivo*

Despite binding to ductin *in vitro* (Figure 3.2), none of the E5 proteins perturbed the activity of the V-ATPase in any of the systems tested. One explanation for this may lie in the differences of *in vivo* binding behaviour of HPV-16 E5 in wild type and *Nephrops* transgenic yeast strains, (Figure 7.1). In the wild type system, HPV-16 E5 was not found in fractions possessing V-ATPase activity or containing enzyme subunits. However, in the *Nephrops* transgenic system, HPV-16 E5 migrated differently, being found in fractions containing both ductin and subunit A, suggesting that it remains bound to the enzyme.

An attempt was made to confirm the *in vivo* association of HPV-16 E5 with *Nephrops* ductin in the vacuole (data not shown), exploiting the hexahistidine tag to purify the protein using Nickel chelated to nitrilotriacetic acid (Ni-NTA), reviewed by Crowe *et al.*, (1994). This overcomes the practical restrictions associated with the production of radiolabelled vacuolar material prior to immunoprecipitation, and the expense of immunoprecipitation and western blotting of unlabelled vacuolar material. Unfortunately, the purification method is designed for hydrophilic proteins, and the columns appeared to bind hydrophobic proteins regardless of whether they possessed a hexahistidine tag. Were sufficient antibody and vacuolar material available, the *in vivo* association of the HPV-16 E5 with ductin and the V-ATPase could be probed in detail by

immunoprecipitation and western blotting of individual fractions from purifications of the wild type and chimeric V-ATPase.

Direct observations of rotational catalysis by the F-ATPase showed that the enzyme is capable of generating high levels of torque (Noji *et al.*, 1997). This torque is suggested to drive the rotation of the critical acidic residue past static subunit a (Jiang and Fillingame, 1998). As the V-ATPase shares structural and mechanistic features with the F-ATPase, it seems likely that a similar torque will be generated in the ductin complex of Vo which is sufficient to dislodge any proteins associated with ductin in the V-ATPase. In addition, if the conformational changes seen upon deprotonation of the Fo subunit c (Rastogi and Girvin, 1999) also occur in ductin, which seem likely given the high levels of structural homology between the two proteins in this region (Pali *et al.*, 1999; Harrison et al., manuscript submitted), then this means that the BPV-1 E5 binding site will be altered during catalysis (Figure 7.2). Therefore, the active V-ATPase may cause the E5 proteins to dissociate from ductin. However, the situation may be different in the *Nephrops* transgenic strain since the Km for ATP is 10 times that of the wild type enzyme and there could be partially active V-ATPase complexes which contain E5 proteins. Nevertheless, even this possibility does not appear to inhibit V-ATPase activity (Figure 6.3).

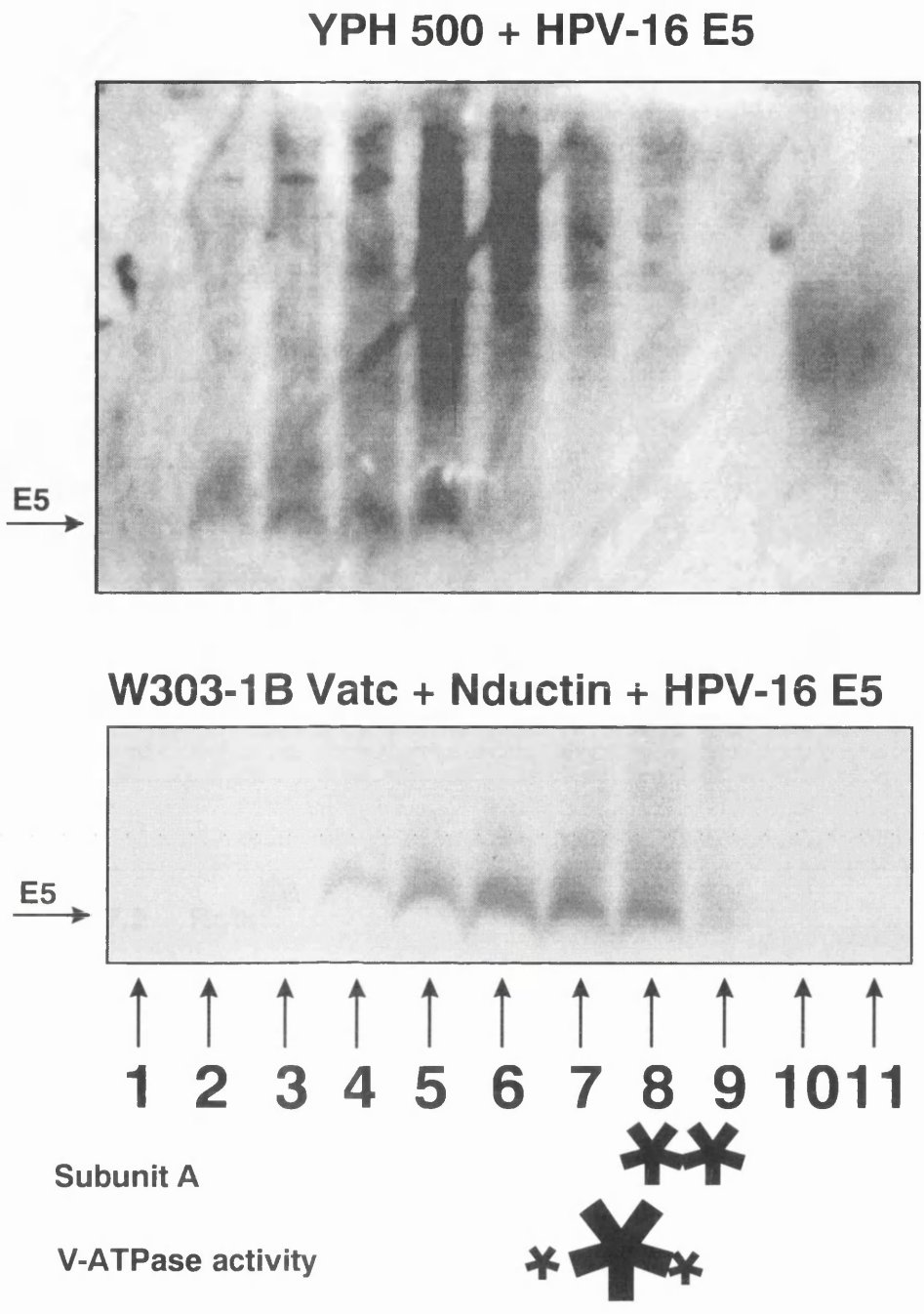


Figure 7.1 HPV-16 E5 appears to dissociate from the active V-ATPase

Comparison of HPV16-E5 localisation with respect to ductin in wild type and Nephrops *ductin transgenic* Saccharomyces cerevisiae. *The location of the two forms of ductin and HPV-16 E5 are revealed by western blotting, and demonstrate differential migration of HPV-16 E5 in the two systems.*

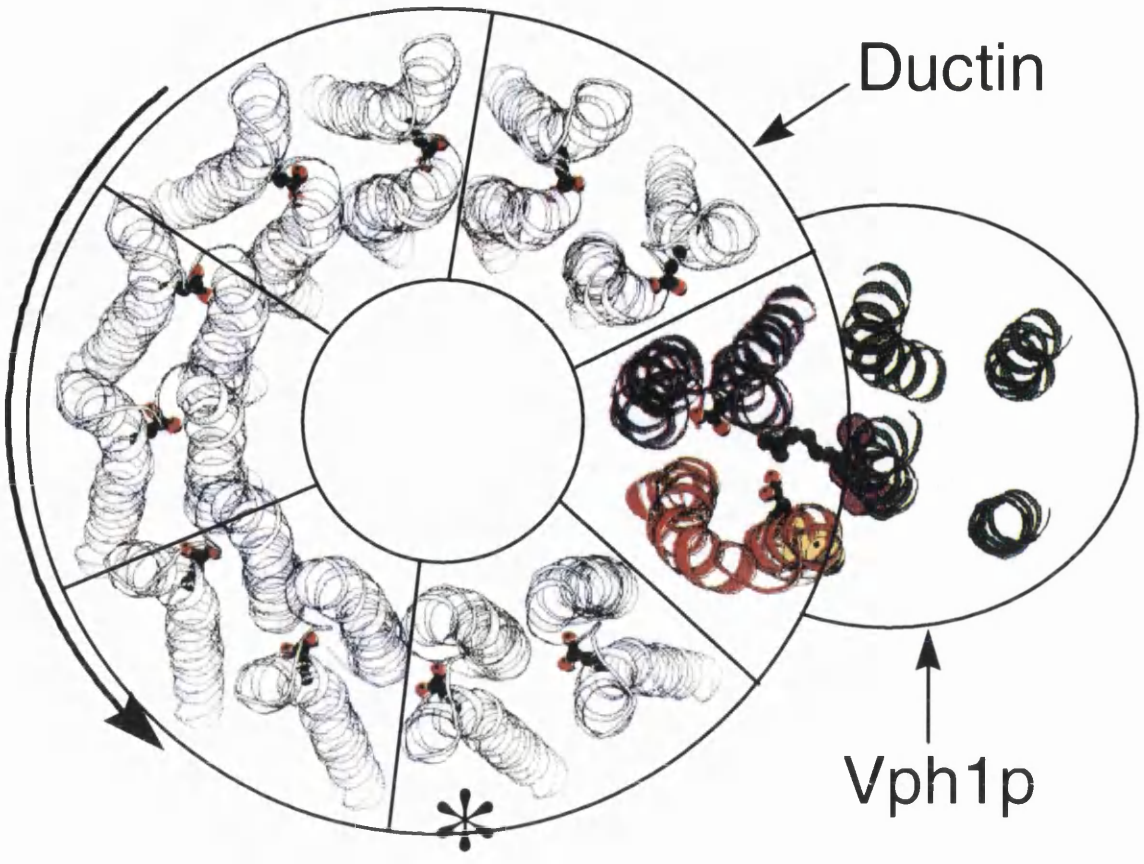


Figure 7.2 Rotational catalysis may cause the dissociation of the E5 proteins from ductin

The organisation of the subunits in the Vo sector of the V-ATPase is inferred from that of the F-ATPase (Rastogi and Girvin, 1999). Ductin is depicted as a four-helix bundle forming a hexameric complex, and the static glycoprotein Vph1p is coloured in green. If the scheme of rotational catalysis is correct, then the ductin hexamer rotates past Vph1p to cause proton translocation, changing conformation as it does so, indicated by the red and purple helices. This conformational change would also affect the proposed BPV-1 E5 binding site (indicated with an asterisk) which, coupled with the amount of torque generated by the F-ATPase (Noji et al., *1997) may be sufficient to disrupt the binding of the E5 proteins.*

The dissociation of the E5 proteins is consistent with their inability to inhibit the V-ATPase and is reminiscent of the association between the Vma12/Vma22p complex with Vph1p. Vph1p is a membrane protein component of the V-ATPase but far from causing inhibition of the enzyme, the binding of the Vma12p/Vma22p assembly complex is actually required for its activity (Graham *et al.*, 1998). Interestingly, Vma12p is of similar size and hydrophobicity to BPV-1 E5 (Jackson and Stevens, 1997), and also appears to dissociate from the active V-ATPase (Graham *et al.*, 1998).

So if the conclusions presented in this thesis are correct, that the E5 proteins do not disturb the activity of the wild type or chimeric V-ATPase, how are the observations that expression of BPV-1 E5 and HPV-16 E5 cause alkalinisation of the Golgi and early endosome respectively (Schapiro *et al.*, 2000; Straight *et al.*, 1995) accounted for? One explanation may lie in the fact that neither yeast nor *Nephrops* are the natural host of the papillomaviruses. However, given the high sequence conservation of ductin, and that all of the E5 proteins tested so far bind to yeast Vma3p and *Nephrops* ductin, this seems unlikely. Therefore, it is more likely that the disturbance of the Golgi and endosome pH is a secondary effect of the E5 proteins.

7.2 Alternative mechanisms of V-ATPase inhibition by the E5 proteins

BPV-1 E5 could inhibit Golgi and vacuolar acidification indirectly (Figure 7.3 G, H), for example Golgi alkalinisation has been observed in response to stimulation of protein kinase A or protein kinase C (Seksek *et al.*, 1995). These kinases are involved in the PDGF-R signalling pathway (deBlaquiere *et al.*, 1994; Li *et al.*,

1994) but transformation of cells with upstream elements of this pathway (the *sis* and *src* oncogenes) did not have any effect on the pH of the Golgi (Schapiro *et al.*, 2000). The pH of the Golgi and the vacuole was not determined in the transformed yeast strains, but the accumulation of red adenine precursors and growth at pH 7.5 (Figure 4.3) suggested that vacuolar function was not compromised.

BPV-1 E5 requires Golgi localisation to cause cell transformation (Sparkowski *et al.*, 1995). Because it, and all of the other E5 proteins are so small, they may lack targeting information that would allow them to exit the ER. Ductin binding might therefore be a mechanism by which the E5 proteins escape the ER (Figure 7.3 A), with rotational catalysis of the V-ATPase ensuring their release, so that they can interact with other cellular targets.

Although Golgi alkalinisation correlates with cell transformation by BPV-1 E5 (Schapiro *et al.*, 2000), ductin binding does non always correlate with cell transformation (Sparkowski *et al.*, 1995; Sparkowski *et al.*, 1996) which is also found for BPV-4 E8 and HTLV-1 $p12^{I}$ (Ashrafi *et al.*, 2000; Koralnik *et al.*, 1995). Further evidence that the E5 proteins do not transform cells by directly inhibiting the V-ATPase comes from studies using the specific V-ATPase inhibitor, bafilomycin A_1 on cultured mammalian cells.

Bafilomycin A_1 was found to inhibit all aspects of receptor downregulation: endocytosis, degradation and recycling (Harada *et al.*, 1996; Johnson *et al.*, 1993; Yoshimori *et al.*, 1991), whereas BPV-1 E5 only inhibited degradation (Waters *et al.*, 1992), and HPV-16 E5 caused only a delay in internalisation and degradation,

and was actually found to increase the rate of recycling (Straight *et al.*, 1993). These studies, as well as those that demonstrated compartmental alkalinisation effects of the E5 proteins (Schapiro *et al.*, 2000; Straight *et al.*, 1995) acknowledged that a direct study would be required to prove that the effects on the enzyme were not due to secondary effects of the proteins. Alternative explanations were also proposed, for example that BPV-1 E5 alters the affinity of EGF-R for EGF (Waters *et al.*, 1992), or that HPV-16 E5 binding to ductin retards the delivery of the V-ATPase to endosomes (Straight *et al.*, 1995).

High levels of bafilomycin A_1 are cytotoxic (Ohkuma *et al.*, 1993), but although BPV-4 E8 is cytotoxic in the absence of other transforming proteins (Pennie *et al.*, 1993) overexpression of BPV-1 E5 is not (Burkhardt *et al.*, 1989). Bafilomycin A_1 and HPV-16 E5 have also been studied in respect of their effects on cell morphology and migration (Thomsen *et al.*, 1999). Although both inhibited cell mobility, they had different effects upon cell morphology, and the cooperativity of their effects upon motility led to the suggestion that they affected the V-ATPase in different ways, or that the action of HPV-16 E5 was indirect.

7.3 Possible mechanisms of cell transformation by the E5 proteins

The data presented in this thesis suggest that the E5 proteins do not directly inhibit the V-ATPase. An alternative reason for binding to ductin has already been discussed, as has the possibility of indirect inhibition of the activity of the V-ATPase. There are also a number of ways in which the proteins could transform cells independently of their effects upon the V-ATPase, summarised in Figure 7.3.

Most of the E5 proteins bind to growth factor receptors (Hwang *et al.*, 1995; Mulloy *et al.*, 1996; Petti and DiMaio, 1992). Although the role of receptor binding for BPV-1 E5 and HPV-16 E5 is disputed (Conrad *et al.*, 1994; Sparkowski *et al.*, 1996), perhaps this binding and subsequent stimulation of mitogenic activity (Figure 7.3 D, J) represents one part of their transforming activity. BPV-1 E5 has also been shown to bind to p125, an α-adaptin protein involved in endocytic vesicle formation (Cohen *et al.*, 1993b) suggesting that it may be able to slow the rate of receptor downregulation independently of effects on the V-ATPase (Figure 7.3 B), but the effects of this interaction have yet to be shown.

The perturbation of EGF-R processing in BPV-1 E5-expressing cells (Waters *et al.*, 1992) may have been due to disruption of the endocytic pathway by inhibition of the V-ATPase, but one of the original suggestions was that E5 expression altered the affinity of EGF-R for its ligand, which in turn reduced the rate at which it was processed (Waters *et al.*, 1992). One possibility is that the alkalinisation of the Golgi by BPV-1 E5 prevented protein maturation (Figure 7.3 I), causing immature growth factor receptors to be expressed at the cell surface which are more resistant to degradation than the mature forms. Disruption of protein maturation in the Golgi has also been suggested to hinder the presentation of major histocompatibility complexes on the cell surface, enabling BPV-1 to escape detection by the immune system (Schapiro *et al.*, 2000). It may also be that the binding of E5 is what alters the affinity to produce a similar resistance to degradation (Figure 7.3 E), which is consistent with the increased rate of recycling seen for HPV-16 E5 expression (Straight *et al.*, 1993).

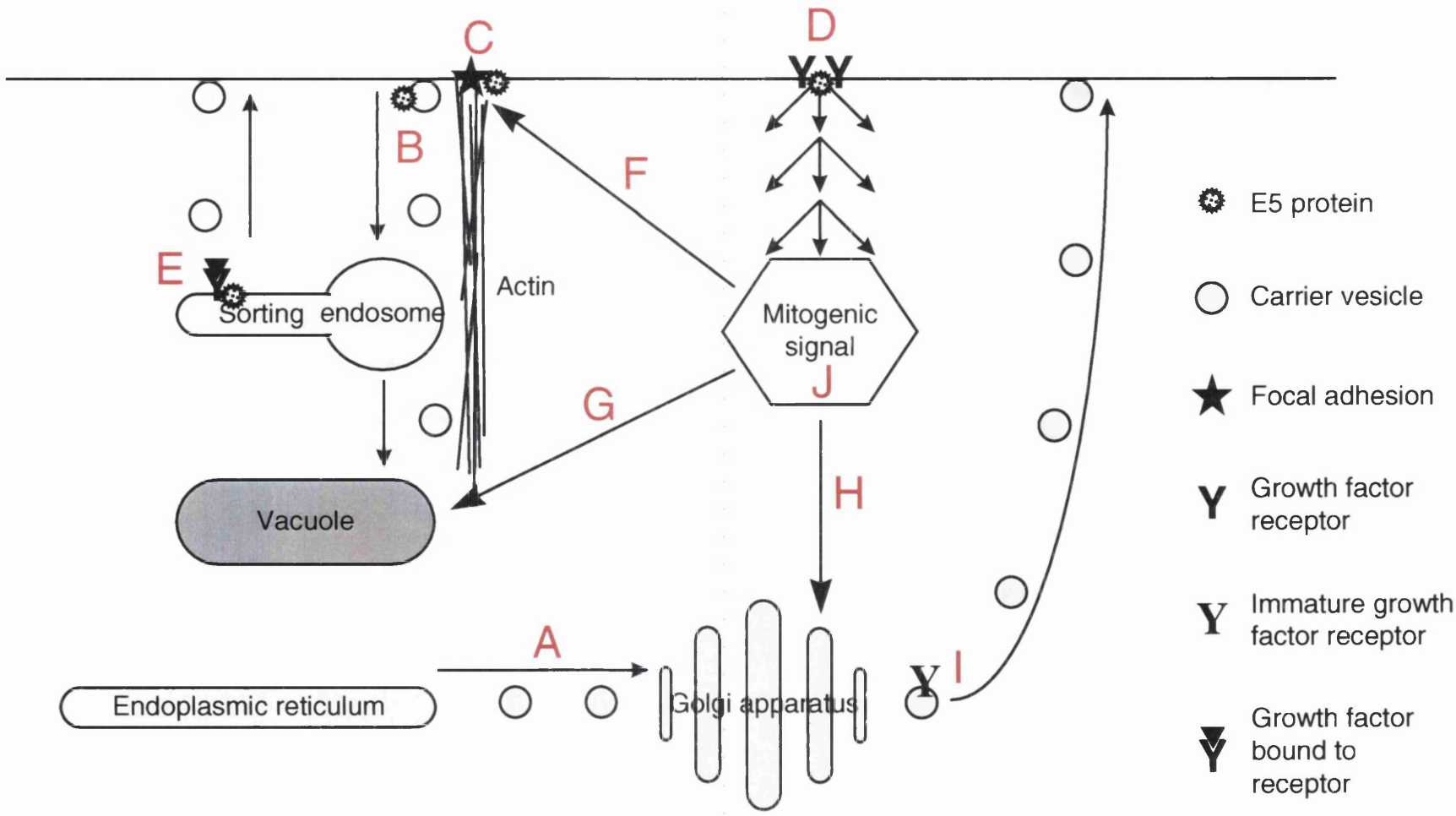
In addition to its roles in the V-ATPase, gap junction and mediatophore, ductin has been shown to bind to β_1-integrin (Skinner and Wildeman, 1999). The binding site is the same as that for BPV-1 E5, so perhaps the E5 proteins compete with, and inhibit the binding of β_1-integrin to ductin (Figure 7.3 C). In addition to this, the PDGF signalling pathway is thought to converge with integrin-mediated signalling at the focal adhesion (Figure 7.3 F), (Rankin and Rozengurt, 1994), which has been suggested to explain some of the activities of the E5 proteins (Skinner and Wildeman, 1999) and may explain the incomplete correlation seen between the various binding activities and transforming potencies (Ashrafi *et al.*, 2000; Sparkowski *et al.*, 1995; Sparkowski *et al.*, 1996).

Integrins link the cytoskeleton to the extracellular matrix (Sastry and Horwitz, 1993) so it was interesting to see that the cellular morphology and contact effects could be altered by overexpression of a form of ductin lacking the fourth helix (Skinner and Wildeman, 1999), and that BPV-1 E5 expression alters actin distribution (Bergman *et al.*, 1988), which is normally regulated by integrins (Defilippi *et al.*, 1999).

Actin is involved in maintenance of the ER and the Golgi, and is suggested to be involved in endosomal vesicle transport (Lamaze *et al.*, 1997), reviewed by DePina and Langford, (1999) so perhaps the cytoskeletal disruption by E5 is what causes the changes in vesicle transport and in the Golgi apparatus.

Figure 7.3 Possible modes of action of the E5 proteins

Ductin binding may represent a mechanism to escape the ER (A) whence it disrupts cellular activity directly (B, C, D) or indirectly (E, F, G, H, I), the eventual effect being to stimulate cell division (J).


C
D
B
F
E
Sorting endosome
Actin
Mitogenic signal
J
G
H
Vacuole
A
Endoplasmic reticulum
Golgi apparatus
I
E5 protein
Carrier vesicle
Focal adhesion
Growth factor receptor
Immature growth factor receptor
Growth factor bound to receptor

7.4 Further work

Despite structural similarities, and the conserved ductin binding of the E5 proteins, it is not known if they transform cells by the same mechanism. For example, it is not known if BPV-4 E8 binds to growth factor receptors, and the ductin-binding domain of HTLV-1 $p12^{I}$ has been shown not only to be dispensable, but to be located in a different region to that of BPV-1 E5 (Koralnik *et al.*, 1995). It might therefore, be useful to determine if the proteins share a common mechanism before continuing with their investigation, by determining if they can be functionally interchanged.

A similar approach could be used to determine their mode of action. Dominant negative ductin has been shown to promote anchorage-independent growth (Andresson *et al.*, 1995), as did an internalisation-defective EGF-R mutant (Wells *et al.*, 1990). If single or multiple combinations of these proteins could overcome the requirement for E5 proteins for normal viral function, then their relative contributions to the mode of E5 protein action could be determined.

The interaction of the E5 proteins with ductin could be studied more extensively in *S. cerevisiae*. In addition to the suggestion in section 7.1, expression of epitope tagged forms of wild-type, human and bovine ductin alongside the epitope-tagged E5 proteins would allow immunoprecipitation in order to determine the extent of interaction in a number of cellular locations, which would indicate if ductin represents their final target or if it acts as an intermediate. The suggestion that the E5 proteins are dislodged by rotational catalysis by the V-ATPase could be tested by determining the V-ATPase activity of these compartments and

correlating this with the level of binding. Alternatively, *VPH1* and *VMA11* mutant strains capable of V-ATPase assembly but not catalysis, as used by (Parra and Kane, 1998) could be used to determine if catalysis causes dissociation of the E5 proteins.

To test the hypothesis that ductin binding is a mechanism of escaping the ER, the effects of mutations that disrupt the ductin binding ability of the E5 proteins could be studied in respect of their effects upon the cellular localisation of the E5 proteins in the cell. Similarly, if this mechanism of exiting the ER is a common feature of small hydrophobic proteins, it may be interesting to see if there are other such proteins that bind to ductin, for example, viral proteins which have been shown to exhibit sequence similarities with the E5 proteins (Garry, 1995).

7.5 Summary

The work presented in this thesis demonstrates the usefulness of yeast for studying the V-ATPase, and shows that the E5 proteins do not directly inhibit the V-ATPase in the vacuole, nor remain associated with the active enzyme. This suggests that observations of compartment alkalinisation (Schapiro *et al.*, 2000; Straight *et al.*, 1995) are due to indirect effects, and that the interaction with ductin may serve another purpose.

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