

**Analysis of the Fowlpox virus
homologue of
Mammalian PC-1 glycoprotein**

by

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with the requirements of the University of London
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Analysis of the Fowlpox virus homologue of Mammalian PC-1 glycoprotein

Abstract

Fowlpox virus (FWPV) is the prototypic member of the genus Avipoxviridae. Although no longer a commercial threat to livestock, in recent years it has been extensively used for expression of foreign antigens, as recombinant vaccines for avian and non-avian targets. Despite this attention, FWPV remains poorly characterised. Sequence analysis of a 6.5kbp region near the left inverted terminal repeat of the FWPV genome had revealed five major ORFs not previously observed in any other virus. One of these ORFs (FP-PC1), exhibited 39% amino acid identity to mammalian PC1 glycoprotein, initially described as an antigen of terminally differentiated B-cells. The full length FP-PC1 gene was cloned directly from the FWPV genome using specific primers. Using the transient dominant selection technique, the resultant transfer vector was used to construct three recombinant FWPV, with different deletions in the FP-PC1 ORF. The FP-PC1 gene was shown to be non-essential for FWPV replication *in vitro*, as FWPV with a full gene deletion in the FP-PC1 ORF was readily isolated. Further characterisation of the mutant FWPV indicated that FP-PC1 did not affect FWPV replication *in vitro*, although a difference in plaque size was observed. A possible involvement of FP-PC1 in the nucleotide salvage pathway was investigated by comparing wt and mutant FWPV growth in nucleotide deficient media. But no difference was observed. Thus further investigation needs to be conducted to investigate a potential role for FP-PC1 as a potential scavenger of nucleotides. Both the full length and extracellular FP-PC1 domains were expressed in an *in vitro* cell free system using rabbit reticulocyte lysates. They were also overexpressed in FWPV infected cells, co-infected with a recombinant FWPV expressing T7 polymerase. Using data from both these systems, full length FP-PC1 was shown to be an N-linked glycosylated integral membrane protein with a type II configuration.

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.c1.Abbreviations

AP1/NPP	Alkaline Phosphodiesterase 1/Nucleotide pyrophosphatase
ATP	Adenosine Triphosphate
cAMP	Cyclic Adenosine Monophosphate
cDNA	complementary DNA
CEV	Cell associated extracellular virus
CPPD	Calcium pyrophosphate deposition disease
crm	cytokine response modifier
CTT	Coupled Transcription Translation
DNA	Deoxyribonucleic acid
ds	Double stranded
EEV	Extracellular enveloped virus
FWPV	Fowlpox virus
GPI	Glycophosphatidylinositol
IBDV	Infectious Bursal Disease Virus
IC	Intermediate Compartment
IFN	Interferon
IMV	Intracellular Mature virus
IR	Insulin receptor
IV	Immature virus
kDa	Kilo dalton
Mab	Monoclonal antibody
MDV	Mareks disease virus
mRNA	Messenger RNA
NIDDM	Non-Insulin dependent diabetes mellitus
NK	Natural Killer
NTPPPH	Nucleotide Pyrophosphohydrolase
ORF	Open reading frame
PHA	Phytohaemagglutinin
PPi	Inorganic phosphate
RNA	Ribonucleic acid
SFV	Shope fibroma virus
ss	Single stranded
TNF	Tumour necrosis factor
TRTV	Turkey rhinotracheitis virus
V V	Vaccinia virus

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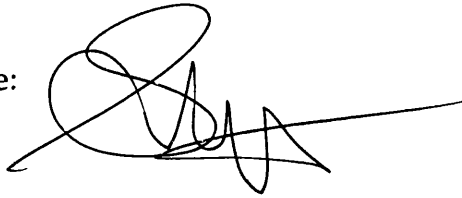
*This thesis is dedicated to my beloved late grandmother
Fatima, from whom I, and all around her, drew their
hopes, their peace and their inspiration, in the hope that
one day, we too, could be a shadow of her - in life and now
in her tranquility.*

Declaration

I declare that the work reported in this thesis was performed by myself except for analysis of FP-PC1 transcripts by northern blotting (fig. 4.6) and PCR amplification of the FP-PC1 ORF across different FWPV strains (fig. 3.3) (both performed by S. Laidlaw). All *in vivo* experiments were carried out under the supervision of P. Green.

Candidate: M. A. Anwar

Signature:



I certify that the work reported in this thesis was performed by M. A. Anwar, except as indicated above.

Supervisor: Dr. M. A. Skinner

Signature:



7-5-99

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Chapter 1

Introduction

1.1 Classification of poxviruses

Poxviruses are divided into two subfamilies, the *Chordopoxvirinae* and the *Entomopoxvirinae*, on the basis of vertebrate and invertebrate host range respectively.

The *Chordopoxvirinae* are by far the best characterised and are subdivided into eight genera as shown below (Table 1.1). They are antigenically and genetically related as they share a common antigen (Woodroffe, 1962), and undergo non-genetic reactivation between genera (Fenner, 1960).

The *Entomopoxvirinae*, remain largely uncharacterised (Table 1.2) and are related to the *Chordopoxvirinae* on the basis of DNA sequence homology to Thymidine kinase, DNA polymerase and nucleoside triphosphate phosphohydrolase 1 (Moss, 1996).

1.2 Poxvirus structure and genome

Definitive characteristics of the *Poxviridae* family include a large double stranded linear genome, ranging in size from 140Kbp to 380Kbp, (*parapoxviruses* and *entomopoxviruses* respectively), cytoplasmic location of replication and large size. Uniquely among viruses they can be filtered and are observable by light microscopy. The virion is brick or ovoid in shape, and in the case of fowlpox virus (FWPV) is 100 x 250 x 300nm in size, the exterior being covered by surface tubule elements. As well as cell associated enveloped virus (CEV), there are two morphologically distinct infective virions, the extracellular enveloped virus (EEV) and intracellular

Table 1.1 Classification of Chordopoxvirinae

Chordopoxvirinae					
Genera	Member viruses	Features			
		GC%	Size	Shape	Host range
Orthopoxvirus	Vaccinia , variola, ectromelia, cowpox	36	~200Kb	Brick	Varied
Parapoxvirus	Orf , chamois, pseudocowpox	64	~140Kb	Ovoid	Ungulates
Avipoxvirus	Fowlpox , turkeypox, pigeonpox, canarypox.	35	~260Kb	Brick	Birds
Capripoxvirus	Sheepox , lumpy skin disease, goatpox.		~150Kb	Brick	Ungulates
Leporipoxvirus	Myxoma , rabbit fibroma, squirrel fibroma.	40	~160Kb	Brick	Leporids and squirrels
Suipoxvirus	Swinepox		~170Kb	Brick	Swine
Molluscipoxvirus	Molluscum contagiosum .	60	~180Kb	Brick	Man
Yatapoxvirus	Yaba monkey tumour , tanapox	33	~145Kb	Brick	Primates and rodents

Table modified from (Moss 1996). Bold type indicates prototypic member of genera.

Table 1.2 Classification of Entomopoxvirinae

Entomopoxvirinae					
Genera	Member viruses	Features			
		GC%	Size	Shape	Host range
Entomopoxvirus A	<i>Melolontha melolontha</i>		~370Kb	Ovoid	Coleoptera
Entomopoxvirus B	<i>Amscata moorei</i>	18.5	~225Kb	Brick	Lepidoptera, orthoptera
Entomopoxvirus C	<i>Chironimus luridus</i>		~380Kb	Brick	Diptera

Table modified from (Moss 1996).

mature virus (IMV), the latter form is usually more abundant, but ratios vary depending on genera and strain. In the *Chordopoxvirinae* the protein associated genome is present in a biconcave core at the centre of the virus, flanked by two lateral bodies of unknown function. The central 60% of the genome in Orthopoxviruses is well conserved whereas considerable variation is seen at the end (Mackett, 1979). The ends consist of inverted terminal repeats of up to 15Kbp (Coupar *et al.*, 1990), and are joined together by an incompletely base paired hairpin loop. Poxviruses have been extensively reviewed (Goebel *et al.*, 1990; Johnson *et al.*, 1993; Moss, 1990; Moss, 1991; Moss & Flexner, 1987; Smith, 1993).

Most genes characterised from poxviruses are from vaccinia virus (VV), 200 potential ORFs are suggested (Johnson *et al.*, 1993), VV ORFs are tightly packed and have no introns (Goebel *et al.*, 1990; Smith *et al.*, 1991). Other poxviruses including variola, Shope fibroma virus, ectromelia virus, myxoma and racoonpox have also been characterised, but to a lesser extent (Cavallaro & Esposito, 1992; Douglass *et al.*, 1994; Ichihashi & Kitamura, 1976; Massung *et al.*, 1994; Mills & Pratt, 1980; Mossman *et al.*, 1995; Sonntag & Darai, 1996; Thomas *et al.*, 1975). In contrast, only a few FWPV genes have been characterised, and are discussed later.

Essential genes tend to be located in central regions of the genome. Examples include enzymes involved in transcription, such as the mRNA capping enzyme (Niles *et al.*, 1989), RNA polymerase subunits (Amegadzie *et al.*, 1991a; Amegadzie *et al.*, 1991b; Broyles & Moss, 1986; Jones *et al.*, 1987) transcription factors (Zhang *et al.*, 1992) and poly A polymerase (Moss *et al.*, 1975) as well as structural proteins (Dyster & Niles, 1991; Kao & Bauer, 1987; Wittek *et al.*, 1984; Yang & Bauer, 1988) and genes involved in DNA replication (Earl *et al.*, 1986). Non-essential genes are usually located in

terminal regions. Notable examples include genes involved in host inhibition, such as, serine protease inhibitors (Boursnell *et al.*, 1988; Kotwal & Moss, 1989; Law & Smith, 1992) and cytokine receptors or other proteins involved in inhibition of the host immune response (Davies *et al.*, 1993; Davies *et al.*, 1992; Kotwal *et al.*, 1990) .

1.3 Viral life cycle

Knowledge of the poxviral life cycle has been accumulated mostly by study of VV, however in general it can be applied to other poxviruses. Evidence that a similar transcription mechanism is involved is suggested as VV promoters work efficiently when introduced into FWPV and vice versa (Boyle & Coupar, 1986; Boyle & Coupar, 1988; Kumar & Boyle, 1990a).

The replication cycle consists of virus entry followed by gene expression and DNA replication, leading to assembly, maturation and release of virions (fig. 1.1).

1.3.1 Attachment and entry

The mechanism of VV attachment and entry is not well characterised and no specific viral attachment protein or a complementary cellular receptor has been conclusively described. The issue is further complicated as there are two infectious forms - IMV and EEV. The latter form has a higher relative infectivity and is required for efficient long distance dissemination (Moss, 1996).

Previous electron microscopy studies on IMV had indicated that entry involved endocytosis or direct fusion at the plasma membrane (Chang & Hertz, 1976; Dales & Kajioaka, 1964). Later evidence implicated pH independent fusion at the plasma membrane as a major mode of entry for IMV and EEV (Janeczko *et al.*, 1987, Doms *et al.*, 1990), as entry and uncoating were not inhibited by addition of chloroquine, methylamine and monensin. Cellular fractionation also showed association of virions and viral polypeptides with plasma membranes and not with endosomal fractions (Janeczko *et al.*, 1987). Fusion of both IMV and EEV occurred at neutral pH and was not acid dependent, with optimal fusion occurring at 37°C (Doms *et al.*, 1990).

It has become progressively evident that IMV and EEV utilise different receptors. IMV fusion was completely inhibited by an antibody to the 14kDa protein, whereas EEV was unaffected (Rodriguez *et al.*, 1987). Attachment of both IMV and EEV were unaffected by the MAB to the 14kDa protein (Doms *et al.*, 1990; Rodriguez *et al.*, 1987).

However, as purified EEV can often become damaged and lead to susceptibility to IMV specific MAb (Ichihashi, 1996), a method utilising confocal microscopy and fluorescent labelling was developed which allowed differentiation between EEV and IMV without purification and the resulting disruption of EEV outer membranes (Vanderplasschen & Smith, 1997). A monoclonal antibody to the B2 trypsin sensitive surface epitope was shown to block IMV infection (Chang, 1995), but had no effect on EEV (Vanderplasschen & Smith, 1997). Further evidence indicated that EEV binding efficiency to BSC-1, RK₁₃ and HeLa cells varied considerably whereas no such variation was observed with IMV, suggesting variation in receptor content between the stated cell lines. Furthermore, pronase

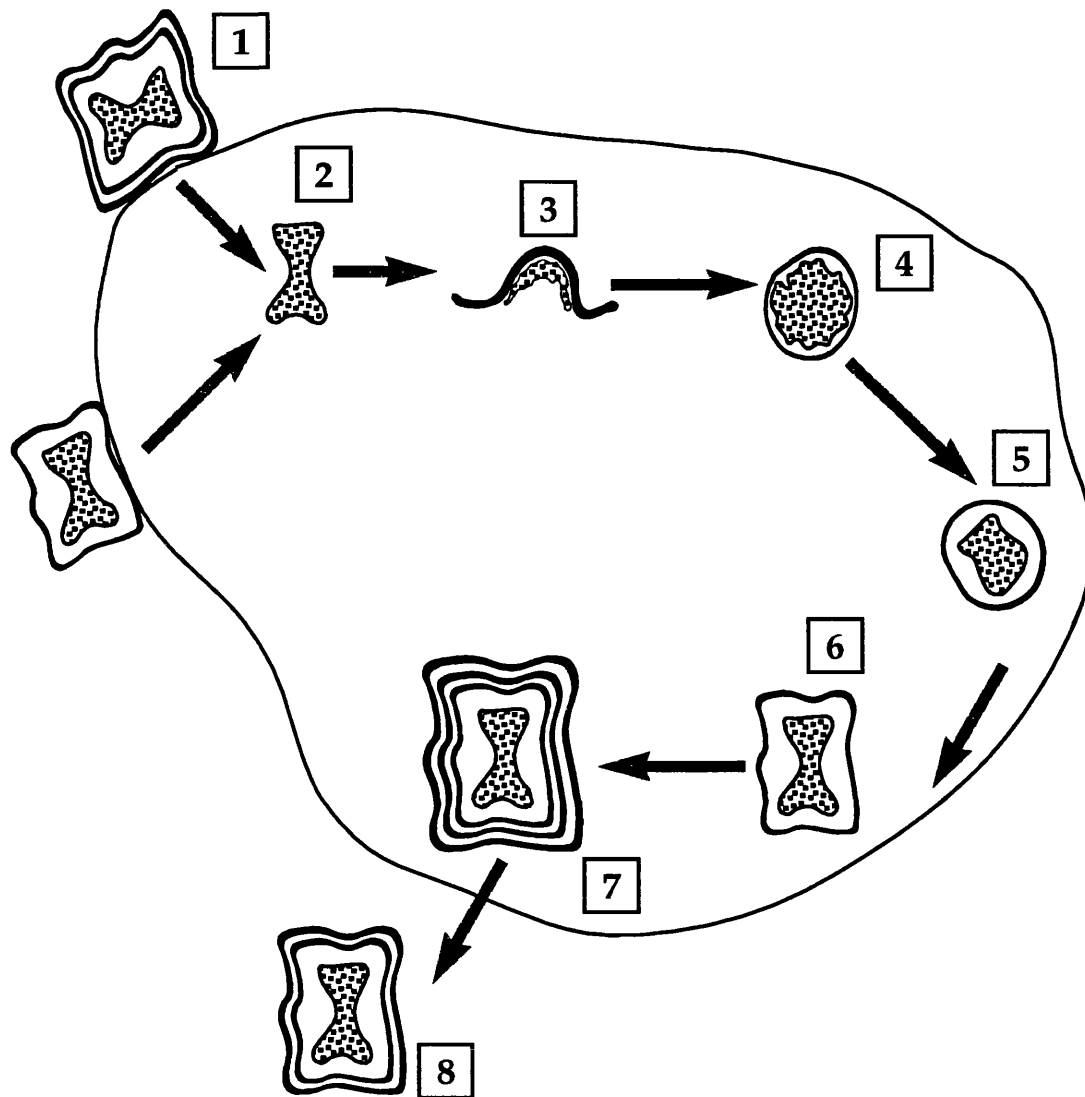
treatment of cells abolished IMV binding, but did not affect EEV, implicating the IMV receptor as a peptide (Vanderplasschen & Smith, 1997).

1.3.2 Gene expression

The relative autonomy of vaccinia virus from the host cell makes it an excellent experimental system for biochemical and genetic studies of transcription. VV gene expression has been divided into early, intermediate and late classes based on the time of expression. Early mRNA is detected within 20 min. of infection, and peaks at about 100 min. Intermediate mRNAs are detected at 100 min and peak at about 120 min; and late mRNAs are detected at 140 min and continue to be expressed for up to 48hrs post infection (Baldick & Moss, 1993b). Gene expression is regulated primarily by the ordered synthesis of class specific viral transcription factors and requirements for unique and specific promoter sequences and trans acting factors (Baldick & Moss, 1993a; Keck *et al.*, 1993). Expression proceeds in a cascade manner where the preceding class of genes encode factors required for the next class.

The elucidation of the mechanism of poxvirus transcription has been aided by *in vitro* assay methods. *In vivo*, the transcription machinery is activated upon entry of the virus into the cytoplasm of the cell. This activation process can be mimicked by treating virions with a reducing agent (DTT) and non-ionic detergent (NP-40). These permeabilised virions will, when provided with NTPs, magnesium, and S-adenosylmethionine, produces functional early mRNAs that are capped, methylated, polyadenylated and extruded into the cytoplasm (Moss, 1996). However, a

Fig.1.1 Proposed model for assembly of poxviruses



After attachment and entry (1), the virus uncoats and early gene expression initiates within cores (2). After a second uncoating event other enzymes and factors required for virus assembly are transcribed. The first morphologically distinct form is a crescent shaped double membrane derived from the intermediate compartment between the golgi and the ER, surrounded by a dense granular viroplasm (3). Crescent circularisation is preceded by entry of the nucleoprotein complex (4), the inner membrane withdraws to form a brick shape and the granulation becomes denser (5). The virion then becomes brick shaped and surface spicules are replaced by surface tubule elements (6), the virion is now categorised as an intracellular immature virus (IMV). A second membrane wrapping event derived from the trans golgi leads to formation of a tetra-membrane virion (7) known as the intracellular enveloped virus (IEV). Some IEV are transported to the plasma membrane where they are released (8) to form the extracellular enveloped virus (EEV), some EEV remain at the surface of the cell and are known as cell associated enveloped virus (CEV).

universal problem is that an *in vitro* system can only loosely emulate an *in vivo* system, in which there are up to 40 genes being transcribed at any one time, (Kates & Beeson, 1970) in both directions. In addition, the genome *in vivo* is not naked, and is probably coated with DNA binding proteins. Finally early genes are being reiteratively transcribed and continue to do so as long as the core is still intact and NTPs are available. All these events would not occur in an *in vitro* system. It is not known how the RNA polymerase recycles or how completed transcripts are extruded from the core (Gross & Shuman, 1996).

1.3.2.1 Uncoating and early gene expression

The macromolecular components necessary for transcription of VV early genes are present in the virion and are brought with it into the host cell during infection (Munyon *et al.*, 1967). Early genes encode proteins involved in DNA replication, intermediate gene expression and host interactions. Components required for early transcription were identified by extraction from vaccinia virions (Golini & Kates, 1985). Proteins required for early gene expression include the viral multi-subunit RNA polymerase, the RNA polymerase associated protein (RAP94), the VV early transcription factor (VETF) and the capping enzyme transcription factor. Additional virus encoded enzymes such as mRNA (nucleoside 2'-O) methyltransferase and poly A polymerase are required for mRNA modification (Moss, 1996).

1.3.2.1.1 Early Initiation and Elongation

Early transcription is by far the best characterised out of the three major classes of VV gene expression. Promoter sequences seem to be conserved between genera, as consistent with the phenomenon of non-genetic reactivation. In addition FWPV promoters have been shown to function effectively in VV and vice versa (Boyle, 1992). VV early promoters are 30bp long and can be divided into three functional regions, the critical region, the spacer region and the initiation region (Davison & Moss, 1989a). The critical region is important for transcriptional strength and definition of location of initiation, and is located at -28 to -13bp relative to the start site in the VV p7.5k early/late gene, and varies only by a few nucleotides in other genes (Davison & Moss, 1989a). The sequence of the 11bp spacer region is less important.

For initiation, only the VV RNA polymerase and the heterodimeric VETF (encoded by genes A7L and D6R) are required. VETF is a general, heterodimeric, sequence specific DNA binding transcription initiation factor with nucleoside triphosphate phosphohydrolyase activity. Purified VETF binds early promoters with high affinity *in vitro* (Broyles *et al.*, 1991). VETF binding is essential for promoter function (Yuen *et al.*, 1987). Promoter bound VETF recruits VV RNA polymerase to the pre-initiation complex (Li & Broyles, 1993). Analysis of the interaction of VETF with the promoter region of the VV p7.5kDa gene has shown that VETF binds to the critical region, and a region at nucleotide positions +8 to +10. (Broyles *et al.*, 1991). This introduces a bend in the promoter region due to the double binding sites and creates a distortion in the duplex DNA, this may potentially allow entry of RNA polymerase into the complex and allow initiation (Hagler & Shuman, 1992).

Early transcription initiation has a mandatory requirement for ATP hydrolysis (Shuman *et al.*, 1980). This is catalysed by VETF with the RAP94 VV RNA polymerase subunit acting as a bridge between VV RNA polymerase and VETF bound at the promoter (Ahn *et al.*, 1994). The fact that transcription termination is coupled to initiation from an early viral promoter raises the question of whether ATP hydrolysis during initiation and termination is performed by the same transcription apparatus. VETF remains associated with the template DNA after the elongating polymerase has cleared the promoter.

1.3.2.1.2 Early Termination

Like initiation, termination is also an energy dependent process (Hagler *et al.*, 1994; Shuman *et al.*, 1980). Virus-encoded-transcription-factor (VTF/capping enzyme), mediates early termination at the TTTTNT sequence, which is actually recognised as a single stranded (Luo *et al.*, 1995) UUUUUNU sequence in the nascent RNA (Shuman & Moss, 1988). VTF functions to transduce the UUUUUNU signal to the elongating VV RNA polymerase (Shuman *et al.*, 1987). Release of the RNA chain from the template engaged RNA polymerase occurs in absence of ongoing elongation, provided that the polymerase has passed at least 20 bases past the termination signal. VTF termination activity is independent of its role in capping and methylating the 5' end of nascent RNA (Luo *et al.*, 1995). Recent evidence has showed that recognition of specific phosphate groups in the termination heptamer is also essential for termination (Deng & Shuman, 1997).

Nucleoside triphosphate phosphohydrolase II (NPH2) was identified as an ancillary early termination factor as its mutants showed that NPH2 was not packaged into cores and lead to defective synthesis and extrusion of early mRNA as well as abnormally long mRNA products (Gross & Shuman, 1996). NPH II is also a NTP dependent helicase that catalyses unidirectional unwinding of 3' tailed duplex RNA in the presence of a divalent cation and any NTP (Shuman, 1993). It has been proposed that NPH II facilitates transcription termination in the virion by prevention of R loop formation behind the elongating polymerase, therefore preventing masking of the TTTTNT signal (Gross & Shuman, 1996).

Resultant early viral mRNAs are of discrete length and like cellular mRNA are capped, methylated and polyadenylated. In vivo, viral RNA can undergo further methylation by cellular enzymes (Boone & Moss, 1977).

1.3.2.2 Intermediate gene expression

Intermediate genes are expressed after DNA replication but before late gene expression. Five intermediate genes have been described so far, these include: three late transcription factors (encoded by genes G8R, A11 and A2L) (Keck *et al.*, 1990) known as VLTF 1, 2 and 3 respectively (Wright & Moss, 1989; Zhang *et al.*, 1992), NPH II (I8R) and a DNA binding protein (I3L) which interacts with ribonucleotide reductase (Vos & Stunnenberg, 1988).

Intermediate stage promoters consist of a 14bp core element separated from the TAAA initiator element by 10 or 11bp (Baldick *et al.*, 1992).

Necessary factors and enzymes for transcription of intermediate promoter templates *in vitro* include VV RNA polymerase, VTF/Capping enzyme (Vos *et al.*, 1991), VV intermediate transcription factor 1 (VITF -1) and VITF-2. VITF-1 is actually the RP030 subunit of VV RNA polymerase and VITF-2 is a cellular transcription factor (Rosales *et al.*, 1994a; Rosales *et al.*, 1994b).

Transcribed mRNAs are of variable length as they do not terminate at the early termination heptamer sequence (Mahr & Roberts, 1984), and have 5' poly A leader sequences of up to 30 additional bases (Baldick & Moss, 1993b).

1.3.2.3 Late gene expression

Activation of vaccinia virus late gene transcription is dependent on DNA replication and the expression of three genes: A1L, A2L, and G8R (Keck *et al.*, 1990). These factors were discovered by use of a novel reverse genetic based approach (Keck *et al.*, 1990), utilising the base analogue cytosine arabinoside and cotransfection of cloned VV genomic fragments and a reporter gene regulated by a late promoter. A1L, A2L, and G8R have now all been characterised as essential late transcription factors VLTF 1-3 (Passarelli *et al.*, 1996; Wright & Coroneos, 1993; Wright *et al.*, 1991). An additional early factor called P3 was also identified as necessary for late transcription, therefore modifying the classical cascade model of gene expression (Kovacs *et al.*, 1994). P3 has now been renamed VLTF-4 and shown to be encoded by the H5R ORF (Kovacs & Moss, 1996).

Additional factors have been suggested for late transcription *in vivo*, including G2R and A18R. G2R mutants exhibit 3' truncation of intermediate and late RNA (Black & Condit, 1996), A18R is a 56kDa DNA dependent ATPase and DNA helicase (Bayliss & Condit, 1995). A18R mutants show promiscuous transcription, leading to transcription of early genes at late times (Bayliss & Condit, 1993); this is identical to the effects of treatment with isatin-B-thiosemicarbazone (IBT). A18R and G2R gene products are hypothesised to act as late, *in vivo*, elongation and termination factors respectively (Condit *et al.*, 1996).

Late promoters consist of a 20bp core region separated by 6bp from a highly conserved and essential TAAAT transcription initiation element, which can frequently overlap the initiation codon (Davison & Moss, 1989b). Late mRNAs have a 5' capped heterogenous length poly A sequence as a result of VV RNA polymerase slippage (Moss, 1996). Most late transcripts are long and heterogenous as the heptameric early termination sequence is not recognised by the late transcription system (Moss, 1996).

1.3.2.4 Constitutive gene expression

Both early and late promoter sequences have been identified upstream of some genes, these are consequently expressed throughout the replication cycle (Cochran *et al.*, 1985) Such a gene has also been found in FWPV (Kumar & Boyle, 1990b)

The VV 7.5k early/late promoter is also reactivated late in infection (Garces *et al.*, 1993). To identify how this happens and why other early promoters cannot be reactivated, mutational analysis of the 7.5k early

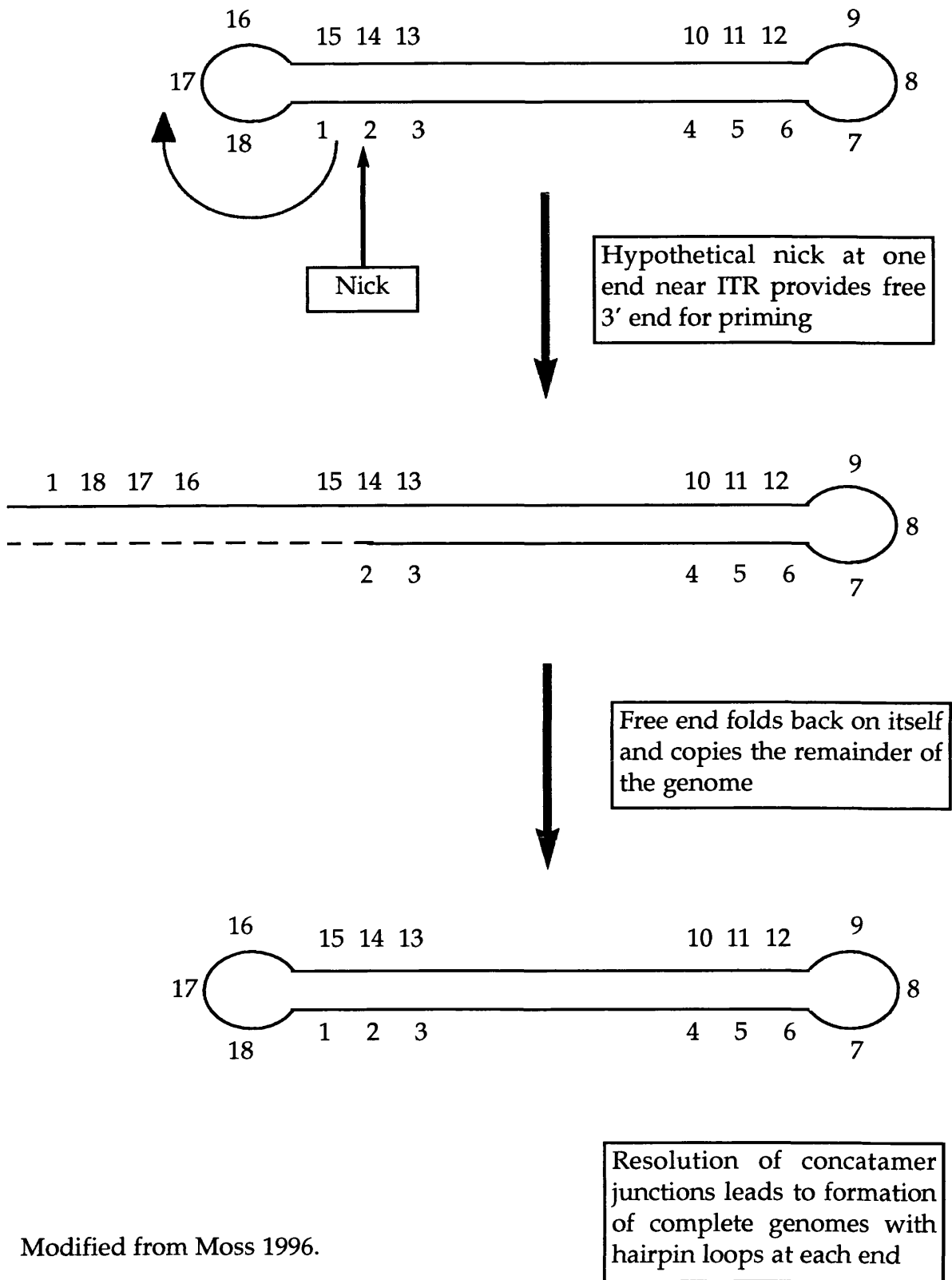
promoter indicated that sequences both upstream and downstream of the initiation site were responsible for reactivation (Masternak & Wittek, 1996). In addition overexpression of VETF lead to suppression of late transcription and reactivation of early promoters which were not normally reactivated (Masternak & Wittek, 1996).

1.3.3 DNA replication

Apart from African swine fever virus, poxviruses are the only other viruses that undergo DNA replication in the cytoplasm, implying near exclusive non-usage of cellular replication machinery (fig. 1.2). Consequently VV encodes a large number of enzymes associated with DNA replication, including enzymes associated with dNTP synthesis (thymidine kinase [J2R] (Hruby & Ball, 1982; Hruby *et al.*, 1983), thymidylate kinase [A48R] (Smith *et al.*, 1989), ribonucleotide reductase [I4L and F4L] (Slabaugh *et al.*, 1988; Slabaugh *et al.*, 1984), deoxyuridine triphosphatase [F2L] (Broyles, 1993)

DNA replication occurs in distinct areas in the cytoplasm called factory areas, occurring between 1-2 hours in VV (Salzman, 1960) and within 12 hours in FWPV (Prideaux & Boyle, 1987). Enzymes required for DNA replication have been identified using complementation groups of ts mutants which undergo early gene expression but exhibit arrested DNA replication. To date four such ts mutant groups have been described, leading to discovery of the VV DNA polymerase [E9L] (Condit *et al.*, 1983); a serine/threonine protein kinase [B1R] (Banham & Smith, 1992); a 90kDa protein of unknown function with an ATP/GTP binding motif [D5R]

Fig.1.2 Mechanism of VV DNA replication



Modified from Moss 1996.

(Evans & Traktman, 1987) and a uracil DNA glycosylase [D4R] (Millns *et al.*, 1994).

VV expresses a 50kDa DNA ligase (Kerr & Smith, 1989), which is homologous to human DNA ligases I, II and III, but is surprisingly non essential for recombination and DNA replication (Kerr & Smith, 1991). The proposed model of VV DNA replication obviates the requirement for a DNA ligase.

The VV genome, is in effect, a covalently closed circle. as the linear dsDNA genome has incompletely base paired terminal hairpins. Shortly after infection a nick occurs close to one of the terminal hairpins (Pogo, 1977) so that theoretically, VV DNA replication can occur by unidirectional leading strand synthesis (Traktman, 1990), requiring only a single ligation step for each progeny genome, which might be carried out by the VV nicking joining enzyme (Reddy & Bauer, 1989). DNA replication involves formation of concatameric intermediates which are resolved into unit length molecules by an unidentified resolvase at the junctions after the onset of late gene expression (Merchlinsky & Moss, 1989b). Each concatamer junction is a precise duplex copy of a T₆N₇₋₉T/CAAAT/A sequence, joined by hairpin loop of 200 bp of less conserved but palindromic sequence (Merchlinsky & Moss, 1986; Merchlinsky & Moss, 1989a).

1.3.4 Virus assembly

Assembly of poxviruses commences after onset of late transcription in distinct granular, electron dense areas of the cytoplasm called viral factories (fig. 1.1) or B-type inclusion bodies (Moss, 1996). Elucidation of

poxvirus assembly has been mainly achieved by use of genetic (conditional, ts and drug resistant mutants), and physical methods (by use of drugs that inhibit assembly without affecting protein synthesis or DNA replication, such as IMCBH and Rifampicin).

The first structure to be visible is a crescent shaped membrane located within the viral factory, which has a brush like array of electron-dense spicules on the convex surface (Moss, 1996). Previous observations indicated that VV undergoes *de novo* membraneogenesis. However, a study using immunostructural ultrastructural localisation and lipid profiling of the early crescents, indicated that the membrane is acquired from the cisternae of the intermediate compartment (IC), between the endoplasmic reticulum and the golgi apparatus (Sodeik *et al.*, 1993). Crescents with proximity and continuity to IC cisternae have also been observed (Sodeik *et al.*, 1993). Acquisition of IC membranes is possibly mediated by A14L, with A17L involved in organisation of these membranes into crescents (Rodriguez *et al.*, 1995; Rodriguez *et al.*, 1997). Early crescents then circularise just prior to entry of the nucleoprotein, to form the double membraned Immature virions (IV), which mature to form ovoid or brick shaped IMV. VETF repression leads to arrest of VV morphogenesis at the IV stage (Hu *et al.*, 1996), repression of D13L, F17R and I7L also lead to arrest of VV assembly at early stages (Kane & Shuman, 1993; Zhang & Moss, 1991; Zhang & Moss, 1992).

Most IMV virions now stay in the cytoplasm until they are released by cell lysis, however some acquire two further cellular membranes derived from the trans golgi network (Schmelz *et al.*, 1994). to form the IEV. These four membraned virions are transported via actin containing microfilaments to the plasma membrane (Cudmore *et al.*, 1995; Cudmore *et*

al., 1996), where-upon fusion occurs. The resultant three membraned virions mostly associate with the cell membrane as CEV, or detach to form EEV. A mechanism involving budding of IMV at the plasma membrane has also been proposed for formation of EEV (Tsutsui *et al.*, 1983). B5R, F13L and A27L are essential for the second wrapping event. The latter protein is IMV associated and therefore probably forms a bridge between the IMV and EEV membranes.

Incorporation of viral proteins into assembling virions and membranes is currently ill defined. Analysis of incorporation of membrane proteins p14 and p32, indicated that p32 is inserted into viral membranes prior to crescent formation whereas p14 was inserted between IV and IMV formation (Sodeik *et al.*, 1995). A further insight was gained upon analysis of an inducible mutant of RAP94, whereupon RNA Polymerase, as well as other enzymes involved in processing of early mRNA, was missing from assembled virus (Zhang *et al.*, 1994). The inference is that RAP94 is involved in formation of a multi-enzyme complex, therefore obviating the need for each protein to have its own targeting signal (Zhang *et al.*, 1994). VETF also seems to form an analogous complex with VV RNA polymerase, VTF/capping enzyme and NPH1 (Li *et al.*, 1994), further substantiating a view of packaging of VV proteins as interdependent .

1.3.5 Virus release

Release of IMV occurs by lysis of infected cells or by entry into microvilli and breakage of their tips (Blasco & Moss, 1992), whereas the mechanism of EEV release is yet to be fully elucidated, but is thought to involve fusion at the plasma membrane (Moss, 1996). EEV specific proteins

are also thought to be involved, as release is blocked when assembly of IEV is arrested by N-isonicotinoyl N' 2-3 methyl-4-chlorobenzoylhydrazine (IMCBH). After release, most of the EEV remains associated with the cell surface as CEV, the ratio between CEV and EEV is dependent upon whether the lectin binding domain of A34R is intact (Blasco *et al.*, 1993).

The EEV envelope contains at least eleven extra proteins, 9 of which are glycoprotein and possibly involved in the entry/release mechanism (Smith, 1993). This was suggested because 1-10mM glucosamine (a glycosylation inhibitor) lead to a 90% decrease in EEV release, whereas production of IMV was unaffected (Payne & Kristensson, 1982). Deletion of A56R [89kDa haemagglutinin], F13L [non-glycosylated 37kDa], B5R [42kDa], A36R [43-50kDa], A34R [22-24kDa] and A33R EEV specific proteins, has a profound affect on virus spread and plaque formation, without affecting IMV production (Moss, 1996; Roper *et al.*, 1996).

1.4 Virus-host interactions

All viruses are obliged to use mechanisms to evade host immune responses that would otherwise lead rapidly to their elimination, as well as employment of mechanisms for manipulation and redirection of host metabolic systems. Specific examples of such redirection are described below.

VV infection *in vitro* leads to shut down of host DNA, RNA and protein synthesis. Conversely, VV stimulates VV growth factor (VGF) mediated hyperplasia *in vivo* (Moss, 1996). Poxviruses have developed methods to shut down host systems as well as counter the immune response. Notable ingenious examples are discussed below.

1.4.1 Interferon

The Interferon (IFN) response is the primary defence mechanism against viruses in mammalian cells. The response is triggered by the presence of large amounts of dsRNA as a result of viral infection (Jacobs & Langland, 1996). The induction of IFN leads to expression of 2'-5' oligoadenylate synthetase and PKR. Activation of these enzymes is dependent on the presence of dsRNA. 2'-5' oligoadenylate synthetase catalyses polymerisation of 2'-5' linkages in oligoadenylates. These oligoadenylates then activate RNase L which is a latent cellular enzyme, which cleaves ssRNA. Activated PKR phosphorylates the α subunit of the eukaryotic translation factor eIF-2 α and I κ B the NF κ B inhibitor.

Poxviruses utilise two key methods to counteract the host IFN response; firstly poxviruses have been shown to encode soluble IFN- γ receptors that inhibit IFN- γ activity, in direct contrast to the highly species specific cellular homologues, the vaccinia IFN- γ receptor has been shown to have broad species specificity (Alcami & Smith, 1996). Secondly, VV encodes proteins encoded by the K3L and E3L ORFs, which interfere with the interferon mediated inhibition of protein synthesis (Chang *et al.*, 1992). K3L and E3L both target the cellular RNA dependent protein kinase PKR. K3L is an eIF-2 α homologue which acts as a viral substrate for PKR, therefore preventing inactivation of the translation initiation factor by PKR (Beattie *et al.*, 1991). E3L is a double stranded RNA binding protein that inhibits the dsRNA dependent autophosphorylation of PKR (Chang *et al.*, 1988).

E3L mutant VV exhibits decreased host range and increased sensitivity to interferon seen at the level of interferon inhibition of protein synthesis and interferon induction of RNA degradation (Beattie *et al.*,

1996). The E3L protein actually represses both the protein synthesis shut off mediated by PKR and activation of the 2'-5' A synthetase pathway.

It was recently shown that PKR induces apoptosis (Lee & Esteban, 1994). Cowpox virus has previously been shown to block apoptosis induced by Fas and TNF α through expression of the crmA gene product which acts as an inhibitor of cellular aspartic proteases. E3L mutant VV induces apoptosis in direct contrast with wt VV in HeLa cells (Lee & Esteban, 1994). Recent evidence has indicated that dsRNA directly initiates the apoptotic response in infected and non infected cells (Kibler *et al.*, 1997).

1.4.2 Chemokines

One of the key features of the early inflammatory response to a viral challenge is the influx and activation of leukocytes – in particular neutrophils, macrophages and NK cells are particularly involved in the first wave of cellular infiltration. All these cell types require directional signals to be directed towards damaged or infected cells. Chemokines are critical for this process. Chemokines can be divided into three classes CXC, CC and C based upon cysteine distribution. Initially it was believed that specific classes affected different immune cells, but recent studies have shown that different classes of chemokines demonstrate overlapping induction of a wide range of leukocytes.

Recently a broad class of secreted poxvirus proteins that interact with a broad class of chemokines *in vitro* have been shown to retard the extent of leukocyte influx into virus infected lesions *in vivo* (Graham *et al.*, 1997). As well as VV, myxoma, SFV, rabbitpox, Cowpox and racoonpox

were shown to express secreted proteins which interact with the CC and CXC families of chemokines (Graham *et al.*, 1997).

1.4.3 Complement

The complement system consists of more than 30 proteins which operate in a precise sequence to eliminate invading micro-organisms. The proposed antiviral effects of the complement pathway include virus neutralisation, opsonisation, lysis of infected cells, and amplification of inflammatory and immune responses.

One group of complement proteins consists of proteins made up of 4-30 short consensus repeats (SCRs) each of which is a 60-70aa repeat. This family includes complement receptors 1 and 2, C4b binding protein, factor-H, membrane co-factor protein (MCP), and VV complement control protein (VCP) (Kotwal & Moss, 1988b). VCP has four SCRs which have homology to C4B binding protein (Kotwal & Moss, 1988b). Purified VCP was shown to bind to C3 and C4, thereby blocking the complement pathway (Kotwal *et al.*, 1990). VCP homologues have also been described in variola and cowpox (Miller *et al.*, 1997).

1.4.4 Polads

POLADS are small untranslated polyadenylated RNAs, which are produced during VV infection and selectively inhibit host protein synthesis by an inhibitory moiety residing on the poly A tail (Bablanian *et*

al., 1986), with the length of the poly A region being proportional to the strength of inhibition (Bablanian *et al.*, 1987). It has recently been shown that during VV infection viral mRNA, host mRNA, host tRNA and small nuclear RNAs are all polyadenylated and may serve as POLADS (Lu & Bablanian, 1996). Polyadenylation of these normally non-adenylated RNA is probably carried out by VV poly A polymerase which acts as a promiscuous polymerase and can prime any RNA without requirement for a specific sequence. It is proposed that POLADS specifically inhibit host protein synthesis by the increased amount of poly A in the cytoplasm leads to sequestering of Poly A binding protein (PAB) which is an essential cellular translation initiation factor. Viral translation can continue normally as it is less affected by poly A (Bablanian *et al.*, 1991).

1.4.5 Tumour necrosis factors and Interleukins

Tumour necrosis factors and interleukins are key cytokines which regulate the inflammatory and immune response. The diploid T2 gene in Shope Fibroma virus (SFV) has been shown to act as a receptor for TNF and $LT\alpha$ by being analogous to the cellular type II TNF receptor. Wt SFV is usually fatal for its host whereas T2 mutant infected rabbits showed two thirds survival (Upton *et al.*, 1991)

Cowpox virus contains another diploid ITR localised gene called cytokine response modifier (crm) B which is expressed early and has identical properties to SFV T2 in terms of receptor mediated inhibition binding (Hu *et al.*, 1994). A different mechanism for countering host cytokines is used by crm A which encodes a 38kDa cytoplasmic protein that inhibits activation of the precursor of IL1 by inactivation of the IL1

converting enzyme (ICE) (Ray *et al.*, 1992). A third member of the crm family called crm C has been recently identified which encodes a soluble secreted protein which specifically binds TNF and completely inhibits TNF mediated cytotoxicity (Smith *et al.*, 1996). But unlike crm A and B, crm C does not bind LT α (Smith *et al.*, 1996).

1.5 Fowlpox

1.5.1 FWPV disease

Fowlpox is characterised by lesions, which are either cutaneous or diphtheritic. The virus enters through damaged skin, the respiratory tract or insect bites. Once inside an epithelial cell, viral proliferation can lead to spread of infection to internal organs and the blood, causing viraemia. Cutaneous lesions progress from papules to pustules, which dry leaving scabs on non-feathered regions. The scabs dry and drop off 2 weeks p.i., containing infective virions which are resistant to desiccation (Jordan, 1990) Diphtheritic lesions are present mostly in the mouth as white nodules which later become raised as yellow plaques. Lesions can also occur in the oesophagus and trachea, leading to eating and breathing problems. Nasal and ocular discharges can result, the latter can lead to blindness (Jordan, 1990).

Mortality can be as high as 50%, but a decrease in egg production and weight gain are the more usual symptoms (Jordan, 1990) Control has been successfully administered by vaccination with attenuated FWPV for many decades, consequently research attention has shifted away from disease eradication into new areas. Early work resulted in FWPV being the

first virus to be grown on the choriollantoic membrane. FWPV virions were seen by light microscopy and termed Borrel bodies, these were present in distinct areas of the cytoplasm termed Bollinger bodies (Simpson, 1969), later renamed A-type inclusion bodies. However, contemporary research has led to elucidation of FWPV molecular virology leading to its use as a recombinant vaccine vector (Boyle & Coupar, 1988; Taylor & Paoletti, 1988a; Taylor *et al.*, 1988b).

1.5.2 FWPV Vaccines

Interest was sparked in use of recombinant poxviruses as vaccines, when it was shown that foreign genes could be introduced into and expressed by VV (Mackett *et al.*, 1982; Panicali & Paoletti, 1982). Poxviruses make good vectors for vaccines as it is relatively easy to insert multiple large sections of foreign DNA into non-essential regions of the viral genome by homologous recombination. Additionally the cytoplasmic location of replication decreases the probability of integration of viral DNA into host DNA. Unlike VV, FWPV only replicates in poultry, making it ideal for use as an avian and non-avian vaccine vector. Consequently, FWPV has been developed for use as a live non-replicating vaccine vector in mammals (Mackett *et al.*, 1982; Panicali & Paoletti, 1982; Taylor *et al.*, 1990; Taylor & Paoletti, 1988a; Wild *et al.*, 1990). In non-avian species replication is abortive, however pre- and post-replicative viral gene expression in different mammalian cell types has been observed (Somogyi *et al.*, 1993).

1.5.2.1 Avian vaccines

The use of fowlpox as a vaccine vector for use in avian and non-avian species was proposed in the late 80's (Boyle & Coupar, 1988; Taylor & Paoletti, 1988). It was proposed that recombinant FWPV could be used as a highly cost effective method for multiple vaccination of poultry. Since then many recombinant vaccines using FWPV have been constructed expressing foreign genes. Candidate vaccines have been developed for Newcastle disease virus (NDV) (Taylor et al., 1990) avian influenza (Beard *et al.*, 1991) turkey rhinotracheitis virus (TRTV) (Qingzhong *et al.*, 1994) and infectious bursal disease virus (IBDV) (Bayliss *et al.*, 1991). (Indeed a commercial NDV recombinant FWPV vaccine has been licensed for use, and is marketed in the United States). Vaccines for NDV and avian influenza utilised viral haemagglutinin as the immunogens, whereas TRTV and IBDV vaccines used viral fusion protein and VP2 coding sequences as immunogens.

1.5.2.2 Non-avian vaccines

FWPV has immense potential for use as a vaccine vector in non-avian species, as it cannot cause disease in humans or other non-target species. Protective vaccines against rabies in mice, cats and dogs (Taylor *et al.*, 1988b) and measles encephalitis in mice (Wild *et al.*, 1990) have been developed. A recombinant FWPV expressing β -galactosidase as a model, tumour associated antigen, has also been shown to protect mice against cancer (Wang, 1995).

1.5.3 Fowlpox genome analysis

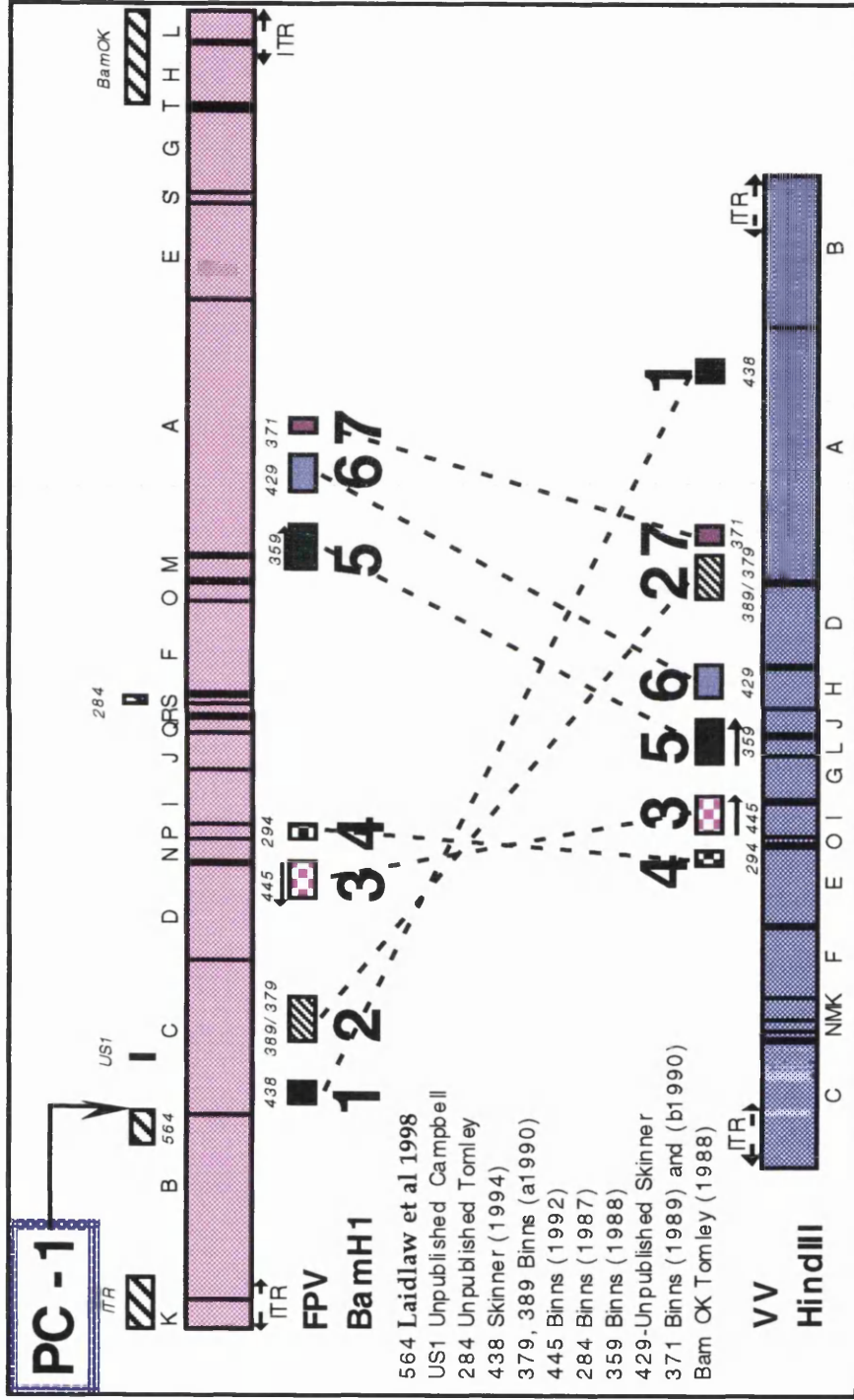
VV and other viruses in the same genus, the *Orthopoxviruses*, exhibit a high degree of genome conservation as indicated by restriction map profiles and DNA hybridisation studies (Esposito & Knight, 1985; Mackett, 1979). But the degree of homology between viruses in different poxvirus genera has only recently been investigated.

The size of the FWPV genome has been derived by restriction endonuclease mapping and pulsed field gel electrophoresis as 254Kbp for strain FP9 (Mockett *et al.*, 1992) The former technique was used to show a size of 309Kbp for FWPV mild vaccine strain (FWPV-M) and 299Kbp for FWPV-M3 (derivative of FWPV-M) (Coupar *et al.*, 1990). The FWPV genome has inverted terminal repeats of 10Kbp in FP9 (Tomley *et al.*, 1988) and 16.5Kbp for FWPV-M and FWPV-M3. Extensive rearrangement of blocks of genes between VV and FWPV has been described (Mockett *et al.*, 1992) Fig. 1.3 shows seven blocks of the FWPV genome which have homology with regions of VV. Blocks 5, 6 and 7 are in the same order, but blocks 1, 2, 3 and 4 are present in an inverted order. FWPV-specific sequences are likely to be present in the terminal regions, as this is the most variable region of poxviral genomes. However rearrangements discussed raise the possibility of FWPV-specific genes in a central region between blocks 4 and 5 (Mockett *et al.*, 1992).

1.5.3.1 VV homologous genes

As expected, analysis of the FWPV genome has revealed several FWPV genes with varying degrees of homology to VV genes; these include:

Fig.1.3 Arrangement of the FWPV genome in comparison to VV



Detailed comparative analysis is presented in section 1.6.3. Blocks of sequence above the FWPV genome have no homologue in VV, the inverse is applicable for blocks below the FWPV genome. Numbers above sequence blocks are arbitrary and represent identification nomenclature only. (Modified from Mockett et al. 1992)

VV DNA polymerase (Binns *et al.*, 1987), VV 4b core protein (Binns *et al.*, 1989), D6 -D13 and A1 (Binns *et al.*, 1990b), thymidine kinase (Boyle & Coupar, 1986), 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase (3 beta-HSD; A44L) and DNA ligase (A50R) (Skinner *et al.*, 1994), and the p39K (A4L) immunodominant protein (Binns *et al.*, 1990a; Boulanger *et al.*, 1998).

1.5.3.2 Fowlpox specific genes

FWPV genes have been identified which are not present in VV or any other poxviruses, several such ORFs were identified in a near terminal 11.2Kbp sequence (Tomley *et al.*, 1988) and a 7.2Kbp fragment (ORF X) (Binns *et al.*, 1992) Sequence analysis of a 11.2Kbp fragment of the MDV genome revealed extensive homology of the MDV sORF2 to FWPV ORF4 (Brunovskis & Velicer, 1995) present in a previously characterised FWPV genome region (Tomley *et al.*, 1988) This was noted as the first example of gene conservation across virus families involving non-cellular genes. Homology extended to 42% identical and 67% similar in a 100 aa region.

FWPV homologues of several cellular genes have been recently identified in a 10Kbp fragment (Laidlaw *et al.*, 1998) these include mammalian SNAP (involved in transport vesicle targeting), 2 uncharacterised *Caenorhabditis elegans* genes and mammalian plasma cell glycoprotein PC-1. None of these genes have been found in any other viruses.

1.6 Mammalian PC-1 - a summary

Mammalian plasma cell glycoprotein was originally defined as a marker of antibody secreting plasma cells (terminally differentiated B-cells) (Takahashi *et al.*, 1970). In the near thirty years since its discovery, the precise physiological role of PC-1 is still unknown. Significant advances in functional characterisation have revealed that PC-1 is a remarkable multi domain protein, with potential multi-enzymatic roles as a kinase, an alkaline phosphodiesterase, a phosphatase and a nucleotide pyrophosphatase. Some of these activities have been directly linked to possible roles as an insulin response regulator, a mitogen and involvement in the nucleotide salvage pathway. In addition PC-1 enzymatic activity has been associated with cancer, obesity, Lowe's syndrome and calcium pyrophosphate dihydrate (CPPD) crystal deposition disease.

1.6.1. Cloning and characterisation of Murine and Human PC-1

Murine PC-1 was first identified during analysis of differential expression of murine thymocyte and lymphocyte cell surface antigens on plasma cells, and defined as a surface antigen by the antiserum made in B6.DBA.F1 mice against the BALB/c myeloma MOPC-70A (Takahashi *et al.*, 1970). PC-1 was categorised as a differentiation antigen, as it was not expressed by thymocytes and lymphocytes, but was present on plasma cells, liver, kidney and brain cells, as determined by the adsorption of polyclonal alloantisera (Takahashi *et al.*, 1970). More precise localisation, utilising an immunohistochemical approach with a monoclonal antibody (Mab), indicated expression of PC-1 in the distal convoluted tubule of the kidney, salivary gland ducts, epididymis, proximal vas deferens, chondrocytes and

brain capillaries (Harahap & Goding, 1988). Recently human PC-1 has also been detected on thymocytes (Deterre *et al.*, 1996).

Cell surface iodination (Vitetta *et al.*, 1971) and immunoprecipitation, confirmed that PC-1 was a cell surface marker protein with a relative molecular mass (Mr) of 105, 000 - 110,000 (Tung *et al.*, 1978). Further structural characterisation indicated that PC-1 was a disulphide bonded homodimer of Mr 115,000 (Goding & Shen, 1982). Partial amino acid sequence was obtained by generation of a monoclonal antibody to PC-1 which allowed purification and sequencing of 7 tryptic peptides (Stearne *et al.*, 1985). These peptide sequences were used to design primers for the isolation of partial (van Driel *et al.*, 1985b) and full length cDNA clones of murine PC-1 (van Driel & Goding, 1987).

The primary structure of murine PC-1 indicated a protein of 905 amino acids (aa) consisting of a short 58 aa cytoplasmic amino terminal, a 21 aa hydrophobic transmembrane domain and a large extracellular carboxyl terminal domain of 736 aa containing 6 predicted N linked glycosylation sites and a 90aa cysteine rich sequence (van Driel & Goding, 1987). The predicted type II membrane orientation was confirmed by the reaction of a 587aa extracellular region of PC-1 with polyclonal serum against PC-1 from NS1 cells, whereas clones with the opposite orientation did not react (van Driel & Goding, 1987).

A 100kDa soluble form of murine PC-1 has also been characterised as a minor fraction of the total protein produced, it was first detectable in the supernatant after about 2h (Belli *et al.*, 1993). The mechanism of formation of the soluble form is currently unknown, but it is proposed that it arises by intracellular proteolytic cleavage between Pro186 and Ala187, and not by alternative splicing (Belli *et al.*, 1993).

Human PC-1 was cloned by screening human foetal liver and placental libraries with probes generated from clones containing murine PC-1 sequence (Buckley *et al.*, 1990). Overall aa sequence homology of 80% was observed between human and murine PC-1, with 88% aa identity at the amino terminal 500aa. Ten N-linked glycosylation sites as opposed to the six sites in murine PC-1 are predicted in human PC-1 (Funakoshi *et al.*, 1992). The murine start codon was also reassigned from earlier studies (van Driel & Goding, 1987) on the basis of sequence alignment between mouse and human PC-1 genes and the absence of an in frame stop codon 5' of the first methionine, therefore reducing the length of the cytoplasmic domain to 24aa (Buckley *et al.*, 1990). However a later study showed that the first methionine was indeed the true initiator, as expression from clones utilising the second methionine was reduced to 15% and 58% of levels obtained from clones using the first methionine (Belli & Goding, 1994). Therefore the sizes of the cytoplasmic domains of the human and mouse PC-1 genes are 76aa and 58aa respectively (Belli & Goding, 1994).

Human PC-1 biosynthesis indicates the presence of an endoglycosidase-H sensitive precursor of about 120kDa, over a period of 2-4 hours the original precursor is processed to an endoglycosidase - H resistant mature form of 130kDa, suggesting the presence of high mannose oligosaccharides in the endoplasmic reticulum which are processed to complex oligosaccharides in the golgi (Belli & Goding, 1994).

1.6.2 Domain structure of PC-1

PC-1 is a type II transmembrane glycoprotein with a short amino terminal cytoplasmic domain, a single membrane spanning domain and a

large multi-domain extracellular domain (fig. 1.4). The extracellular domain is separated into the somatamedin-B homologous cysteine rich domain suitable for intramolecular disulphide bond formation (Patthy, 1988), a region homologous to the active site of bovine intestinal 5'-nucleotide phosphodiesterase 1 (Skinner, 1991), an ATP binding site (Oda *et al.*, 1991) two EF-hand domains (Belli *et al.*, 1994; Buckley *et al.*, 1990) and two adenine binding sites (Uriarte *et al.*, 1995).

1.6.3. Functional characterisation

The domains described below have all been implicated in possible roles for PC-1. The continual rediscovery of PC-1 due to its multiple domains, leading to multi-enzymatic functions, has been a notable feature during characterisation of this protein.

1.6.3.1. Alkaline phosphodiesterase / Nucleotide pyrophosphatase activity

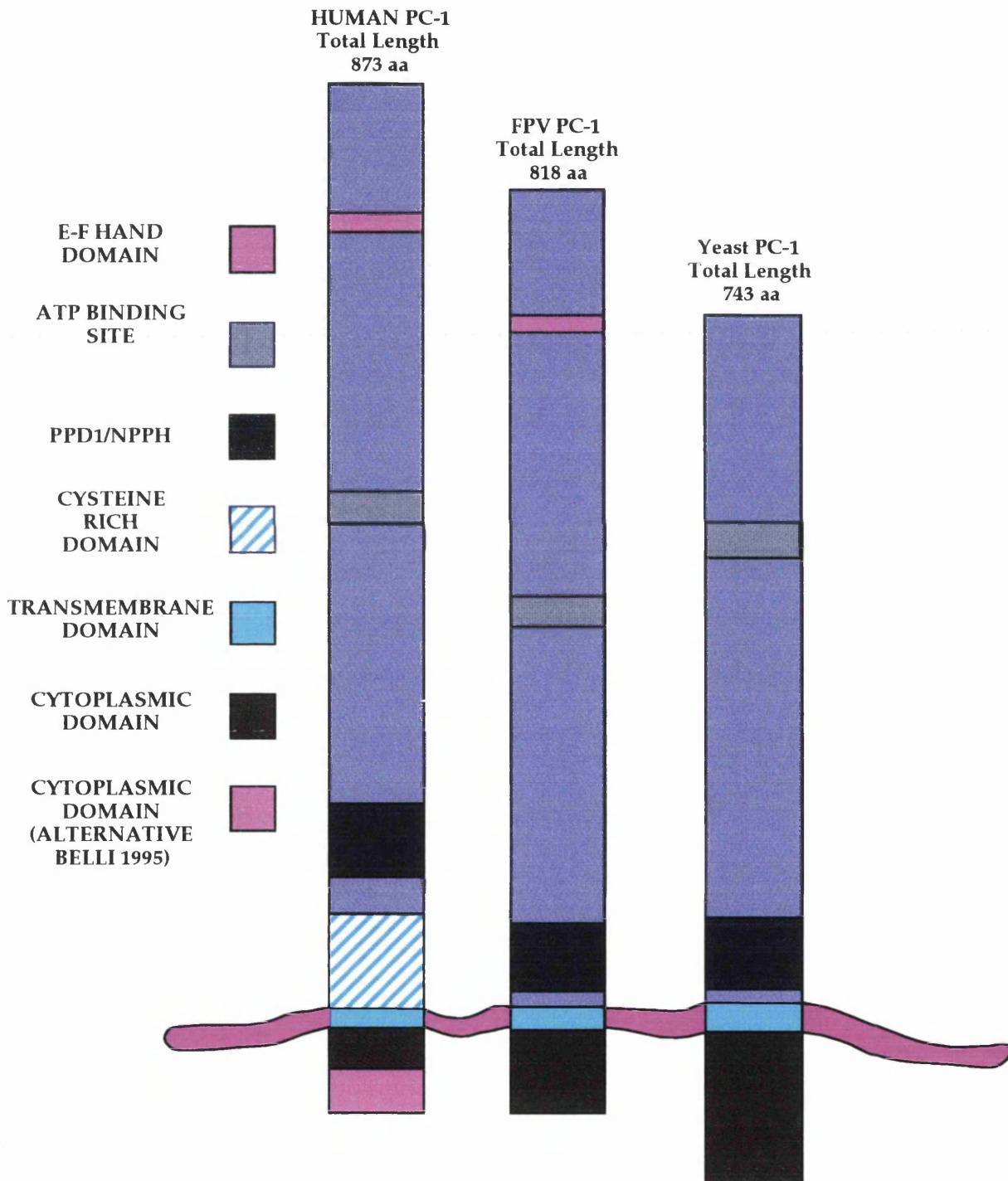
Type 1 alkaline phosphodiesterases (AP1) are capable of hydrolysing phosphodiester bonds at the 3' end of RNA and single stranded DNA as well as nucleotides such as cAMP (Kelly *et al.*, 1975; Landt & Butler, 1978). Nucleotide pyrophosphatases (NPP) can hydrolyse pyrophosphate bonds of nucleotides such as ATP and ADP (Kelly *et al.*, 1975; Landt & Butler, 1978) and nucleotide sugars such as UDP-galactose (Evans *et al.*, 1973).

AP1/NPP activities were initially ascribed to murine PC-1 as antibodies to purified AP1/NPP specifically recognised PC-1 expression

clones and other antibodies raised against a recombinant PC-1 fusion protein recognised AP1/NPP, there was also a correlation in levels of PC-1 mRNA expression and cell lines known to express different levels of AP1/NPP (Rebbe *et al.*, 1991). Conclusive proof that murine PC-1 has AP1/NPP activity was obtained as levels of AP1 activity in B-cell lines at progressive stages of differentiation mirrored levels of PC-1 mRNA, and COS-7 cells transfected with recombinant murine PC-1 expressed both AP1 and NPP activity (Rebbe *et al.*, 1993). This also suggests that both AP1 and NPP activities are expressed by the same protein (Rebbe *et al.*, 1993). Elevated levels of NPP activity have also been observed in cultured skin fibroblasts from patients with Lowe's syndrome (Funakoshi *et al.*, 1992).

The presence of chelating agents such as EDTA lead to a reduction in the alkaline phosphodiesterase, nucleotide pyrophosphatase and autophosphorylation enzymatic activities of PC-1, suggesting that they are indirectly or directly modulated by divalent cations (Rebbe *et al.*, 1991). Furthermore, the likelihood that divalent cations would be implicated, was suggested as murine and human PC-1 contain two predicted EF-hand domains, one is near the C-terminal and adheres closely to the consensus EF-hand sequence DXDXDGXXD (Buckley *et al.*, 1990), whereas the second EF-hand is located towards the centre of the gene and is diverged from the consensus (Belli *et al.*, 1994). The presence of divalent cations protects PC-1 against thermal denaturation and proteolytic degradation, suggesting that binding of divalent cations leads to structural stability and therefore preservation of enzymatic activity (Belli *et al.*, 1994).

Fig.1.4 FP-PC1 gene structure comparison with human and yeast PC1



It has been shown that AP1/NPP activity can be released from rat, porcine and tumour tissues by treatment with phosphatidylinositol specific phospholipase C, indicating certain forms of AP1/NPP activity produced by proteins are anchored via glycosylphosphatidylinositol (GPI) anchors at the C-terminus (Nakabayashi & Ikezawa, 1984; Nakabayashi & Ikezawa, 1986a; Nakabayashi & Ikezawa, 1986b; Nakabayashi *et al.*, 1994; Nakabayashi *et al.*, 1993a; Nakabayashi *et al.*, 1993b). However, to date no sequence information is available for these GPI anchored proteins; in addition, no GPI anchor domain has been identified in mammalian PC-1 genes. The possibility that one of the growing family of PC-1 homologous proteins could be GPI anchored was suggested by the apical localisation of gp130^{RB13-6}, in contrast to the basolateral location of PC-1 (Scott *et al.*, 1997). This is because the GPI anchor was reported to be the only apical sorting signal for membrane proteins (Lisanti *et al.*, 1989). PD-1 β , however, which is a type II transmembrane glycoprotein, has been localised to the apical membrane of pancreatic acinar cells (Terashima *et al.*, 1995).

1.6.3.2. Kinase activities of PC-1

There is considerable controversy surrounding the ability of PC-1 to act as an intrinsic kinase i.e. the ability to phosphorylate proteins other than itself. PC-1 has an ATP binding site characteristic of protein kinases, which follows the consensus sequence **GXGXXG...A/GXK**, but does not show close overall aa identity to characterised kinases (Oda *et al.*, 1991). PC-1 purified from bovine liver seemed to possess acidic fibroblast growth factor (aFGF) and Mn²⁺ stimulated, threonine specific autophosphorylation and phosphorylation, as histone and myelin basic proteins were phosphorylated (Oda *et al.*, 1991). PC-1 purified from rat liver membranes

also showed a similar autophosphorylation and phosphorylation profile, but activity was not stimulated by aFGF or Mn^{2+} (Uriarte *et al.*, 1993). Further analysis indicated that PC-1 purified from rat liver membranes had a third enzymatic activity as a threonine phosphatase, based on the evidence that the three activities co-purified, and by the presence of two adenine binding sites which conform to the H/R/KX(5-8) ϕ X ϕ ϕ D/E sequence motif (where ϕ represents a hydrophobic residue) and may mediate dephosphorylation of PC-1 (Uriarte *et al.*, 1995).

In direct contrast, analysis of kinase activity using recombinant PC-1 failed to show intrinsic kinase activity as phosphorylation of histones 1-4 did not occur (Belli *et al.*, 1995). It was suggested that previous studies showing kinase activity attributable to PC-1 (Oda *et al.*, 1991; Uriarte *et al.*, 1993; Uriarte *et al.*, 1995) were in fact due to contaminating kinases (Belli *et al.*, 1995). Recently a second protein with AP1/NPP activity, called gp130^{RB13-6} has been cloned from rat liver (Deissler *et al.*, 1995; Scott *et al.*, 1997) this could explain the discrepancy between results obtained from different groups (Belli *et al.*, 1995; Uriarte *et al.*, 1993; Uriarte *et al.*, 1995).

Analysis of the active site of PC-1 indicated that mutation of the active site threonine to tyrosine, serine or alanine reduced AP1 and autophosphorylation activity to undetectable levels (Belli *et al.*, 1995).

Earlier studies had indicated that PC-1 was capable of autophosphorylation as indicated by labelling with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at the active site threonine (Oda *et al.*, 1991; Uriarte *et al.*, 1993; Uriarte *et al.*, 1995). However this labelling could also be attributed to the formation of the adenylated covalent intermediate which is a step in the catalytic mechanism of alkaline phosphodiesterase I, as indicated by labelling of intact cells expressing PC-1 with $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ and $[\text{S}\text{-}^{35}]\text{dATP}$ (Belli *et al.*,

1995). However recent analysis utilising assays which are claimed to allow differentiation between autophosphorylation and adenylation of PC-1 suggest kinase and phosphatase activities (Stefan *et al.*, 1996a), as well as indicating a mechanism for regulation of PC-1 based upon reversible phosphorylation (Stefan *et al.*, 1996a)

1.6.3.3 PC-1 and the nucleotide salvage pathway

Nucleotides are key ubiquitous biochemicals involved in a wide variety of roles: as regulators of metabolic and hormonal pathways (ATP and cAMP); as sources of energy (nucleoside triphosphates); as coenzymes (NAD and FAD); and as the monomeric building blocks of nucleic acids (Voet & Voet, 1995). Cellular catabolic processes lead to the release of nucleotides into extracellular fluids, these nucleotides can be re-used when *de novo* synthesis is blocked, or nucleotides are scarce, by use of the salvage pathway for nucleotide recovery (Voet & Voet, 1995; Zubay, 1988). As nucleotides cannot be taken up directly into the cell, they are hydrolysed to nucleosides and then transported into the cell via a nucleoside transporter (Belli *et al.*, 1993).

When a soluble form of PC-1 was discovered, it was proposed that it might be involved in the nucleotide salvage pathway, by hydrolysis of extracellular nucleotides into nucleosides (Belli *et al.*, 1993). It was proposed that autophosphorylation of PC-1 on Thr-238 at low ATP concentrations serves as an auto regulatory mechanism that makes Thr-238 unavailable for participation in the hydrolysis of extracellular nucleotides when they become scarce (Stefan *et al.*, 1996a).

Recent studies have provided more direct evidence of the role of PC-1 in the nucleotide salvage pathway. Strong mitogenic stimulation of lymphocytes by phytohaemagglutinin (PHA), causes impairment of de novo nucleotide synthesis and leads to cells switching to the salvage pathway for recovery of nucleotides (Deterre *et al.*, 1996). Under these conditions expression of PC-1, CD38 and CD73 was elevated, suggesting that they may recycle extracellular NAD by degrading it to adenosine and nicotinamide (Deterre *et al.*, 1996).

1.6.3.4. Homology to Vitronectin Somatamedin-B domain

The extracellular cysteine rich region consists of 2 imperfect repeats of 41 and 44 aa (van-Driel & Goding, 1987) with 37% and 29% homology respectively to the somatamedin-B domain of vitronectin (Patthy, 1988). Vitronectin promotes cell adhesion by binding to proteoglycans and glycosaminoglycans at an Arg-Gly-Asp motif. The binding domain of vitronectin is adjacent to somatamedin B which is released as a free mitogenic serum peptide upon degradation of vitronectin (Suzuki *et al.*, 1985). PC-1 may also be implicated in a similar mitogenic role, although there is no evidence indicating this role. A Somatamedin B homologous domain is also found in the novel immature T-cell marker TCL-30 (Baughman *et al.*, 1992).

1.6.4 Mammalian PC-1 and disease

1.6.4.1. PC-1 and Type II Non-Insulin dependent diabetes mellitus (NIDDM)

Insulin controls blood glucose levels by stimulation of glucose influx in muscle and adipocytes and inhibition of gluconeogenesis in the liver (White & Kahn, 1994). The insulin pathway involves binding of insulin to the α subunit insulin receptor at the cell surface which leads to tyrosine kinase autophosphorylation of the intracellular β subunit of the insulin receptor (IR), which in turn tyrosine phosphorylates insulin receptor substrate 1 (IRS 1). IRS 1 is a cytoplasmic protein with multiple tyrosine phosphorylation sites and SH2 domains, therefore allowing mediation of the original signal to enzymes such as phosphatidylinositol-3-OH kinase (PI3K) and raf, MAP and S6 kinases (Kahn, 1995). Ultimately this leads to stimulation of glycogen, lipid and protein synthesis and translocation of glucose transporters to the surface of muscle cells and adipocytes (Kahn, 1995).

Individuals with NIDDM produce normal levels of insulin but are resistant to its action. Around 20% of cases of NIDDM are due to direct defects in genes in the primary insulin signalling system, hence research has been directed towards discovery of inhibitory chemicals (Kahn, 1995). Recently two inhibitors of IR tyrosine kinase have been described: TNF- α and PC-1 (Kahn, 1995; Maddux *et al.*, 1995). However, PC-1 overexpression does not seem to cause inhibition of IR tyrosine kinase in pseudo-acromagalic patients (Whitehead *et al.*, 1997).

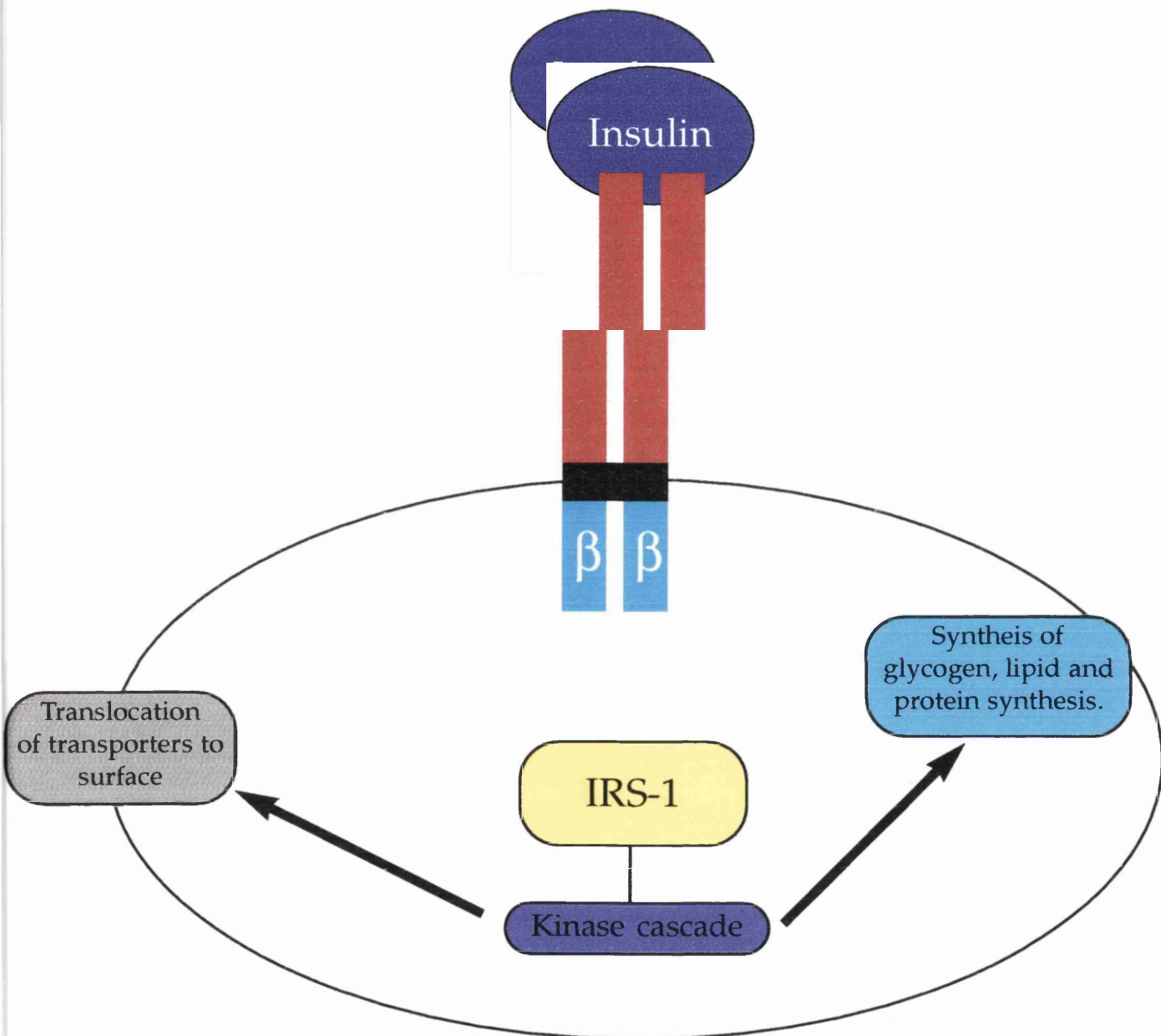
One of the ramifications of NIDDM is impaired IR kinase activity, but this is not as a result of defects in the IR gene, as IR content was normal and purified IR had normal levels of tyrosine kinase activity (Maddux *et*

al., 1995). Muscle cells and fibroblasts had increased expression of the IR tyrosine kinase inhibitor, which was identified as PC-1, as activity was increased in NIDDM patients and over expression of PC-1 in transfected cells reduced insulin stimulated kinase activity (Maddux *et al.*, 1995). There is currently no evidence implicating phosphorylation of IR as a means of arresting its activity (Grupe *et al.*, 1995).

PC-1 associated insulin resistance is categorised as post IR resistance as patients with IR mutations do not show higher expression levels of PC-1 (Whitehead *et al.*, 1995). In fact PC-1 activity is dramatically reduced in patients with mutations in IR and IRS-1 (Whitehead *et al.*, 1997). Recently PC-1 has also been shown to diminish insulin stimulated ribosomal S6 kinase activity, but not PI3K or ras (Kumakura *et al.*, 1998).

Another possible mechanism of PC-1 mediated inhibition, by increasing adenosine levels leading to a decrease in ATP levels, was also ruled out, as agents that change levels of adenosine had no affect on PC-1 inhibition of kinase activity (Grupe *et al.*, 1995). Furthermore, mutation of the active site threonine which abolishes AP1/NPP activity (Belli *et al.*, 1995) had no affect on inhibition of kinase activity (Grupe *et al.*, 1995). PC-1 has also been shown to act as an inhibitor of other kinases as well as IR, including phosphorylase kinase, protein kinase A and casein kinase 1 (Grupe *et al.*, 1995). In contrast recent studies have shown that IR tyrosine kinase inhibition is not PC-1 specific and results from the hydrolysis of ATP (Stefan *et al.*, 1996b).

Fig.1.5 The Insulin pathway



A simplistic outline of the initial stages in the insulin signalling pathway is represented. Insulin is the principle hormone controlling blood glucose levels. Insulin binds to the insulin receptor at a binding site in the alpha subunit causing tyrosine phosphorylation at the *trans* beta subunit. The insulin receptor is also involved in internalisation of insulin. This leads to tyrosine phosphorylation of the principal receptor of the insulin receptor known as insulin receptor substrate 1 (IRS 1), and leads to downstream signal transduction, largely by a cascade of phosphorylation events. Mammalian PC1 is thought to inhibit the tyrosine phosphorylation activity of the insulin receptor.

1.6.4.2. PC-1 and cancer

In situ hybridisation with human metaphase chromosomes localises the human PC-1 gene to chromosome 6q22 to q23, a site which is frequently deleted in lymphoid tumours (Buckley *et al.*, 1990). The proto oncogene MYB has also been localised to this region of human chromosome 6 (Buckley & Goding, 1992). Murine PC-1 was also co-localised with proto oncogene MYB on mouse chromosome 10 (Buckley & Goding, 1992) hence suggesting a common pattern of conserved gene linkage in murine and human genome organisation. PC-1 over expression in MDA-MB321 breast cancer cells is associated with inhibition of IR tyrosine kinase activity (Belfiore *et al.*, 1996) as shown previously in diabetic and obese individuals (Maddux *et al.*, 1995; Youngren *et al.*, 1996).

1.6.4.3. PC-1 and Lowe's syndrome

Lowe's syndrome is an X-linked disorder characterised clinically by congenital cataracts, hypotonia, mental retardation and renal tubular Fanconi syndrome (Lowe *et al.*, 1952). These symptoms have been attributed to undersulphation of glycosaminoglycans due to the depletion of adenosine 3'-phosphate-5'phosphosulphate (PAPS) due to pyrophosphatase activity (Fukui *et al.*, 1981; Yamashina *et al.*, 1983). The identity of the protein responsible for PAPS degradation was identified as PC-1 (Funakoshi *et al.*, 1992).

1.6.4.4 PC-1 and calcium pyrophosphate dihydrate crystal deposition disease

Calcium pyrophosphate dihydrate (CPPD) crystal deposition disease is a heterogeneous disorder in which CPPD crystals are deposited near chondrocytes and tendons (Terkeltaub *et al.*, 1994). CPPD deposition disease can be associated with increased extracellular inorganic pyrophosphate (PP_i) production due to nucleotide pyrophosphohydrolase (NTPPPH) activity by hydrolysis of the nucleotide monophosphate -pyrophosphate bond in nucleotides (Caswell *et al.*, 1987).

Association of NTPPPH with CPPD deposition disease was suggested as PC-1 overexpression lead to increased PP_i generation *in vitro* (Terkeltaub *et al.*, 1994). PC-1 expression in osteoblast-like osteosarcoma cell line U20S was upregulated by transforming growth factor β (Huang *et al.*, 1994) and basic fibroblast like growth factor (bFGF); and indirectly down regulated by interleukin 1 β (Lotz *et al.*, 1995). Recent evidence directly implicates PC-1 as the NTPPPH enzyme in U20S cells as immunoadsorption studies using PC-1 antibody severely depleted NTPPPH activity (Solan *et al.*, 1996).

1.6.5. PC-1 Homologues

As well as human and murine PC-1, PC-1 homologues in rat, canine, avian, bovine and simian sources have been suggested (Buckley *et al.*, 1990). There is also direct evidence of the PC-1 gene in rat and hamster (van Driel *et al.*, 1985a).

1.6.5.1 PC-1 homologues

Recently a wide range of genes have been characterised which seem to belong to a growing family of PC-1 homologous proteins. These include the human tumour cell motility factor autotaxin (Murata *et al.*, 1994); PD-1 α which is a brain specific protein with AP1/NPP activity (Narita *et al.*, 1994); PD-1 β a rat intestinal AP1/NPP (Terashima *et al.*, 1995); and the rat neural and differentiation tumour cell antigen gp130^{RB13-6} (Deissler *et al.*, 1995). However it has been suggested that human autotaxin and PD-1 α are the same protein (Scott *et al.*, 1997; Terashima *et al.*, 1995). A PC-1 homologue called ycr026c is also present on *Saccharomyces cerevisiae* chromosome III (Bork, 1992).

1.6.5.2 Fowlpox PC-1 (FP-PC1)

To date no homologue of PC-1 has been reported in any virus apart from FWPV (Laidlaw *et al.*, 1998). Detailed sequence analysis of FP-PC1 is presented in chapter 3. Briefly, however, the FP-PC1 gene has 38% amino acid identity with murine PC-1 over a 728 aa span as determined by FASTA. The FWPV gene also has a similar domain organisation apart from the absence of the cysteine rich somatamedin-B homologous region.

1.7 Aims

Prior to commencement of this project the only information available about FP-PC1 was the predicted full length gene sequence. Hence

the two key aims were to show if the gene was essential for FWPV replication *in vitro*, and secondly to express the protein in recombinant protein expression systems to allow both structural and functional characterisation. Key points to be answered using these two distinct approaches are described below:

1. Does the predicted full length sequence actually encode a protein that is expressed by FWPV during infection?
2. Does the FP-PC1 protein have a similar structural configuration compared to human and murine PC-1.
3. If the gene is essential, does this extend to the entire coding sequence or only a specific region. If non-essential are there any phenotypic ramifications, *in vitro* or *in vivo*, as a result of removal of the gene from the virus?
4. Is the FP-PC1 protein packaged into the virus, if not does it localise to a specific sub-cellular region?
5. Does FP-PC1 have similar functional characteristics as human and murine PC-1 proteins?

In summary, the ultimate aim of this study was elucidation of the possible role that FP-PC1 may play in FWPV, which in turn could assist in

elucidation of the actual physiological role of mammalian PC-1, which to this day remains undefined.

Chapter 2

Materials and Methods

2.1 Abbreviations, Birds, Bacteria, Viruses and Oligonucleotides

2.1.1 Abbreviations

AMPS	Ammonium persulphate
AP1/NPP	Alkaline phosphodiesterase 1/Nucleotide pyrophosphatase
BCIP	5-bromo-4-chloro-3'-indolylphosphate-p-toluidine
CAT	Chloramphenicol acetyltransferase
CEFs	Chick embryo fibroblasts
CTT	Coupled transcription translation
dFCS	Dialysed foetal calf serum
DTT	Dithiothreitol
EDTA	Ethyldiamine tetra acetic acid
EGTA	Ethyleneglycol tetra acetic acid
EW	Elga Water
FCS	Foetal calf serum
FWPV	Fowlpox virus
GPT	Xanthine-guanine phosphoribosyltransferase
IPTG	Isopropyl b-D-thiogalactopyranoside
LMP	Low melting point
M.O.I	Multiplicity of infection
MEM	Minimal essential medium
NBCS	New born calf serum
NBT	Nitro-blue tetrazolium
PBSa	Phosphate buffered saline
P. f. u.	Plaque forming unit
PMSF	Phenylmethylsulphonylfluoride
RIPA	Radioimmunoprecipitation
SAP	Shrimp Alkaline phosphatase
SDS	Sodium dodecyl sulphate
TEMED	N,N,N', N'-Tetramethylethylethylenediamine

2.1.2 Birds

Rhode Island Red and Light Sussex chickens were used in all experiments.

2.1.3 Bacterial Strains, and Plasmids

2.1.3.1 Bacterial strains

<i>E.coli</i> strain	Source	Genotype
JM109	Promega	<i>rec A1, sup E44, end A1, hsd R17, gyr A96, rel A1, thi, D(lac-proAB), (rk⁻·mk⁺), [F', traD36, proAB, lac I^q ZDM15]</i>
TG1		<i>supE, hsd₅, thi, _(lac-proAB), F'[traD36 , pro AB⁺, lac^q, lacZ_M15]</i>
XL1-Blue	Stratagene	<i>D(mcrA)183, D(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA, gyrA96, relA1, lac, [F' proAB, lacI^qZDM15, Tn10 (Tet^r)]</i>

2.1.3.2 Plasmids

Plasmid	Source	Comments
pBS SK ⁻	Stratagene	Derived from pUC19, F1 origin, β-galactosidase selection, T3 and T7 promoters, ampicillin resistant
pSHT	J. W. Goding (Madison and Bird 1992)	Derived from pSVT7, M13 ori, SV40 early promoter, T7 promoter, Haemagglutinin secretion signal, ampicillin resistant.
pNEB193GPT	NEB	Derived from pNEB193, ampicillin resistant.
pFLAG-CTS	IBI	Ampicillin resistant, ompA signal sequence.
pFTM2	D. Hot	Derived from pTM1, T7 promoter, kanamycin resistant.

2.1.3.2 Media for bacterial propagation

Media	Contents per litre	
SOB	Bacto-tryptone	20g
	Bacto-yeast extract	5g
	Sodium Chloride	0.5g
	Potassium Chloride	1.86g
	Sodium Hydroxide (5M)	20ml
LB broth	Bacto-tryptone	10g
	Bacto-yeast extract	5g
	Sodium Chloride	10g
LB agar	Bactoagar diluted in 1 litre LB broth	15g

2.1.4 Viruses, Eukaryotic Cells and Media

2.1.4.1 Viruses

All analysis of the FP-PC1 gene in this thesis was conducted using FWPV strain HP1-438 (FP9). This strain is derived from the original pathogenic progenitor strain HP1, that has been attenuated as a result of 6 passages on CEFs followed by 2 passages on chorioallantoic membranes and a further 438 passages in tissue culture using CEFs.

2.1.4.2 Cells

Primary CEFs were provided by F. Puttock and S. Duggan (tissue culture lab, I.A.H., Compton), and were produced from Rhode Island Red chickens.

2.1.5 Oligonucleotides

2.1.5.1 Cloning of full length FP-PC1 and deletion studies

Primer	Sequence	Orientation	Reference
F1	CCCCGCATGCATCATGACA GGAAGTAAAAC	Forward	C134713
F2	GAAGAAAGAATAAATACC GTATTGAGGTGG	Forward	G150592
R1	CCCGGATCCGTACATATAC TACATGTAAG	Reverse	G150485
R2	ATCTACATTAGACATACCA TGATCCGATAC	Reverse	C152551
Z	TCTATAATATGATGTGTG	Forward	G208765

2.1.5.2 Expression of full length and domains of FP-PC1

Primer	Sequence	Orientation	Reference
B1	GGGGGATCCCCAGCCATGA GAGATTCGAGGTTAGGG	Forward	G256081
B2	GGGGGATCCCCAGCCATGT CTATGAAAAATAG	Forward	G256046
B1sht	CCCGGGGATCCAAGAGATT CGAGGTTAGG	Forward	GIBCO 117161B
RB1	GGGGGATCCTCGAGAGATC GATCTTCAC	Reverse	C225010
C	CCCCCGAGATAGAGATTC GAGGTTAG	Forward	G172551
J	CCCCGGTACCCATGTAAGTT TTAAGTAAGC	Reverse	C221452
D	CCCCCTCGAGCGATGAATG GATTTAGATATG	Forward	G172753
K	CCCCGGTACCTTCATTATCC GTTATACC	Reverse	C221412

2.2 Cloning techniques

General techniques applied for manipulation and cloning of recombinant DNA molecules were followed as described in Molecular Cloning - A laboratory manual (Sambrook *et al.*, 1989).

2.2.1 Restriction digests of DNA

Digests were carried out using 10 units (u) of enzyme per 10 μ l of reaction mixture comprising of the appropriate enzyme buffer at the temperature recommended by the manufacturer.

2.2.2 Removal of protein from DNA samples by phenol/chloroform extraction

An equal volume of Tris-HCl buffered Phenol/Chloroform/Iso-amyl alcohol (48:50:2) was added to the sample and vortexed briefly. The sample was then centrifuged at 12,000rpm in a Sorvall MC 12V (DuPont) for 5 minutes (mins.), (this step will now be referred to as microcentrifugation). The upper aqueous phase was removed and stored, an equal volume of TE (10mM Tris-HCl pH8.0 and 1mM EDTA) was added to the remaining phenol, and centrifugation and upper phase removal were repeated. Aqueous samples were pooled and an equal volume of chloroform added and microcentrifuged for 5 mins., the aqueous phase was removed and stored.

2.2.3 Ethanol precipitation of DNA

DNA was precipitated by addition of Sodium Acetate (pH 5.2) to a final concentration of 0.3M and 2.5 volumes of absolute alcohol. After storage for 30 mins. at -20°C, precipitated DNA was pelleted by microcentrifugation for 15 mins., washed in 200µl 70% ethanol by microcentrifugation for 5 mins. and the pellet air dried and resuspended in water or TE (10mM Tris HCl pH 8.0, 1mM EDTA).

2.2.4 Titration to establish optimum levels of phosphatase for dephosphorylation of 5' compatible ends of DNA fragments

Five μg of the appropriate plasmid were digested with the appropriate enzyme/s and split into aliquots, after phenol/chloroform extraction and ethanol precipitation of DNA, various numbers of units of USB shrimp alkaline phosphatase (SAP) were added and incubated with the supplied buffer for 1hr at 37°C. After enzyme deactivation by incubation at 65°C for 20 mins. an appropriate aliquot was ligated, transformed and plated onto LB agar plates containing 100mg/ μl ampicillin. SAP units giving the lowest numbers of colonies were used for cloning.

2.2.5 Resolution of DNA fragments by agarose gel electrophoresis

DNA samples were mixed with 0.2 volumes of DNA sample buffer (4M Urea, 50% sucrose; 50mM EDTA and 0.1% Bromophenol blue; diluted in distilled water) and resolved by electrophoresis through agarose gels at 0.7 - 1.5% in 1 x TAE or TBE containing ethidium bromide at 10 μgml^{-1} in 1 x TAE or TBE buffer containing a similar amount of ethidium bromide. Electrophoresis was carried out in a Pharmacia LKB GNA 100 gel tank for 2-3 hours. Ten μl of $\text{ØX174}/\text{HaeIII}$ and $\lambda/\text{Hind III}$ marker mix (500mg $\lambda/\text{Hind III}$, 40mg $\text{ØX174}/\text{HaeIII}$, 125 μl DNA sample buffer and 65 μl TE) were run alongside samples as size markers. DNA was visualised by ultra-violet illumination using an UVP dual density transilluminator and photographed using an UVP Imagestore 5000 system.

2.2.6 Isolation of DNA fragments from agarose gels

2.2.6.1. Gene clean kit II

After LMP agarose (BRL 5517UA) gel electrophoresis in 1 x TAE buffer the DNA band was excised, weighed and 3 volumes of NaI solution added (Bio 101- cat 1001-401). This was heated at 50°C for 5 mins. and mixed with 20µl of glassmilk solution and left overnight on a roller (Denley spiramix 5) at 4°C. Glassmilk beads were pelleted by microcentrifugation for 5 seconds, supernatant was discarded and the beads resuspended in 500µl New Wash (Bio 101) and microcentrifuged similarly. The supernatant was discarded and beads resuspended and pelleted in New Wash in a similar fashion two more times. Beads were then resuspended in TE and incubated at 50°C for 10 mins. Beads were then pelleted by microcentrifugation for 30 seconds and the supernatant collected and stored.

2.2.6.2. β-agarase enzyme digestion

After LMP agarose (BRL 5517UA) gel electrophoresis in 1 x TAE buffer, DNA bands were excised, weighed and resuspended in 0.1 volume β-agarase buffer (NEB), gel slices were then melted by incubation at 65°C for 10 mins. and cooled at 40°C for 15 mins. before addition of 2 units of β-agarase enzyme per 200µl of gel slice. This was incubated at 40°C for 2 hours. The mixture was then equilibrated to give a final NaCl concentration of 0.5M and placed at 4°C for 15 mins., undigested agarose was then removed by microcentrifugation for 5 mins. and the supernatant resuspended in 2.5 volumes of isopropanol and DNA precipitated in a similar fashion as previously described.

2.2.6.3. Gen-elute columns

Gen-elute columns (Supelco) were prepared by addition of 100 μ l TE to the membrane and microcentrifuged for 5 seconds, flow through was discarded and a non LMP agarose (Gibco-BRL 15510-027) slice containing DNA was placed on top of the membrane and microcentrifuged for 10 mins., DNA was then precipitated and resuspended in TE by previously described methods.

2.2.7 DNA ligations with cohesive ends

After appropriate digestion of DNA, relative insert and vector yields were analysed by standard agarose gel electrophoresis and appropriate ligation mixtures set up to give a 3:1 vector : insert molar ratio. This was incubated with T4 DNA ligase (Boehringer) and 10 x T4 DNA ligase buffer (Boehringer) made up with Elga Water (EW) to give a final concentration of 0.1u T4 DNA ligase per μ l of reaction and 1 x T4 DNA ligase buffer. Ligation was carried out at room temperature for 1 hour.

2.2.8 Transformation of *E.coli*

2.2.8.1. Preparation of chemically competent *E.coli*

E.coli strain XL1 blue/TG1 or JM109 was inoculated from frozen stock into 10ml of SOB medium and incubated overnight at 37°C with moderate agitation, 1ml of this culture was inoculated into a 1litre conical

flask containing 100ml SOB medium, and incubated under similar conditions until an OD at 680nm of 0.25 was reached (Pye-Unicam). The culture was then collected into 50ml tubes and chilled on ice for 15 mins., before centrifugation at 2000 r.p.m for 15 mins. at 4°C (IEC Centra 3R). The pellet was resuspended in 35ml 0.1M CaCl₂ and incubated on ice for 90 mins. and pelleted as stated previously. Cells were then resuspended in 8ml of 0.1M CaCl₂ and incubated on ice for 15 mins. before being split into aliquots and flash frozen using liquid nitrogen and stored at -70°C.

2.2.8.2. Transformation of chemically competent *E.coli*

Plasmid DNA equivalent to 20 to 200ng was added to 100µl of chemically competent cells and incubated on ice for 30 mins., heat shocked for 45 seconds at 42°C and returned to ice for a further 2 mins. The cells were made up to a total volume of 1ml in SOC medium (SOB +: 10mM MgCl₂, 10mM MgSO₄ and 20 mM Glucose) and incubated with moderate agitation for 60 mins. at 37°C before being plated out on to LB agar plates containing ampicillin at 100µg/ml or kanamycin at 25µg/ml and incubated overnight at 37°C.

2.2.8.3. Preparation of electrocompetent *E.coli*

E.coli strain XL1 blue was inoculated from frozen stock into 50ml of LB broth medium and incubated overnight at 37°C with moderate agitation, all of this culture was inoculated into 250ml of LB broth medium in a 1litre conical flask and incubated under similar conditions until an OD

at 550nm of 0.5 – 0.6 was reached (Pye-Unicam). Cells were then split into 4 aliquots in 250ml centrifuge bottles and kept on ice for 30 mins. Each aliquot was then pelleted by centrifugation at 3,500 r.p.m. for 15 mins. at 4°C (Sorvall RC-5B using a Sorvall GSA rotor). Cells were then slowly resuspended in 250ml ice cold EW, whilst keeping on ice at all times, (in all subsequent steps cells were kept on ice at all stages between centrifugation). Centrifugation was repeated as previously and cells resuspended in 150ml ice cold water. Cells were re-pelleted as before and resuspended in 10ml 10% ice cold glycerol and transferred to 50ml centrifuge bottles and pelleted by centrifugation at 5,800 rpm for 15 mins. at 0°C (Sorvall RC-5B using a Sorvall SS34 rotor). Glycerol was then decanted and cells resuspended in 500µl 10% ice cold glycerol by vortexing. 40µl aliquots were then placed immediately on dry ice and stored at -70°C until required.

2.2.8.4. Transformation of electrocompetent *E.coli*

Transformation of electrocompetent *E.coli* was carried out using the Invitrogen electroporator II apparatus with 1mm gap cuvettes (Invitrogen). Prior to electroporation; electroporation cuvettes, plasmid DNA (up to 200ng in 2µl EW) and SOC medium were placed on ice. The electroporator capacitance and resistance were set to 50µF and 150ohms respectively. The power supply voltage, current and power were set to 1500V, 25mA and 25W respectively. The electroporator was discharged twice without a cuvette: with the arm/disarm dial in the armed position the electroporator was switched from pulse to charge mode. After 30 seconds, the pulse mode was engaged. Forty µl of electrocompetent cells were mixed with plasmid DNA and placed into the gap in the electroporation cuvette. Cuvettes were tapped to remove air bubbles and

dried with a tissue to remove external condensation before placing in the electroporator, ensuring that the arm/disarm dial was in the disarmed position. The arm/disarm dial was then moved to the armed position, and the electroporator switched from pulse to charge mode. After 30 seconds, the pulse mode was engaged and the arm/disarm dial moved back to disarm before removing the cuvette. 1ml SOC medium was added to the cuvette and mixed with the electrocompetent cells/plasmid DNA mixture and transferred to a sterile bijoux and incubated for 60 mins. at 37°C with moderate agitation before being plated out on to LB agar plates containing ampicillin at 100µg/ml or kanamycin at 25µg/ml and incubated overnight at 37°C.

2.2.9 Preparation of plasmid DNA

2.2.9.1. Small scale plasmid preparations - Alkali Lysis

A 2.0ml overnight culture was microcentrifuged for 1 min and the supernatant discarded. Pelleted bacteria were resuspended in 150µl solution I (50 mM glucose, 25 mM Tris-HCl (pH8.0), 10 mM EDTA) by gentle vortexing. Two hundred µl solution II (200 mM NaOH, 1% SDS) was added and mixed by gentle inversion and incubated for 5mins. at room temperature. Then 150 µl of ice cold solution III (60 ml 5M KAc (pH4.8), 11.5 ml glacial acetic acid, 28.5 ml water) were added and mixed by inversion and incubated at room temperature for a further 5 mins. Following microcentrifugation for 5 mins., the supernatant was extracted using standard phenol chloroform extraction and DNA pelleted and resuspended in 45µl TE following ethanol precipitation. RNase (Sigma)

was then added to a final concentration of 10 μ g/ml and incubated at 37°C for 10 mins.

2.2.9.2. Large scale plasmid preparation

A 500ml overnight LB *E. coli* culture was pelleted by centrifugation at 10,000r.p.m. for 5 mins. at 4°C in a Sorvall RC-5B using a Sorvall GSA rotor and cells resuspended in 7.5 ml GTE solution (50 mM glucose, 25 mM Tris-HCl pH8.0, 10 mM EDTA pH8.0) before addition of 15ml room temperature solution II (0.2M NaOH, 1% SDS) and incubated on ice for 10mins. After addition of 11ml solution 3 (60 ml 5M potassium acetate pH4.8, 11.5ml glacial acetic acid, 28.5ml sterile water) and incubation on ice for 10mins., the preparation was centrifuged at 15,000r.p.m. for 20 mins. at 4°C in a Sorvall RC-5B using a Sorvall SS34 rotor. The supernatant was decanted and 0.6 volume isopropanol added. This was incubated at room temperature for 30 mins., before centrifugation at 10,000r.p.m. for 20 mins. at 4°C in a Sorvall RC-5B using a Sorvall SS34 rotor. Pelleted material was resuspended in 2ml TE and CsCl (1.2g/ml) and split into two eppendorfs and microcentrifuged for 5mins. The supernatant was placed into 6ml ultracentrifuge tubes (Sorvall 03945) and CsCl content adjusted to a final density of 1.7g/ml. This was then centrifuged for 16 hours at 50,000r.p.m. at 25°C in a Sorvall OTD 65B using a Sorvall TV1665 vertical rotor.

Plasmid DNA was then recovered by side puncture and ethidium bromide extracted with isopropanol saturated with sodium chloride and water. Three volumes of TE were added to the plasmid DNA which was pelleted by ethanol precipitation and finally resuspended in TE.

2.2.10 Bacterial glycerol stocks

Overnight culture of *E. coli* in LB broth was taken and 60% (0.2µm filter sterilised) glycerol solution added to give a final concentration of 30%. Cultures were then stored at -70°C for up to six months.

2.3 Amplification of DNA by the polymerase chain reaction (PCR)

2.3.1. Oligonucleotide primers

2.3.1.1 Synthesis

Oligonucleotide primers were synthesised by K. Mawditt and supplied as an ammonia buffered solution which was incubated at 50°C overnight in 1.5ml eppendorf tubes surrounded by sand in a test-tube using a heated block. Ammonia was removed by evaporation using a vacuum centrifuge (Uniscience univap) and resuspended in EW water.

Oligonucleotides were also supplied by Gibco-BRL and Cruachem, as desiccated samples and resuspended in EW water as described above.

2.3.1.2 Purification of oligonucleotide primers

Oligonucleotides were purified by electrophoresis through a 12% acrylamide (19:1) TBE gel using a Bio Rad Sequi-Gen sequencing cell. Twenty µl of oligonucleotide was mixed with 10 µl formamide stop buffer (98% deionised formamide, 10 mM EDTA (pH8.0) 0.025% xylene cyanol and

0.025% bromophenol blue) and incubated at 100°C for 3 mins. This was then loaded onto the gel and was run until the first dye front was 2/3 of the way down the gel. The gel was removed from the sequencing cell and wrapped in cling film. The oligonucleotide band was visualised by UV shadowing and the band excised. The oligonucleotide was then eluted overnight at 4°C into 0.8 ml 0.5M NH₄Ac/1mM EDTA elution buffer, before ethanol precipitation as described previously (section 2.2.3).

2.3.2 PCR reactions

PCR was performed using a programmable Techne Progene apparatus in 200µl tubes. Reaction mixtures were made up to 50µl with chilled, UV irradiated EW consisting of the appropriate PCR buffer, 2.5u Taq polymerase, 1µl of each oligonucleotide at 10pmol, 2.0mM MgCl₂, 1.0mM dNTPs and approximately 100ng of template DNA. PCR cycling consisted of 1 cycle of denaturation for 5 mins. at 94°C, followed by 25 cycles composed of three steps: (i) denaturation for 1 minute at 94°C, (ii) annealing of oligonucleotide for 1 minute at 37-45°C depending on optimum temperature calculated using Primer v 0.5 (Whitehead Institute for Biomedical Research) and (iii) extension at 72°C for 1-5 mins. according to length of proposed product. A final extension at 72°C for 10 mins. was used to complete the reaction.

2.4 Protein analysis

2.4.1 Resolution of proteins by SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were resolved by electrophoresis through denaturing polyacrylamide gels using the Tris/glycine discontinuous buffer system, using the Bio-Rad Mini Protean II apparatus. A resolving gel (375mM Tris-HCl pH 8.8, 0.1% SDS, 0.75% AMPS, 0.0066M TEMED and 7.5-17.5% acrylamide) was allowed to set on top of which a stacking gel (125mM Tris-HCl pH 6.8, 0.1% SDS, 0.75% ammonium persulphate, 0.0066M TEMED and 5.0% acrylamide) was poured and an 8 or 13 well comb added prior to setting. Protein samples were prepared before loading by addition of 0.2 volume of SDS-PAGE sample buffer (62.5mM Tris pH 6.8, 2% SDS, 2% mercaptoethanol, 10% glycerol and 0.01% bromophenol blue) and boiled at 100°C for 5 mins. Protein size markers were run alongside (LC 5625 Novex) and electrophoresis conducted at 100V for 2-3 hours in SDS-PAGE running buffer (0.192M glycine, 0.1% SDS and 0.025M Tris-HCl) .

2.4.2 Coomassie blue staining of SDS-PAGE gels

Acrylamide gels were placed in Coomassie brilliant blue stain solution (0.1% Coomassie brilliant blue, 50% methanol, 7% acetic acid) and rotated gently on a R100 rotatest shaker (Luckham) for 10 mins., this was decanted and proteins visualised by gentle rotation in destain solution I (50% methanol, 7% acetic acid) for 10 mins. For further clarification destain solution II (7% acetic acid) was added and left overnight on a rotator as stated above.

2.4.3 Fixing of SDS-PAGE gels

Gels containing radiolabelled proteins were fixed using 4% acetic acid solution for 15 mins., the gel was then soaked and agitated in Amplify (Amersham - Amp 100) for 30 mins., dried for 2 hours at 60-80°C using a vacuum gel drier (Bio-rad 443) and exposed to film (Kodak X-Omat AR diagnostic film) at -70°C.

2.4.4 Immunoblot analysis

2.4.4.1. Electrophoretic transfer of proteins to nitro-cellulose and Western Blotting

Protein to be analysed was resolved by standard SDS-PAGE electrophoresis and transferred to a nitro-cellulose membrane (Hybond-C Amersham) using the Bio-Rad Mini-Transblot kit at 100V for 2 hours at room temperature or 16 hours at 4°C in Transfer buffer mix (20% methanol, 10% Transfer buffer [0.192M glycine and 0.025M Tris-HCl - stock concentration] diluted in distilled water). The nitro-cellulose membrane was then removed and immersed overnight at 4°C in 5% milk powder (Marvel) in TBS (50mM Tris-HCl pH 8.0 and 150mM NaCl). The milk solution was then removed and antibody added at a 1:1000 ratio in TBS containing 2% milk powder and placed on a rotator for 1hour, the membrane was then washed in TBS three times and TBS/Tween (0.1%) twice, ending with a TBS wash. Conjugate (anti mouse IgG alkaline phosphatase Sigma A5153) was added at a 1:1000 ratio in TBS/2% milk powder and placed on a rotator for 1hour. Excess conjugate was removed by washes as described for removal of excess antibody. Epitope-antibody-

conjugate complexes were detected by use of a western blot detection system based upon NBT/BCIP (Pierce 34042).

2.5 Expression and purification of FP-PC1 in *E. coli*.

2.5.1 Screening of transformants for flag fusion proteins

Cultures of individual colonies were grown up overnight at 37°C with moderate agitation in 5ml LB broth containing 50µg/ml ampicillin and 0.4% glucose. Overnight cultures were then diluted 1:100 in fresh medium and further incubated until an OD at 600nm of 0.2 was recorded, cells were induced by addition of IPTG to 0.5mM and incubated similarly for a further 2 hours. A 500µl aliquot was then microcentrifuged for 1 minute and resuspended in 50µl SDS-PAGE sample buffer and boiled for 5 mins. Samples were then analysed by western blot using the M2 antibody for the flag epitope (IBI Kodak).

2.5.2 Analytical fractionation of flag fusion proteins

In order to establish the fraction containing the highest yield of protein; overnight cultures were grown up and induced as stated above, the whole cell sample was prepared as in 2.5.1. The remaining culture was split into two aliquots and pelleted at 5000rpm for 10 mins. at 10°C, culture supernatant was stored and analysed as representing protein fractionating to the culture medium, by standard immunoblotting.

2.5.2.1 Periplasmic fraction

The first of the two pellets from section 2.5.1 was resuspended in 40ml/g (by wet weight of cells) periplasmic shock buffer (0.5M sucrose, 0.03M Tris-HCl pH 8.0 and 1mM EDTA). The cells were pelleted by centrifugation at 3500 rpm for 10 mins. at 10°C, supernatant discarded and cells rapidly resuspended in 25ml/g wet weight of cells in distilled water at 4°C. Cells were then repelleted by centrifugation at 3500 rpm for 10 mins. at 4°C. Supernatant was immediately collected and resuspended in an appropriate volume of SDS-PAGE buffer and analysed by immunoblotting.

2.5.2.2 Whole cell soluble and insoluble fraction

The second pellet attained in 2.5.2 was resuspended in 5ml of extraction buffer A (50mM Tris-HCl pH8.0, 5mM EDTA, 0.25mg/ml lysozyme and 50µg/ml sodium azide) and incubated at room temperature for 5 mins. An equal volume of extraction buffer B (1.5M NaCl, 0.1M CaCl₂, 0.1M MgCl₂, 0.02 mg/ml DNase 1 and 0.05 mg/ml ovumucoid protease inhibitor) was added and the sample incubated for a further 5 mins. The sample was then centrifuged at 18,000 rpm for 60 mins., the supernatant was analysed by immunoblotting as the soluble whole cell sample, whereas the pellet was resuspended in 5ml extraction buffer A and a 50µl aliquot added to an equal volume of SDS-PAGE buffer and analysed by immunoblotting.

2.5.3 Optimisation of expression of flag fusion proteins

In order to maximise yield of protein, the following ranges of the stated variables were used, quantification of yield of protein was approximated by intensity indicated by resultant bands after immunoblotting.

Variable	Range
Temperature of incubation	25, 30 and 37°C
Length of induction in minutes	30, 60, 120 and 240
Final concentration of IPTG used during induction	0.005-10mM

2.5.4 Large scale periplasmic preparation of flag fusion proteins

Overnight cultures were grown up and induced at optimum growth and induction conditions and harvested by centrifugation at 5,000rpm for 10 mins. at 10°C. Cells were resuspended in 40ml/g of cells of freshly made 10mM Tris-HCl pH 8.0 and cells pelleted by centrifugation at 3,500rpm for 10 mins. at 10°C. Supernatant was decanted and cells resuspended and repelleted as in the previous step, before being resuspended in 40ml/g of cells periplasmic shock buffer and pelleted by centrifugation at 3,500rpm for 10 mins. at 10°C. Supernatant was discarded and the pellet resuspended in distilled water at 4°C and cells repelleted by centrifugation at 3,500rpm for 10 mins. at 4°C. An equal volume of 2 x TBS was then added to the supernatant and centrifuged at 25,000rpm for 60

mins. at 4°C, supernatant was then filtered using a 0.2µm filter and stored at -20°C.

2.5.5 Purification of flag tagged fusion proteins

M2 affinity gel (IBI-Kodak) was prepared by pelleting of a 0.5ml aliquot by microcentrifugation at 2,000 rpm for 10 mins., the supernatant was then removed and resuspended in 1ml TBS and the beads repelleted as stated. Resuspension in TBS and pelleting was repeated and the 5ml of protein sample was added and rolled overnight in a rotator (Denley spiramix 5) at 4°C. Beads were then pelleted and washed in 1ml aliquots of TBS five times. Protein was then eluted from the beads by two successive washes in 0.1M glycine pH 3.0. Eluant was added to 5µl 1M Tris-HCl pH 8.0 and stored at -70°C. Beads were regenerated by four washes in TBS and stored at 4°C.

2.6 Expression and analysis of FP-PC1 in an in vitro reticulocyte based coupled transcription/translation system (CTT)

2.6.1 Translation procedure

Translation reactions (Promega L4610) were set up on ice as shown below in 1.5ml screw top microcentrifuge tubes, (the addition of rabbit reticulocyte lysate and [³⁵S] methionine was performed last):

Component	Amount
Rabbit reticulocyte lysate	25 μ l
Reaction buffer	2 μ l
T7 RNA polymerase	1 μ l
1mM Amino acid mixture (-methionine)	1 μ l
[³⁵ S]-methionine (1000Ci/mmol) at 10mCi/ml (Amersham)	4 μ l
Rnasin Ribonuclease Inhibitor (40u/ μ l)	1 μ l
Plasmid DNA (1 μ g/ μ l, dissolved in nuclease free water)	1 μ l
Nuclease free water	15 μ l

Reactions were incubated for 60 mins. at 30°C prior to analysis by SDS-PAGE electrophoresis and autoradiography as previously discussed.

2.6.2 Translation of Proteins in the presence of canine pancreatic microsomes

Translation reactions (Promega L4610) were set up on ice (in the order and quantities shown below), in 1.5ml screw top microcentrifuge tubes.

Component	Amount
Rabbit reticulocyte lysate	12.5 μ l
Reaction buffer	0.5 μ l
1mM Amino acid mixture (-methionine)	0.5 μ l
Rnasin Ribonuclease Inhibitor (40u/ μ l)	0.5 μ l
T7 RNA polymerase	0.5 μ l
[³⁵ S]-methionine (1000Ci/mmol) at 10mCi/ml (Amersham)	2.0 μ l
Nuclease free water	5.5 μ l
Plasmid DNA (1 μ g/ μ l, dissolved in nuclease free water)	0.5 μ l
Canine microsomal membranes (Promega Y4051)	2.5 μ l

2.6.3 Radioimmunoprecipitation of CTT translation products

Protein A sepharose CL4B beads (0.7g, Pharmacia 17-0780-01) were mixed with 50ml of 0.1M sodium phosphate buffer (pH 8.1). Sepharose beads were pelleted by centrifugation at 2,000 rpm for 5 mins., resuspended as before and repelleted, before suspending in 7ml of 0.1M sodium phosphate buffer and stored at 4°C. Fifty µl of this slurry was incubated with 10µl of mouse immunoglobulin conjugate (Dako Z259) for 30 mins. at room temperature. The slurry was pelleted and washed twice with 500µl 0.1M sodium phosphate buffer (pH 8.1). Ten µl of M2 flag antibody (IBI-Kodak) or 6x histidine monoclonal antibody (Clontech) was added to the slurry and incubated at 4°C on a roller (Denley spiramix 5) for 60 mins. The slurry was washed twice as before, and pre-adsorbed with 500µl of unlabelled, uninfected CEF cell lysate for 16 hours at 4°C. Beads were then re-pelleted and the supernatant discarded. Twenty µl of translation products from a CTT reaction were then added to the sepharose slurry and incubated at 4°C for 3 hours on a roller (Denley spiramix 5). The sepharose was washed five times with RIPA I buffer (0.15 M NaCl, 0.05 M Tris-HCl pH 7.2, 1% Triton-X-100, 0.1% SDS, 1% sodium deoxycholate) and twice with RIPA II buffer (0.15 M NaCl, 0.05 M Tris-HCl pH 7.2, 1% Triton-X-100). The samples were then analysed by SDS PAGE.

2.6.4 Protease protection assay

CTT translations were carried out with and without canine microsomal membranes. Translation products were cooled on ice and stabilised by addition of CaCl₂ to a final concentration of 10mM. Preincubated (15mins. at 37°C) Proteinase K (PK) solution (10mg/ml PK,

10mM Tris.HCl and 10mM CaCl₂) was added to the translation mixtures to a final concentration of 1mg/ml and the mixture incubated on ice for 60 mins. before addition of PMSF to a final concentration of 5mM for 10 mins. before analysis of products by SDS PAGE as described previously. Controls were incubated with 1% Triton X-100 in addition to PK.

2.6.5 Glycosylation assay

After translation, microsomes were pelleted by centrifugation at 12,000 rpm for 10 mins. and resuspended in 10ul PBSa and SDS to 0.5% and boiled for 2 mins. The mixture was then cooled to 37°C before addition of 90ul of ENDO F/PNGF buffer mix (50mM Sodium Acetate (NaAc) pH 5.2, 25mM EDTA, 1% octylglucoside and 1% β-mercaptoethanol) and 0.2 units of ENDO F/PNGF (Boehringer). The reaction was then incubated at 37°C overnight and products analysed by SDS PAGE.

2.7 Construction of recombinant FWPVs

2.7.1 Infection of Chicken embryo fibroblasts (CEF) monolayers

FWPV infection of CEFs was performed by removal of the culture medium from cell monolayers in flasks from 25cm² to 175cm² or 20cm² dishes, virus stocks diluted in serum free Medium 1 x 199 (10% 10 x 199, 10% tryptose phosphate broth, 4.4% sodium bicarbonate, penicillin 100 u/ml, streptomycin 100µg/ml and mycostatin 25u/ml) were used to infect cells at m.o.i. levels between 0.001 and 50 using sterile 10ml glass pipettes.

Cells were incubated for 1-2 hours at 37°C in 5% CO₂, before the viral inoculum was replaced with Medium 1 x 199 containing 2% new born calf serum (NBCS) and incubated for a further 72-96 hours.

2.7.2 Transfections

The culture medium from monolayers of 80% confluent CEF cells was removed from 25cm² flasks and monolayers infected with 2.0×10^5 pfu per cm² of cells (virus titre: 1×10^7 pfu/ml), to give an moi of 1-2 and incubated for 2 hours as previously described. 4.5ml of Medium 199 containing 5% NBCS were added to the flasks which were incubated for a further 2 hours. Prior to transfection, 10µg of plasmid DNA and 20µl of transfection reagent (Lipofectin Gibco) were diluted into separate bijoux containing 100µl of serum free Medium 199, both bijoux were then combined and mixed slowly at room temperature for 15 mins. During this period flasks were washed twice with 2ml serum free Medium 199. Three ml of serum free Medium 199 were added to the plasmid DNA/lipofectin mix which was subsequently added to each flask and incubated overnight at 37°C The medium was then discarded and replaced with 5 ml GPT selective Medium 199 (mycophenolic acid 25µg/ml, xanthine 250µg/ml and hypoxanthine 15µg/ml) containing 2% NBCS, flasks were incubated for a further 72 hours before harvesting virus.

2.7.2 Selection and plaque purification of FWPV recombinants

Transfection medium containing virus was freeze thawed three times to allow cell lysis and release of virus. Twenty cm² dishes of 80% confluent monolayers of CEF cells were infected with 100ul of serially diluted virus from 10⁻¹ to 10⁻⁴ and incubated for 1 hour. Viral inoculum was then removed and overlaid with 5ml of GPT selective Medium MEM (50% Low melting point agarose, 10% 10 x MEM, 10% tryptose phosphate broth, 4.4% sodium bicarbonate, penicillin 100u/ml, streptomycin 100µg/ml, mycostatin 25u/ml, mycophenolic acid 25µg/ml, xanthine 250µg/ml and hypoxanthine 15µg/ml), allowed to set at room temperature and then incubated for 72-96 hours until plaques were observed. Plaques were picked into 0.5ml serum free Medium 199 using plugged pasteur pipettes and freeze thawed three times before repeating two further plaque purifications in GPT selective media. Virus was then plaque purified twice in non-GPT selective before screening for the loss of the GPT gene by further rounds of plaque purification in GPT selective and non-selective medium.

2.7.3 Amplification of plaques, isolation of viral DNA and identification of FWPV recombinants

One hundred µl of freeze thawed plaque material from the final plaque purification were used to infect a 25cm² flask of CEFs and incubated for 72 hours, the flask was then freeze thawed and 1ml of medium was used to inoculate a 175cm² flask which was similarly incubated and freeze thawed. Cells were pelleted by centrifugation at 1,500 rpm for 10 mins. (Centra 3R centrifuge) and virus containing medium decanted into 50ml centrifuge bottles. Virus was then pelleted by centrifugation at 18,000 rpm

for 1hr at 4°C and resuspended in 0.5ml 10mM Tris-HCl (pH 9.0). An equal volume of 2 x lysis buffer (100mM Tris-HCl pH 8.0, 200mM β -mercaptoethanol, 2% [w/v] SDS and 54% [w/v] sucrose) was added and incubated at 50°C for 1 hour before leaving overnight at 4°C in 15ml blue topped falcon tubes. Phenol/chloroform extraction and ethanol precipitation of DNA were carried out as described previously except that during the former procedure falcon tubes were shaken for 6 hours. Viral DNA was then used in PCR reactions using primers that would allow identification of recombinants. PCR reactions were subjected to hot starts to allow thorough denaturation of viral DNA by incubation of PCR samples at 94°C for 5 mins. before addition of Taq polymerase.

2.8 Analysis of FWPV recombinants

2.8.1 Growth curves

Medium from confluent 20cm² dishes was discarded and monolayers washed with warmed phosphate buffered saline (PBSa). One ml of mutated or wt FWPV (at m.o.i levels between 0.01 and 50) diluted in serum free Medium 199 were added to the dishes using a P1000 pipette (Gilson) and left to adsorb for exactly 60 mins. at 37°C. The viral inoculum was removed and monolayers washed twice with warmed PBSa, before addition of 5ml of Medium 199 containing NBCS at 2% with a P5000 pipette (Gilson). Dishes were incubated as previously described, and viral samples were taken in triplicate at intervals between 0 and 96 hours.

Total virus samples were taken by freeze thawing dishes and storing the resultant at -70°C. Samples representing the extracellular

sample were taken by storing 1ml aliquots (taken with a P1000) from the culture medium of each dish and store at -70°C . The remaining 4ml were discarded and cells washed once in PBSa as described previously, before addition of 1ml of serum free Medium 199. The dish was then freeze thawed three times before placing at -70°C prior to titration by plaque assay.

2.8.2 Plaque assays

Titration of each viral sample was carried out in triplicate as described in section 2.7.2, except that in all stages the use of a glass pipette was exchanged for a P1000 or P5000 pipette.

2.8.3 Plaque size assay

CEF monolayers in 20cm^2 dishes were infected as described in section 2.8.1. After various time points p.i. the viral inoculum was discarded and monolayers fixed in 10% formaldehyde in PBSa for 10 mins. before discarding the agarose overlay and repeating the fixing step for a further 2 mins. over the bare monolayer. Plaques were then visualised by staining with crystal violet stain (2% [w/v] Gentian violet and 20% ethanol, made up in PBSa) for 5 mins, after which monolayers were liberally washed in distilled water to remove excess staining.

2.8.4 Analysis of role in the Nucleotide salvage pathway

Viral plaques were amplified and virus pelleted before resuspending in PBSa. Virus was then split into two aliquots and diluted in either Medium MEM or 199. Growth curves and titration of virus was carried out as described previously (sections 2.8.2 and 2.8.3).

2.8.5 In vivo analysis

Four week old Rhode Island Red (RIR) and Light Sussex (LSx) chickens were infected by scarification at both wing webs, with 50 μ l (approximately 5×10^7 p.f.u.) of purified wt or mutant FWPV (FP9 [wtFWPV], Δ PC1-b [FP-PC1 full gene knockout FWPV, see chapter 4] or pBSA [negative control]). Pock size and morphology were assessed at various time intervals. Tissue and blood samples were also taken at various time intervals.

2.9 Expression of FP-PC1 in a FWPV expression system

2.9.1 Transient expression using T7 polymerase recombinant FWPV

Confluent CEF monolayers in 25cm² flasks were infected at an m.o.i. of 3 with 1ml recombinant FWPV (rFWPV) (in serum free Medium 199) expressing T7 RNA polymerase. After incubation at 37°C for 2 hours, 4ml of Medium 199 containing NBCS at 2% were added and flasks incubated similarly for a further 2 hours. Cells were then transfected with

20µg of plasmid DNA as described in section 2.8.2 and incubated for 22 hours before cells were scraped, resuspended in 0.5ml 0.25M Tris HCl (pH 7.0) and freeze thawed three times before analysis of proteins by immunoprecipitation and western blotting.

2.9.2 Expression from recombinant FWPV

Recombinant FWPV containing flag tagged FP-PC1 driven by the T7 promoter was co-infected with rFWPV expressing T7 RNA polymerase at a m.o.i. of 10 into 80% confluent CEF monolayers and incubated for 24 hours in GPT selective Medium 199. The growth medium was discarded and scraped cells resuspended in 0.5ml PBSa. Cells were freeze thawed three times before analysis.

2.9.3 Tunicamycin treatment

Twenty-five cm² flasks of CEF monolayers were co-infected as described before. After 1hr the viral inoculum was removed and replaced with 5ml of GPT selective Medium 199. After 22 hours the medium was discarded and monolayers washed with methionine free MEM (Gibco) and incubated for 2 hours in methionine free MEM containing 2% dialysed foetal calf serum (dFCS) and tunicamycin at 0, 0.5 and 5µg/ml. This medium was replaced with 2ml of methionine free medium containing 100µCi of [³⁵S] methionine and the same concentrations of tunicamycin and dFCS. Two hours later the medium was discarded and washed twice with PBSa. Cells were lysed by incubation at room temperature for 10 mins. in

2ml RIPA buffer 1 and 0.01mM PMSF, before analysis of proteins by SDS PAGE and autoradiography.

2.9.4 Radio-immunoprecipitation

Virus was inoculated into CEFs as described in section 2.9.3, except that tunicamycin was absent. Radioimmunoprecipitation was performed as in section 2.6.3.

2.9.5 Pulse chase analysis of FP-PC1

CEF monolayers were infected and labelled as described in section 2.9.3 except that tunicamycin was not used and after labelling with [³⁵S] methionine, unlabelled methionine was added to a final concentration of 1mM. Samples from the supernatant and lysed cells were taken at 0, 4 and 8 hours. Labelled protein was analysed by radioimmunoprecipitation and SDS PAGE as described above.

2.9.6 Immunofluorescence

CEFs were cultured to 60-80% confluency on microplate chamber slides (Nunc), cells were infected with wt and/or rFWPV diluted in serum free Medium 199 at an m.o.i. of 5. One hour after infection an equal volume of 4% NBCS Medium 199 was added to each chamber. At 12 and 24

hours post-infection (p.i.) viral inoculum was discarded and cells washed with ice-cold PBSa and dried, cells were then fixed with ice-cold methanol at -20°C for 30 mins. Cells were incubated with the primary antibody (diluted in PBSa) for 30 mins. at 37°C, excess unbound antibody was then removed by 3, 10 minute washes in PBSa. The secondary antibody (Dako F261) was incubated and unbound antibody removed as described for the primary antibody. Slides were washed once in tap water before being dried by a combination of patting against towel paper and a 30 minute incubation in a 30°C oven (Heraeus). Cells were observed either by fluorescent light or confocal microscopy.

2.10 Indirect and direct assays for AP/NP1 activity

2.10.1 Assay for Alkaline phosphodiesterase activity

PPD assay reaction buffer (0.15M NaCl, 20mM Tris-HCl pH 8.0. 1 mM Ca^{2+} or 1mM EDTA/EGTA) containing p-nitrophenyl phenylphosphonate (Sigma) to 5 mg per 10ml of reaction buffer was added to an equal volume of substrate and incubated at 37°C until a yellow colour was observed. The reaction was stopped by addition of DTT at 1mM, and OD measured at 400nm.

2.10.2 Assay for autophosphorylation using $\gamma^{32}P$ ATP

Cells were grown to a density of $1-2 \times 10^6$ (semi-confluent) in 25cm² flasks. Growth medium was then discarded and infected and non-infected

controls were set up. Virus was added to 10pfu/cell and incubated for 1hr at 37°C. The viral inoculum was then discarded and 5ml Medium 199 + 2% NBCS were added and incubated at 37°C to allow the following time points: 0Hr, 6Hr, 12Hr, 18Hr and 24Hr, all +/- Ara-C 40µg/ml. Cells were then washed twice in 3ml 145mM NaCl, and 2ml of serum-free and phosphate-free MEM(ICN:16/227/49) was added and cells incubated for 30 min. at 37°C in 5% CO₂. Cells were then held on ice and 5µl γ-³²ATP was added using plugged tips behind perspex screens (1µCi per µl: 4000Ci/mM ATP). Flasks were left on ice behind perspex screens for a further 30 min. MEM and γ-³²ATP were then removed and discarded. One ml TBS buffer was added to the cells and the cells scraped from the flask using a cell scraper. Cells were then pelleted by centrifugation at 10,000 rpm for 1min. at 4°C and resuspended in SDS-PAGE sample buffer containing 50mM DTT, samples were then boiled for 2 mins., prior to loading on a 10% SDS-Page gel at 100V and dried using a gel drier. The gel was then placed in a phosphotungstate screen and exposed to film at -70°C.

2.11 Assay for CAT activity

CAT activity was measured by a colourimetric enzyme based immunoassay system (Boehringer 1 363 727). Samples (200µl) to be assayed were added to 96 well micro-titre plates (MTP) pre adsorbed with anti-CAT antibodies. MTPs were covered with foil and incubated for 1hr at 37°C. Samples were then removed by shaking, and wells washed by rinsing each well 5 times with 250µl "washing buffer". After careful removal of washing buffer, 200µl of anti-CAT-DIG (digoxigenin labelled antibody to CAT) was added to each well and incubated for 1hr at 37°C. Wells were then emptied by shaking, and washed again by rinsing each well 5 times with 250µl

“washing buffer”. After careful removal of washing buffer, 200µl of anti-DIG-POD (antibody to digoxigenin conjugated to peroxidase) was added to each well and incubated for 1hr at 37°C. Samples were then removed by shaking, and wells washed by rinsing each well 5 times with 250µl “washing buffer”. After careful removal of washing buffer, 200µl of POD substrate (ABTS) was added and incubated at room temperature until a green colour was observed. Sample absorbance was measured at 405nm using a microtitre plate reader.

Chapter 3

Sequence analysis of the FP-PC1 ORF

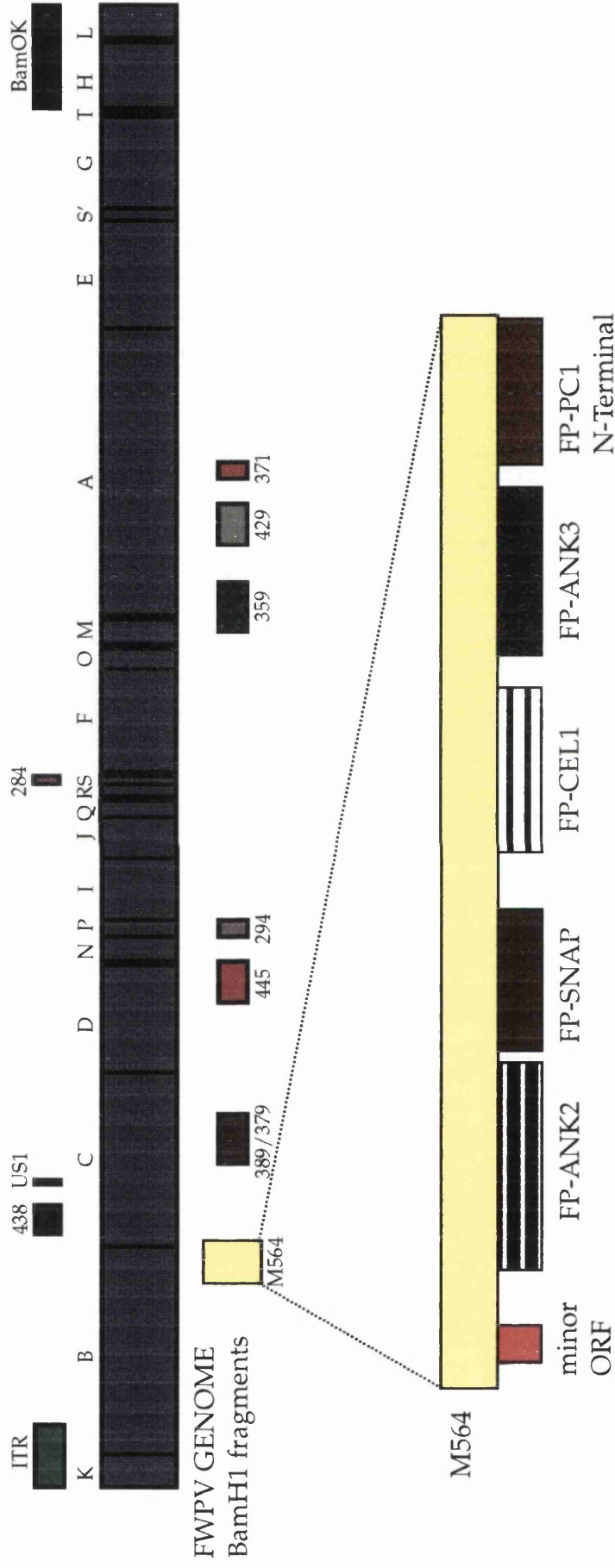
3.1 Introduction

As described in chapter 1, more than two thirds of the 260kbp FWPV genome remains unsequenced (fig.3.1). In addition, the potential ORFs identified within sequenced regions remain largely uncharacterised. However, recent sequence analysis of a previously uncharacterised 8.5kbp region of the FWPV genome (partially represented in fig. 3.1), revealed the presence of five ORFs (ORFs 1-5) which exhibited varying degrees of homology to various eukaryotic proteins (Laidlaw *et al.*, 1998). ORFs 1 and 4 encode proteins containing nine and five ankyrin repeat domains respectively; ORF 2 encodes a homologue of the yeast Sec17p and mammalian SNAP; ORF 3 shows homology to DNase II; and ORF 5 is homologous to mammalian plasma cell glycoprotein PC-1 (Laidlaw *et al.*, 1998).

Interestingly, no other virus has been previously shown to encode cellular homologues of the genes encoded by FWPV ORFs 2, 3 and 5 (Laidlaw *et al.*, 1998), therefore potentially indicating a role for these proteins specifically in infection of avian cells by FWPV.

The data presented in this chapter covers analysis of the FP-PC1 ORF and the predicted FP-PC1 protein, with discussion of possible structural and functional implications. In addition, the FP-PC1 sequence is compared to the growing number of eukaryotic proteins that belong to the emerging PC1 gene family, in an attempt to allow elucidation of sequence, structural and functional inter-relationships.

Fig.3.1 Genomic location of FP-PC1



Coloured blocks above and below the upper diagram representing the FWPV genome, indicate sequenced regions of the FWPV genome, with the letters immediately above the genome block representing fragments arising from digestion of the FWPV genome with BamH1. The lower diagram is an expanded view of the 6.5kbp M564 genomic fragment, containing 1 minor ORF and 5 major ORFs as shown. (Diagram modified from Mockett et al. 1992)

3.2 Cloning history of FP-PC1

The presence of a FWPV homologue of mammalian PC-1 was revealed following sequence analysis of a random M13 FWPV genomic DNA clone, MFP504, that showed homology to the 5' nucleotide phosphodiesterase active site region of murine PC-1. The sequence at the 5' end of the FP-PC1 gene was obtained by hybridisation screening of a HindIII FWPV genomic library in pAT153 using a probe generated from clone MFP504, which hybridised to clone M564, a 6.5kbp HindIII FWPV genomic DNA fragment. The remaining downstream FP-PC1 sequence was obtained from a 3.8kbp SmaI/BglII FWPV genomic DNA clone K03, cloned into pUC19, which overlapped with M564 from the SmaI site present within the FP-PC1 ORF (Laidlaw *et al.*, 1998).

3.3 Assembly and confirmation of the FP-PC1 ORF by PCR

Full length FP-PC1 was assembled into pNEB193-GPT by PCR directly from the FWPV genome (FWPV strain FP9) using gene specific flanking primers F1 and R1. As predicted by sequence analysis of clones M654 and KO3, the resultant PCR product was approximately 2.6kbp (fig. 3.2). Additional analysis using various combinations of both internal (F2 and R2) and flanking (F1 and R1) gene specific primers, also gave PCR products of the correct predicted sizes (fig. 3.2 and table 3.4). This data therefore confirmed the size of full length FP-PC1.

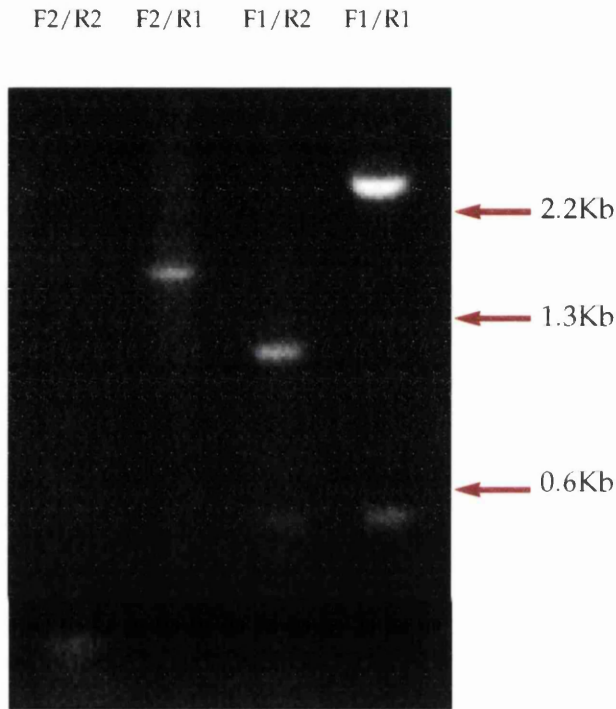
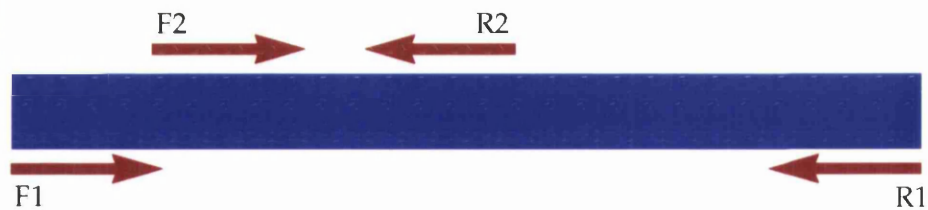


Table 3.4 Product sizes with FP-PC1 gene specific primers

F1/R1	2612bp
F1/R2	1160bp
F2/R1	1704bp
F2/R2	252bp



Following isolation of genomic viral DNA, FP-PC1 ORF gene specific flanking primers F1 and R1, and gene internal primers F2 and R2, were used to confirm the the FP-PC1 sequence. The position and orientation of the primers is indicated above. The sizes of the predicted product from various combinations of both flanking and internal gene primers is shown in table 3.4. The product sizes shown in the PCR reaction match closely with those predicted using CAD gene 2.0.

3.4 Conservation of the FP-PC1 gene across FWPV strains

(PCR courtesy of Steve Laidlaw)

All analysis of the FP-PC1 gene in this thesis was conducted using FWPV strain HP1-438 (FP9). This strain is derived from the original pathogenic progenitor strain HP1, that has been attenuated as a result of 6 passages on CEFs followed by 2 passages on chorioallantoic membranes and a further 438 passages in tissue culture using CEFs (Mayr & Danner, 1976). In order to exclude the possibility that FP-PC1 was acquired as a result of passage attenuation during tissue culture, PCR analysis using FP-PC1 flanking gene specific primers F1 and R1 was conducted across a wide range of FWPV strains as well as other avipoxviruses. As shown in fig. 3.3, the presence of FP-PC1 by PCR was indicated in all FWPV strains analysed, including the virulent progenitor FWPV strain, HP1, as well as commercial FWPV vaccine strains such as "chick-n-pox". This indicates that the FP-PC1 ORF was not just acquired during repeated tissue culture passage, but actually a gene which is present in both virulent and attenuated FWPV strains. However, FP-PC1 was not detected by PCR in other avipoxviruses such as canarypox or turkeypox (Laidlaw *et al.*, 1998).

3.5 Characteristics of the FP-PC1 ORF

3.5.1 Introduction

The FP-PC1 gene spans 2641bp, with approximately 150 to 174 bp of untranslated sequence depending on the optimum translation initiation codon. Although there has been no extensive study of FWPV promoters,

VV promoters have been shown to function in FWPV and vice versa (Boyle, 1992), therefore for the purposes of this thesis it will be assumed that the consensus sequences described for VV promoters (Baldick *et al.*, 1992; Baldick & Moss, 1993a) are similar to those of FWPV promoters. By this rationale there is evidence of 3 possible early promoter sequences which adhere closely to the VV consensus sequence for early promoters. In addition, there are no VV early transcription termination sequences (TTTTNT (Shuman & Moss, 1988) within the FP-PC1 ORF, therefore again suggesting that the gene could be transcribed early, as well as late. In contrast, early termination signals are present within ORFs 2 and 3 (Laidlaw *et al.*, 1998), indicating that these genes are probably only transcribed at intermediate or late stages.

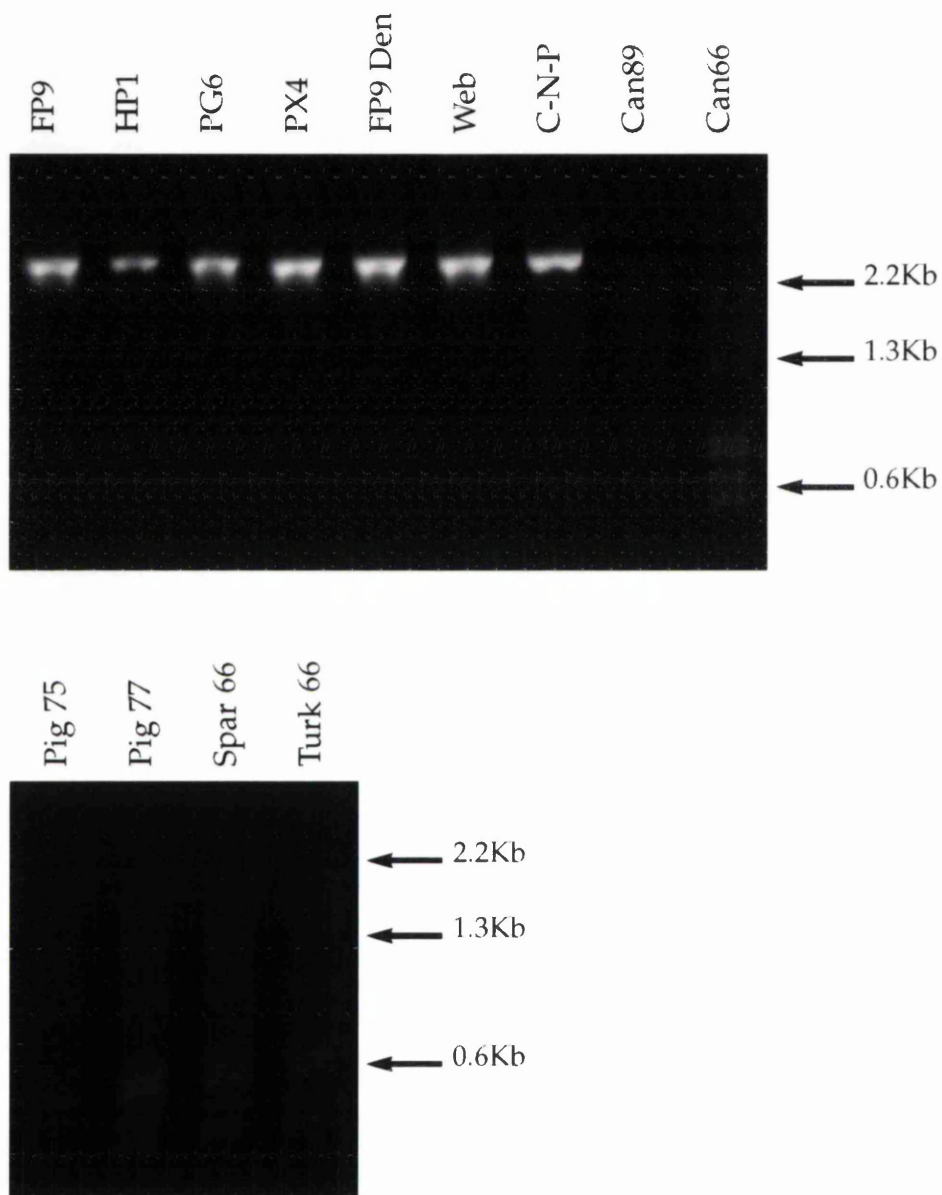
The FP-PC1 translated sequence spans 2454bp, leading to potential translation of a 818aa polypeptide, this assumes that the first potential translation initiation codon (nucleotide position 150) is the true initiator. The sequence around this methionine (CGAATGTCT) agrees poorly with the consensus sequence described for eukaryotic initiation domains (A/GCCCAUGG) (Kozak, 1987) , as neither the A/G at position -3 or the G at +4 are present. The same can be stated for the next downstream methionine at nucleotide position 156 (TCTATGAAA). However, the next methionine at nucleotide position 174, agrees closely with the consensus sequence (ACT ATG GAT) and is probably more likely to be the true initiator, therefore giving a potential polypeptide of 810aa. As FP-PC1 expression was planned in a cell free expression system using rabbit reticulocyte lysates, in which translation from internal initiation sites is common, all theoretical translation initiation codons were assessed for likelihood of being effective translation initiators, in order to allow size prediction of secondary polypeptides (Table 3.1).

Primary sequence analysis of the FP-PC1 ORF indicates that the predicted protein has a short hydrophilic amino terminus, a single hydrophobic transmembrane domain, and a large carboxyl terminal domain. The latter domain spans the 5' nucleotide phosphodiesterase active site, followed by an ATP binding site and an EF-hand domain. Details of these domains are described below.

3.5.2 Topological assignment of FP-PC1 based upon sequence data

The FP-PC1 sequence, like other type II transmembrane proteins, does not possess a classical N-terminal signal sequence. As suggested earlier, sequence analysis of the theoretical protein encoded by the FP-PC1 ORF, indicates that it traverses the plasma membrane only once, based upon analysis using a Kyte-Doolittle hydrophobicity plot attained using DNA Strider 1.2 (fig. 3.4). Assuming that the first methionine codon is the true initiator, the predicted transmembrane sequence is 21aa long and stretches from amino acid position 76 to 96. As discussed in detail in chapter 6, this transmembrane sequence has a dual role as an internal signal sequence, allowing the carboxyl terminal domain to traverse the hydrophobic lipid bilayer, as well as serving as a membrane anchor sequence.

Experimental data suggesting the actual orientation of FP-PC1 is presented in chapter 6. However, analysis of potential acceptor tripeptides for N-linked glycans reveals that there are 3 such potential tri-peptides in the amino terminal domain, and six in the carboxyl terminal domain, two of which are conserved between human and murine PC1 (fig. 3.5). It is likely that the carboxy terminal is extracellular, as the conserved location of



FPV-PC1 ORF gene specific flanking primers F1 and R1 were used to confirm the presence of the FP-PC1 gene using genomic DNA from various FWPV and other avipoxviruses (FP9 is FWPV strain HP1 passaged 438 times as described in Chapter 2; HP1 is a virulent FWPV strain; PG6, PX4, FP9 Den and Websters are other FWPV strains; C-N-P is a commercial FWPV vaccine strain; Can 89 and 66 are both Canarypox strains; Pig 75 and 77 are Pigeonpox strains; Spar 66 is a Sparrowpox strain and Turk 66 is a Turkeypox strain. The negative control was no DNA. The results indicate that the FP-PC1 is present in all FWPV strains and was not acquired as a result of repeated passage *in vitro*.

Table 3.1 Potential translation initiation sites within the FP-PC1 ORF scored according to the Kozak consensus sequence for eukaryotic messenger RNAs

Amino Acid Position	Protein size dalton	Kozak Score
1	-	0
3	-	0
9	93,064	3
19	91,886	2
87	-	0
125	79,836	2
147	77,047	1
155	-	0
218	-	0
243	-	0
266	63,247	1
311	-	0
333	55,853	2
387	49,875	3
449	42,763	2
477	39,593	2
478	39,462	2
621	23,236	2
624	22,850	2
663	-	0
675	-	0
692	14,575	3
713	-	0
797	2,565	2

All methionines in the FP-PC1 ORF were scored for closeness to the Kozak consensus sequence for eukaryotic messenger RNAs (A/GCCCAUGG), with 2 points allocated for A/G at -3 and 1 point for a G at +4. Protein sizes for the most likely initiators were calculated using DNA Strider 1.2.

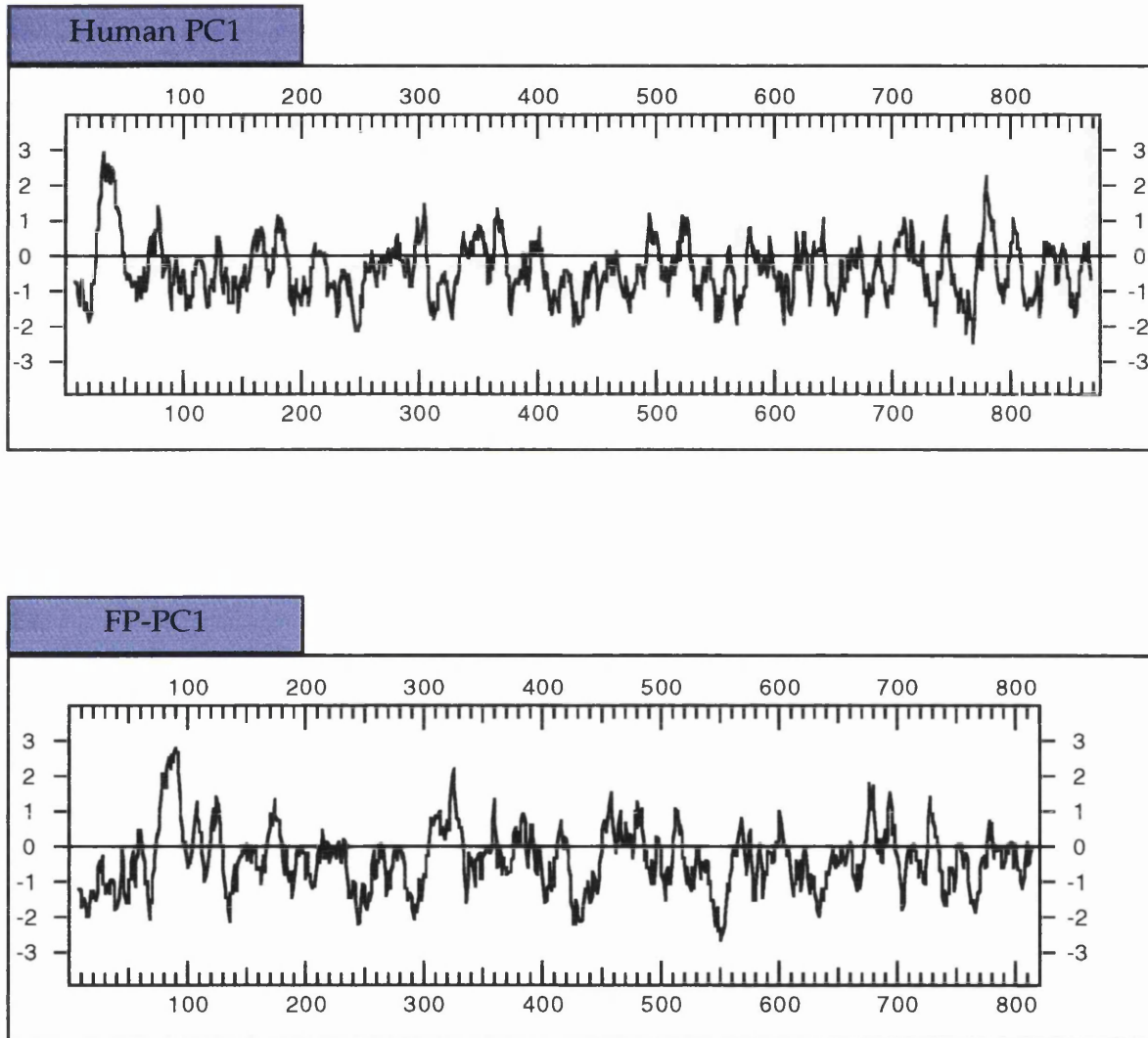
the N-linked glycosylation sites would be consistent with FP-PC1 being a type II transmembrane protein. In addition, all the key known functional and structural domains identified by primary sequence analysis, including the 5' nucleotide phosphodiesterase active site, the ATP binding site and the EF-hand domain are located in the carboxy terminus domain (fig. 3.5). Likewise, all 11 of the cysteine residues which are conserved between human PC1 and FP-PC1 are also present in the carboxy-terminal domain. As cysteine residues only form disulphide bonds in the oxidising environment outside the cell or in the endoplasmic reticulum, this is further evidence of the carboxyl terminal domain being extracellular. Taken together this data suggests that the FP-PC1 protein, like its cellular homologues, has a type II orientation; with a short, intracellular amino-terminal domain; and a large extracellular carboxy-terminal domain, containing all the key functional groups and domains that have been previously characterised in cellular homologues.

3.5.3 Comparison of FP-PC1 sequence with cellular homologues

3.5.3.1 Overall comparison

The FP-PC1 protein shows 38/39% amino acid identity over a 731 to 728aa region with human and murine PC1 respectively. As described below, the highest levels of homology are shown in the key functional domains, whereas the levels of homology are very low in the cytoplasmic and transmembrane domains. Interestingly, FP-PC1 lacks the double repeat cysteine rich domains, homologous to somatamedin-B, which are present in mammalian PC1 genes. A comparison of FP-PC1 amino acid identity to homologous genes is shown in table 3.2. Detailed sequence comparison and

Fig.3.4 Kyte-Doolittle hydrophobicity plot of Human PC1 compared to FP-PC1



Kyte Doolittle hydrophobicity plots were generated using DNA Strider 1.2. As shown both human and FP-PC1 have only one region with sufficient length and hydrophobicity to traverse the lipid bilayer. The human transmembrane domain stretches from residues 25 to 46, and the FP-PC1 domain from residues 72 to 97.

a graphical presentation of the lineage of genes homologous to FP-PC1 is shown in figs. 3.5 and 3.6 respectively.

Table 3.3 General characteristics of FP-PC1

	Full length human PC1	Full length FP-PC1
Protein length	873 aa	818 aa
Protein size	99,865 D	93,994 D
N-linked glycosylation sites	12	9
Cysteines	37	17

3.5.3.2 Alkaline phosphodiesterase active site domain

The active site domain of alkaline phosphodiesterase has been previously defined by analysis of a 61aa cyanogen bromide peptide from bovine intestinal 5'-nucleotide phosphodiesterase, which contains the active site threonine residue that is phosphorylated during catalysis (Culp *et al.*, 1985). Sequence comparison between the active site of bovine intestinal 5'-nucleotide phosphodiesterase as defined above, and a comparable region of murine PC1 indicates 74% aa identity over a 61aa span (Skinner, 1991). The alkaline phosphodiesterase active site domain spans amino acid residues 126 to 190 in FP-PC1 (human PC1 residues 166-231) (fig. 3.5), with amino acid identity extending to 56% between FP-PC1 and human PC-1, this figure increases to 84% if conservative changes are also taken into account. The level of amino acid identity is highest around the active site threonine residue (Culp *et al.*, 1985), of 16 residues on either

Table 3.2 Cross comparison of amino acid identity between FP-PC1 and members of the PC1 gene family

	Murine PC1	Rat RB13-6	Human Autotaxin	Rat Pd1 α	FP-PC1	Rice PPD	Yeast YCR6	Yeast YEB6	<i>C. elegans</i> 1330365
Human PC1	80 (873)	50 (876)	38 (827)	37 (857)	39 (731)	40 (395)	38 (129)	30 (236)	28 (116)
Murine PC1		47 (876)	38 (830)	36 (857)	38 (728)	41 (398)	37 (131)	30 (236)	25 (132)
Rat RB13-6			46 (832)	35 (843)	35 (730)	36 (263)	35 (128)	35 (128)	27 (317)
Human Autotaxin				91 (593)	31 (608)	36 (262)	37 (108)	34 (121)	28 (88)
Rat Pd1 α					35 (318)	36 (263)	36 (108)	32 (123)	28 (88)
FP-PC1						36 (395)	29 (174)	32 (239)	32 (119)
Rice PPD							32 (176)	32 (176)	28 (328)
Yeast YCR6								36 (499)	22 (263)
Yeast YEB6									25 (248)

Accession numbers for genes shown in table above are listed in the legend to fig. 3.5. Figures indicate the percentage amino-acid identity between two protein, with the figure in brackets representing the span of the homology.

side of the active site threonine, 14 are identical between human and FP-PC1.

There has been extensive controversy surrounding the categorisation of PC1 as a kinase (Belli *et al.*, 1995; Oda *et al.*, 1991). It has been proposed that murine PC1 is capable of autophosphorylation as well as phosphorylation of other proteins (Oda *et al.*, 1991; Uriarte *et al.*, 1993). Only murine and human PC1 adhere exactly to the ATP binding site consensus sequence **GXGXXG...A..K**. However, the other mammalian PC1 homologues and FP-PC1 are only diverged from this consensus sequence at the first glycine residue. Twenty residues downstream of the last conserved glycine in this motif a typical lysine residue is present in all the mammalian PC1 genes (Belli *et al.*, 1995) but not in FP-PC1 where it is replaced with a leucine. However, only murine PC1 and FP-PC1 share the typical alanine two residues upstream of the conserved lysine. This data does not implicate one way or the other the likelihood of any of the genes in the PC1 gene family, including FP-PC1, of having any intrinsic kinase activity, as there is no overall homology to known kinases or clear functional data suggesting any kinase activity (see chapters 1 and 7).

3.5.3.4 Potential EF - hand domains

The EF-hand domain is a “structural pocket” which co-ordinates calcium ions, leading to possible structural and functional ramifications for the protein, as well as possibly acting as a signalling mechanism (reviewed (Chazin, 1995; Kretsinger, 1996). The canonical EF-hand consists of an α helix (E: residues 1-10), a loop wrapped around the Ca^{2+} ion (residues 10-21) and a second α helix (F: residues 19-29) (Kretsinger, 1996). In earlier studies

Human PC1	136	KSWVEEPCESINEPQCPAGFETPPPTLLFLSFLDGG	167
Murine PC1	136	KSWVEETCESIDTPECPAEFESPPPTLLFLSFLDGG	167
Hu. autotaxin	142	SHWVDDDCIEIKAAECPPAGFVVRPPPLIIFSVVDGG	173
Rat PD1-alpha	141	SHWVDDAARN-QSSEC-LQVCPPPPLIIFSVVDGG	170
Rat RB13-6	138	VPWVTEACASSQEPQCEPGEFDFQPPVILFSLMDGG	169
FP-PC1	100	RL--GGCISFQVT-LEFEFERPPLILIAMNG	127
Yeast YCr6	149	GSPEFDPHYVKYFNGTHEF---FPLTIVISLIDGG	177
Yeast Yeb6	57	PRTIANRNSNTYFNGTHDF---KTLTILISIDGG	85
Rice Ppd	69	TP-LLSAAPAPAAAAARAQVKLEKPVVILISIDGG	99
C. elegans	4	VLILLGL-LVLSAARDPIG---QNLIVILADGG	31

Human PC1	168	FRAEYLL-H---TWGGLLPVISKLLKKC-----	189
Murine PC1	168	FRAEYLL-H---TWGGLLPVISKLLKNC-----	189
Hu. autotaxin	174	FRAEYLL-H---TWGGLLPVISKLLRSC-----	195
Rat PD1-alpha	171	FRAEYLL-H---TWGGLLPVISKLLRSC-----	192
Rat RB13-6	170	FRAEYLL-Q---TWGGLLPVINLKLKTC-----	191
FP-PC1	128	FBYDYLL-N---KWEPTIKDLMEH-----	149
Yeast YCr6	178	FHPSLISK---RNTPFLHDLYEELKYDGG-----	201
Yeast Yeb6	86	FHPRLLIDA---KYTPFLYNLHNLRSPLY-----	109
Rice Ppd	100	FRFGYQH---KAAT---PHIHRLLIGN-----	119
C. elegans	32	YGATLLNNTKPDATFGMDTGDIRMTEICSLGI	63

Human PC1	190	---GTYTKN-MRPVYPTKTTFPNHYSIVVTGL	215
Murine PC1	190	---GTYTKN-MRPMYPTKTTFPNHYSIVVTGL	215
Hu. autotaxin	196	---GTHSPY-MRPVYPTKTTFPNLYTLATGL	221
Rat PD1-alpha	193	---GTHVPY-TRPVYPTKTTFPNLYTLATGL	218
Rat RB13-6	192	---GLHSKY-MRAMYPTKTTFPNHYTIVVTGL	217
FP-PC1	150	---GVTAP-MRPVYPTNTTFPNLYSIVVTGL	174
Yeast YCr6	202	---GMNITSTPFMIIPSFPTETTFPNHWTLVVTGQ	230
Yeast Yeb6	110	---DMNITTAPYMIIPSFPTQTFPNHWSMVTGK	138
Rice Ppd	120	---GTSAAATGLVPIFPFTLTFPNHYSIATGL	146
C. elegans	64	RHLATNGVQVDYVTPSFPTHTWVQWMSLSTGP	95

Human PC1	216	YPEESHGIIIDNKMYDPKMNASFSLKSKKEKFNPN	246
Murine PC1	216	YPEESHGIIIDNKMYDPKMNASFSLKSKKEKFNPN	246
Hu. autotaxin	222	YPEESHGIVGNSMYDPVFDATFHLRGRGKFNH	252
Rat PD1-alpha	219	YPEESHGIVGNSMYDPVFDASFHLRGRGKFNH	249
Rat RB13-6	218	YPEESHGIIIDNNMYDVYLNKNFSLSSVEKSNP	248
FP-PC1	175	YPIISHGITDNEFIDRGTDEFT-IASEETE EV	205
Yeast YCr6	231	YPIHHGIVSNVFWDPDLNEEFHPGVLDPRIW	261
Yeast Yeb6	139	YPIEHGIVSNI FWDNFTSSEFR-PNNLDARIW	169
Rice Ppd	147	YPS SHGIIINNYFPDPISGDYFT-MS SHE--P	174
C. elegans	96	NDTE---DVWWD-DAKAPL--WYTAGKAGV	119

Human PC1	247	EWYKGGEPVWVTAKYQ---GLKSGTFFFWPGS	273
Murine PC1	247	LWYKGGQPIWVTAHQ---EVKSGTYFFFWPGS	273
Hu. autotaxin	253	RWWGGQPPLWITATKQ---GVKAGTFFFW---	276
Rat PD1-alpha	250	RWWGGQPPLWITATKQ---GVRAGTFFFW---	273
Rat RB13-6	249	AWWSGQPPIWLTAMYQ---GLKAASYYWLGSS	275
FP-PC1	206	EWFGGEPVWTTIMKN---GFKSATFFFWPGS	232
Yeast YCr6	262	NNNDTEPIWQTVQSAFDGDI PFKAAATHMWPGS	293
Yeast Yeb6	170	-SNTADPIWQLLQTESQGE--YKVATHMWPGS	198
Rice Ppd	175	KWWLGEPLWVTAADQ---GIQAATYFWPGS	201
C. elegans	120	---DVHCYW---FA-----	127

Human PC1	274	DVEIN-----GIFPDIYKM	287
Murine PC1	274	DVEID-----GILPDIYKV	287
Hu. autotaxin	277	-----	276
Rat PD1-alpha	274	-----	273
Rat RB13-6	276	DVAVN-----GSFPNIYRN	289
FP-PC1	233	DKVVP-----RKRPMTMYRS	246
Yeast YCr6	294	DVNYTKYKTEEKLQPEHKKPIAREERTPFYFDE	325
Yeast Yeb6	199	EVVY-----EDHGDVPRERMPFYFGK	219
Rice Ppd	202	EVKKG-----SWDCPKYCRH	217
C. elegans	128	-HCHRAFYDMVVQVPEKRWANLDDQHQT DNL	157

Human PC1	288	YNGSVVPFEERI L AVL LQWLQ - LPK - DERPH HFY T	317
Murine PC1	288	YNGSVVPFEERI L AVL LEWLQ - LPS - HERPH HFY T	317
Hu. autotaxin	277	- SVVIPHERRI L T L LQWLT - LPD - HERPS SVY A	305
Rat PD1-alpha	274	- SVSIPHERRI L T L LQWLS - LPD - NERPS SVY A	302
Rat RB13-6	290	YNSVVPYER I A T L LQWLD - LPK - AERPS SFY T	319
FP-PC1	247	YKSVVPYER I N T V L R W L K - MDT - GYR P Y F Y A	276
Yeast YCr6	326	FNAKEPLSQKLSKI I EYVD - MST LNERPQL I L	356
Yeast Yeb6	220	FNQWEK LQDKLAQ I FRY I D - MPQLKDRPEL V I	250
Rice Ppd	218	YNGSVVPFEER V D A I L G Y F D - LPS - DEM P Q F L T	247
C. elegans	158	R D I F P E I A N R I S K Y Q V Y K Q Q M - - - - - F L I	181

Human PC1	318	LYLEEPDSSGHSYGPVVS - - - - - SEVIKAL LQR	343
Murine PC1	318	LYLEEPDSSGHSYGPVVS - - - - - SEVIKAL LQR	343
Hu. autotaxin	306	FYSEQPDFS GHKYGPFVFG - - - - - PEMT NPLRE	331
Rat PD1-alpha	303	FYSEQPDFS GHKYGPFVFG - - - - - PEMT NPLRE	328
Rat RB13-6	320	IYVEEPD SAGHKSGPVS - - - - - AGVIKAL LQL	345
FP-PC1	277	LYLEEPGSSGYEYGTDD - - - - - ERV GKAL L EK	302
Yeast YCr6	357	GYV PNVDAFGHKHGY PSESEYY YEDFTET LGE	388
Yeast Yeb6	251	SYI PNVDSYGH SFGY - - - - - DL RDKRLQL IGE	278
Rice Ppd	248	LYFEDPDHQGHQVGPDD - - - - - PAITEAVVR	273
C. elegans	182	RY - A N I G N A Q K E H G P E S - - - - - D E V E Q E V A R	206

Human PC1	344	VDGMVGM LMD - - - - - GLKELN LHRCL - - - - - LNLI	367
Murine PC1	344	VDRLVGM LMD - - - - - GLKDLGLDKC - - - - - LNLI	367
Hu. autotaxin	332	IDKTVGQLMD - - - - - GLKQLK LHRCL - - - - - VNV I	355
Rat PD1-alpha	329	IDKTVGQLMD - - - - - GLKQLK LHRCL - - - - - VNV I	352
Rat RB13-6	346	VDDAFGLME - - - - - GLKQRNLHNC - - - - - VNV I	369
FP-PC1	303	VDKAIALLMK - - - - - GLKDLQL IGD - - - - - ANLI	326
Yeast YCr6	389	VDTF LKQLVE - - - - - SLQERNL L T S F - - - - - TNLV	412
Yeast Yeb6	279	VDGFFLDLIE - - - - - GLQKRN L LKI - - - - - SNVM	302
Rice Ppd	274	IDEMIGRLIA - - - - - GLEERGV FED - - - - - VNV I	297
C. elegans	207	FDLYINE LQQ - - - - - LLED RGL FSS - - - - - TNLV	230

Human PC1	368	LISDHGM EQGSC KKYIYL NK - - - - - YLGDVKN I K	396
Murine PC1	368	LISDHGM EQGSC KKYVYL NK - - - - - YLGDV NNVK	396
Hu. autotaxin	356	FVGDHGM EDVTC DRTEFLSN - - - - - YLTNVDDIT	384
Rat PD1-alpha	353	FVGDHGM EDVTC DRTEFLSN - - - - - YLTNVDDIT	381
Rat RB13-6	370	VLA DHGM DQ TSC DRVEYMTD - - - - - YFPEI - NFY	397
FP-PC1	327	LVSDHGM S S D P K K I V N L K D - - - - - Y I T N - N D V V	354
Yeast YCr6	413	IVSDHGM SDIVV - - - - - PSNV I I W E D L L D E K L R K D	442
Yeast Yeb6	303	IVSDHGM SNV N A N D G E H V V W E R V F P A D A M S A	334
Rice Ppd	298	LVGDHGM VGTCDK KLVFL DELAPWIKLEE DWV	329
C. elegans	231	VMSDHG Y T P L Q K E E Q F F M E Q C L P D Y S L V K K V V	262

Human PC1	397	VIY - - - - - GPAARLRPSDVPDKYYSFNY - EGI	422
Murine PC1	397	VVY - - - - - GPAARLRPTDVPETYYSFNY - EAL	422
Hu. autotaxin	385	LVP - - - - - GTLGRIRSKFSNNA - - KYDP - KAI	408
Rat PD1-alpha	382	LVP - - - - - GTLGRIRAKSINNS - - KYDP - KTI	405
Rat RB13-6	398	MYQ - - - - - GPAPRIRTRNIPQDFFTFNS - EEI	423
FP-PC1	355	IKP - - - - - GATPVIKPQN LNHI RL - FDY - DGI	379
Yeast YCr6	443	YVSHAYLEGPMMALSLKDSGNINEVYHNLKTS	474
Yeast Yeb6	335	FISHLYNEGPM MMVC LKNPRDKQWICDLIEAQ	366
Rice Ppd	330	LSM - - - - - TPLLAIRPPDMSLPDVVAK - MNE	355
C. elegans	263	NS - - - - - HSMIMVFTNPEDEGTVHYEFSVC EVW	290

Human PC1	423	ARNLSCREPNQH F - KPY - - - - - LKHFLPKR	446
Murine PC1	423	AKNLSCREPNQH F - RPY - - - - - LKPF LPKR	446
Hu. autotaxin	409	IANLTCCKPDQH F - KPY - - - - - LKQH LPKR	432
Rat PD1-alpha	406	IANLTCCKPDQH F - KPY - - - - - MKQH LPKR	429
Rat RB13-6	424	VRLSCKRKS DQH F - KPY - - - - - LTPD LPKR	447
FP-PC1	380	ISSTSCVMDDQPF - IVS - - - - - YRSRLPKR	403
Yeast YCr6	475	IDED - - - - - KY - TVY - - - - - VNGNFPKE	491
Yeast Yeb6	367	LEKAYGDEISRKE - HV - - - - - LKEDFDPS	390
Rice Ppd	356	GLGSGKV ENGEY L - RMY - - - - - LKED LPSR	379
C. elegans	291	SPMGDYDENDTPFVKTY - - - - - RMS ELPDD	315

Human PC1	447	L H F A K - - - - - S D R I E P L T F Y L D P Q W Q L A -	469
Murine PC1	447	L H F A K - - - - - S D R I E P L T F Y L D P Q W Q L A -	469
Hu. autotaxin	433	L H Y A N - - - - - N R R I E D I H L L V E R R W H V A R	456
Rat PD1-alpha	430	L H Y A N - - - - - N R R I E D I H L L V D R R W H V A R	453
Rat RB13-6	448	L H Y A K - - - - - N V R I D K V H L M V D R Q W - L A -	469
FP-PC1	404	L H Y G S - - - - - G F R T E I L G V Y L E E G W Q S T D	427
Yeast YCr6	492	W N F N D G K N H - - - - - H M A S I W I V P E P G Y A V M K	517
Yeast Yeb6	391	W K Y F Q Y D N R K H R Y D D R V G D I W I L A D E Y Y A I V -	421
Rice Ppd	380	L H Y A D - - - - - S Y R I P P I I G L P E E G Y K V E M	403
C. elegans	316	L H W K N - - - - - S R F M S G V V L I T K P G T S V - -	337

Human PC1	470	- - - - - L N P S E R K Y C G S G F F H G S D - N V	488
Murine PC1	470	- - - - - L N P S E R K Y C G S G F F H G S D - N V	488
Hu. autotaxin	457	K P L - - - - - D V Y K K P S G K C F F Q G D H G F D - N K	480
Rat PD1-alpha	454	K P L - - - - - D V Y K K P S G K C F F Q G D H G F D - N K	477
Rat RB13-6	470	- - - - - Y R N K G S S N C E G G T H G Y N - N E	488
FP-PC1	428	- - - - - E N G N L K H R S G G F F H G S D - N S	445
Yeast YCr6	518	K E Q L K K V A K G D H K D K N E D N V F T I G S H G Y D - N N	548
Yeast Yeb6	422	- - - - - K E M G D V P I G I M G T H G Y N F N N	441
Rice Ppd	404	- - - - - K R S D K N E C G G A H G Y D - N A	420
C. elegans	338	- V T K E - - - - - L P T V P H S G D P T	352

Human PC1	489	- - - - -	488
Murine PC1	489	- - - - -	488
Hu. autotaxin	481	- - - - -	480
Rat PD1-alpha	478	- - - - -	477
Rat RB13-6	489	- - - - -	488
FP-PC1	446	- - - - -	445
Yeast YCr6	549	- - - - -	548
Yeast Yeb6	442	- - - - -	441
Rice Ppd	421	- - - - -	420
C. elegans	353	- - - - - I D A K Q A S G W - - - - -	361

Human PC1	489	- - - - - F S N M Q A L F	496
Murine PC1	489	- - - - - F S N M Q A L F	496
Hu. autotaxin	481	- - - - - V N S M Q T V F	488
Rat PD1-alpha	478	- - - - - V N S M Q T V F	485
Rat RB13-6	489	- - - - - F K S M E A I F	496
FP-PC1	446	- - - - - F Q D M T A V F	453
Yeast YCr6	549	- - - - - A I D M R S V F	556
Yeast Yeb6	442	- - - - - C S D M A S I F	449
Rice Ppd	421	- - - - - F F S M R T I F	428
C. elegans	362	- - - - - E P Y H D D M K G I F	372

Human PC1	497	V G Y G P G F K H G I E A D T F E - N I E V Y N L M C D L L N L	527
Murine PC1	497	I G Y G P A F K H G A E V D S F E - N I E V Y N L M C D L L G L	527
Hu. autotaxin	489	V G Y G P T F K Y K T K V P P F E - N I E L Y N V M C D L L G L	519
Rat PD1-alpha	486	V G Y G P T F K Y R T K V P P F E - N I E L Y N V M C D L L G L	516
Rat RB13-6	497	L A H G P S F K E K T V I E P F E - N I E V Y N L L C D L L H I	527
FP-PC1	454	L G Y G P A F L D D V R V P T F D - N I E L Y N M M C E I L G I	484
Yeast YCr6	557	I G M G P Y F P Q G Y - I E P F Q - N T E I Y N L L C D I C G V	586
Yeast Yeb6	450	I G M G P M F N N E V - V P P F E - N I E V Y N M L I K A S A L	479
Rice Ppd	429	I A H G P R F E G G R V V P S F E - N V E I Y N V I A S I L N L	459
C. elegans	373	V A R G P A F R E N E R F G P T E I - V D V Y Q M L L N I L S I	403

Human PC1	528	T P A - P N N G T H G S L N H L L K N P V Y - T P K H P K E V H	557
Murine PC1	528	I P A - P N N G S H G S L N H L L K K P I Y - N P S H P K E E G	557
Hu. autotaxin	520	K P A - P N N G T H G S L N H L L R T N T F - R P T M P E E V T	549
Rat PD1-alpha	517	K P A - P N N G T H G S L N H L L R T N T F - R P T M P D E V S	546
Rat RB13-6	528	Q P A - P N N G S H G S L N H L L K A P F Y - Q P S H A E E L S	557
FP-PC1	485	N P A - N N N G T V G S L N N I L R N S R Y T H V S S L D I I T	515
Yeast YCr6	587	A E K - D R N S N D G T - - - G M L M N Q L R E P I Q S S E E V E	614
Yeast Yeb6	480	L G E - E K T K K E K S - - - L L Q - - - - - - - - -	493
Rice Ppd	460	E P A - P N N G S S S F P D T I L L P S E - - - - - - - - -	479
C. elegans	404	E P A H P H N G T W T N V E N M L S E - - - - - G W E N R	427

Figure 3.5 - By utilising the BLAST sequence analysis programme, proteins that showed the highest level of amino acid homology (**Human PC1**:ac.no.p22413; **Murine PC1**:ac.no.p06802; **Human autotaxin**:ac.no. q13827; **Rat PD1 alpha**:ac.no. q15117; **Rat RB13-6**:ac.no.a57080; **FP-PC1**:ac.no.e1311430; **Yeast YCr6**:ac.no. p25353; **Yeast Yeb6**:ac.no.p39997; **Rice Ppd**:ac.no.q42974 and **C.elegans 453aa**:ac.no. q22129) to the predicted FP-PC1 protein were aligned using PILEUP, using default parameters. Visualisation of homologous sequences was carried out using the SeqVu programme. Residues found in 55% or more of the sequences are boxed. Residues are shaded in blue if they show 85% or more homology to human PC1.

Key:






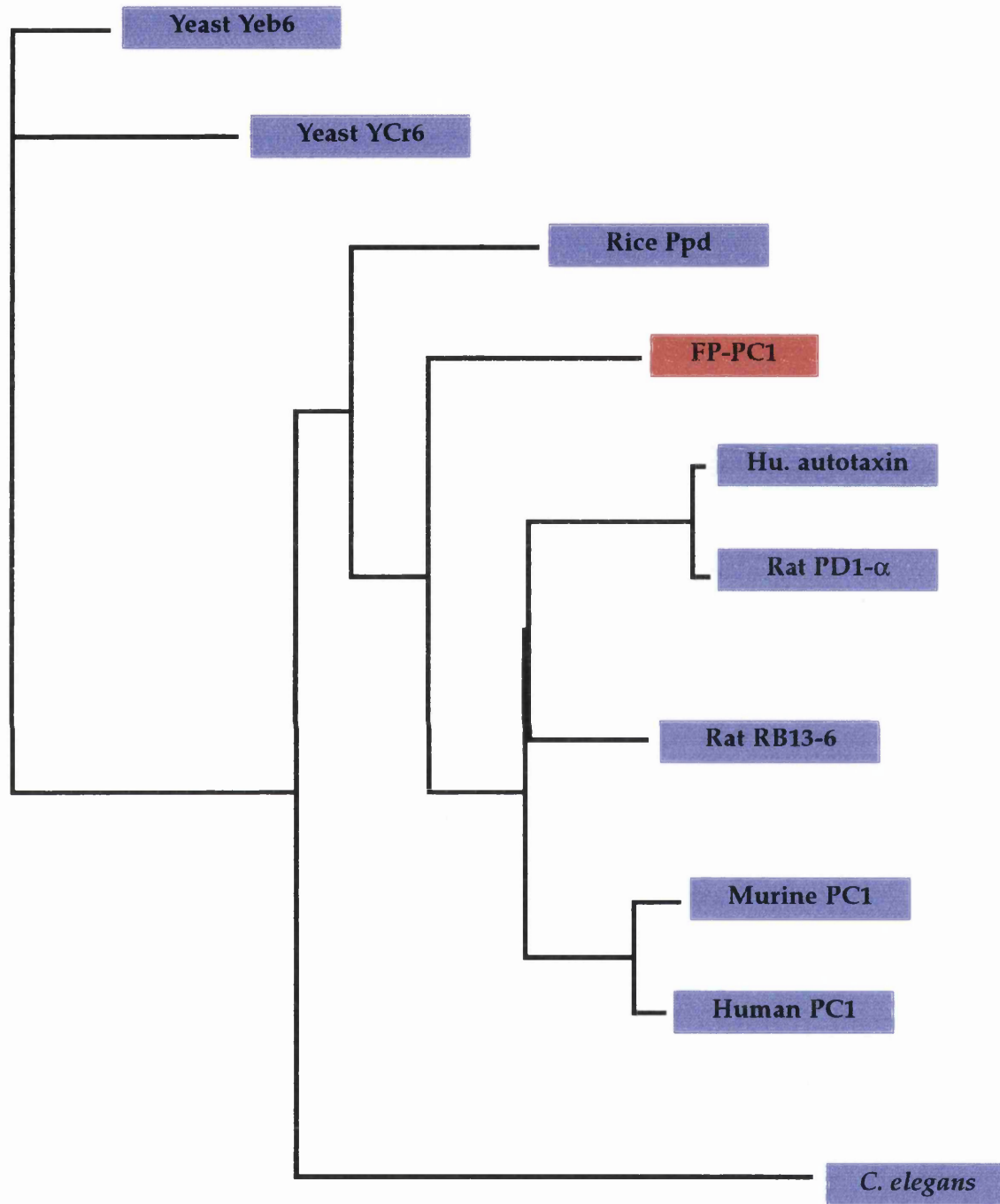
1. Conserved cysteines including FP-PC1 sequence 
2. Conserved N-linked glycosylation sites - overlined 
3. PPD domain - overlined 
4. EF-Hand domain - overlined 
5. PPD active site threonine 

Fig.3.6 Lineage of FP-PC1 in comparison to PC1 gene family cellular homologues



0.1

Sequences described in fig. 3.5 were analysed using PILEUP. Gene phylogeny was visualised using TREEVIEW. The horizontal distances are proportional to the expected percentage amino acid changes based on the observed data using PAM dayhoff tables for amino acid substitution rates.

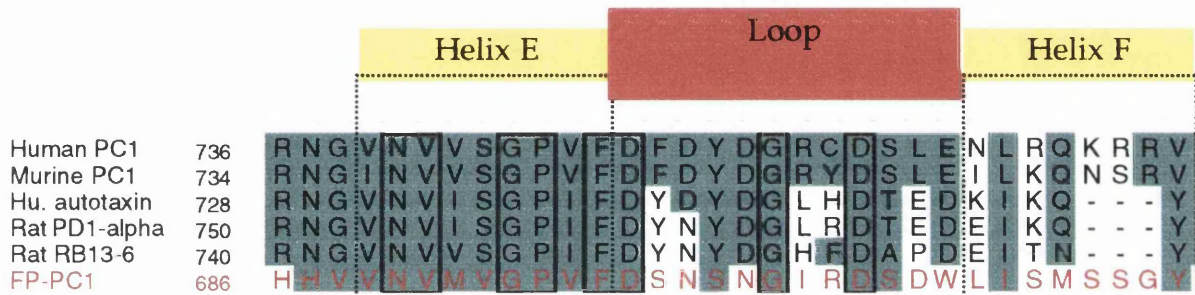
attempts were made to assign consensus sequence rules for identification of potential EF-hand domains in otherwise uncharacterised proteins. However as the number of proteins identified with functional EF-hands continues to increase, it has become increasingly evident that there is no absolute method for identification of EF-hands based purely upon motif analysis utilising a pre-defined consensus sequence (Kretsinger, 1996). Indeed it has been suggested that structural similarities should be used instead of sequence homology, when scanning for EF-hands (Kretsinger, 1996).

The primary EF-hand domain identified in both human (Buckley *et al.*, 1990) and murine PC1 adheres well to the EF-hand domain present in parvalbumin (Belli *et al.*, 1994). Although there is no direct evidence that the PC1 EF-hand domain actually partakes in binding calcium ions, the presence of divalent cations has been shown to impart greater structural and thermal stability to murine PC1, as well as leading to conformational alterations which affect the binding of PC1 specific antibodies (Belli *et al.*, 1994). These findings suggest that calcium ions do bind to murine PC1 and lead to an alteration in its structure (Belli *et al.*, 1994).

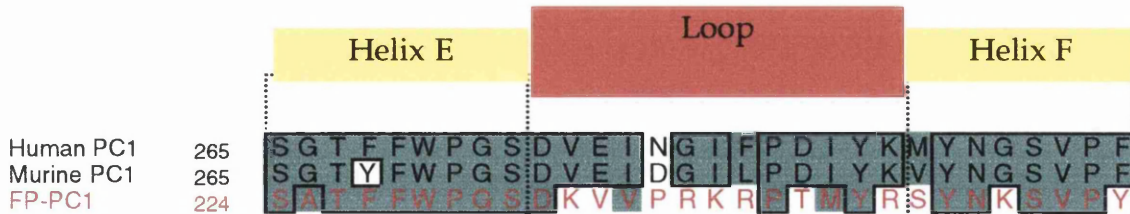
As shown in fig. 3.7, FP-PC1 homology to the EF-hand loop section of murine and human PC1 extends only to 2 out of the 4 key aspartic acid residues and to the central glycine residue. In the variant PC1 EF-hand, only 1 aspartic acid residue is conserved (fig. 3.7). Although, at first sight FP-PC1 does not appear to abide closely to the conserved EF-hand sequence, aspartic acid residues in the loop section of EF-hands may be substituted for either asparagine, glutamine, glutamic acid, serine or threonine residues, as they are all thought to be capable of using an oxygen atom as a Ca^{2+} ligand. In FP-PC1 the two central, non-conserved aspartic acid residues are replaced

Fig.3.7 Sequence detail of conserved and variant EF-hand domains in FP-PC1 and cellular homologues

EF-Hand domain



Variant EF-Hand domain



By utilising the BLAST sequence analysis programme, **Human PC1:ac.no.p22413**; **Murine PC1:ac.no.p06802**; **Human autotaxin:ac.no. q13827**; **Rat PD1 α :ac.no. q15117**; **Rat RB13-6:ac.no.a57080** and **FP-PC1:ac.no.e1311430**; were aligned using default parameters to Human PC1 using PILEUP. Visualisation of homologous sequences was carried out using the SeqVu programme. Residues found in 55% or more of the sequences are boxed. Residues are shaded in blue if they show 85% or more homology to human PC1. Gaps were inserted at strategic positions in order to attain the closest alignment of FP-PC1 with the variant EF-hand domains described for human and murine PC1 (Belli, 95).

by asparagine, indeed one of these aspartic acid residues is also replaced by an asparagine in PD1 α and RB13-6. Similarly, the non-conserved aspartic acid residue in the variant EF-hand loop domain is replaced with a threonine. The FP-PC1 variant EF hand shows higher levels of amino acid identity to human and murine PC1 proteins in the alpha helices bordering the loop domain (7/8 in helix E and 5/8 in helix F) when compared to the canonical EF-hand (7/9 in helix E and 1/8 in helix F) (fig. 3.7).

3.6 Summary

The detailed implications of the discovery of a FWPV gene homologue of mammalian plasma cell glycoprotein PC1 are discussed in chapter 7. The salient observation to be drawn from the analysis presented above is that conservation of genes amongst diverse species implies the importance or significance of a particular domain or protein. The acquisition and maintenance of a host gene by FWPV implies a presumed selective pressure to maintain a particular gene, due to a specific role which conveys a useful function, and potentially gives a particular virus a selective advantage over another. Areas in which such an advantage could arise include virus replication inside the cell, evasion of host immune systems and spread/infection to other hosts. The fact that a PC1 homologue was not identified in other avipoxviruses does not rule out the likelihood of these viruses having a PC1 homologue, it is likely that if other Avipoxviruses do possess a PC1 homologue the gene sequences may have diverged. This is specially relevant as PCR screening for the FP-PC1 ORF was carried out using oligonucleotides designed to prime on flanking regions, an alternate approach may have been to use oligonucleotides designed to prime to regions of the FP-PC1 gene which show high degrees

of homology to cellular PC1 genes, such as the phosphodiesterase active site domain. Subject to probable problems with false positives, this strategy may have allowed identification of PC1 homologues in other avipoxviruses. Progressing from this possibility, the fact that no other virus has been shown to encode a homologue to PC1, indeed nor to the FP-SNAP or FP-Cell1 (Laidlaw *et al.*, 1998), it is tempting to propose that these genes have functions that are specific to FWPV infection.

The actual orientation of a membrane protein can only be determined using actual experimental data, but as outlined above, amino acid sequence can allow protein orientations to be predicted. As shown by Kyte-Doolittle hydrophobicity analysis there is only one region of the FP-PC1 protein which is of the size and hydrophobicity required to traverse the lipid bilayer. The orientation is suggested as all the key functional and structural domains that would normally be associated with playing roles in interacting with extracellular signals, are located in the large carboxyl terminal domain.

Chapter 4

FP-PC1 Gene deletion studies

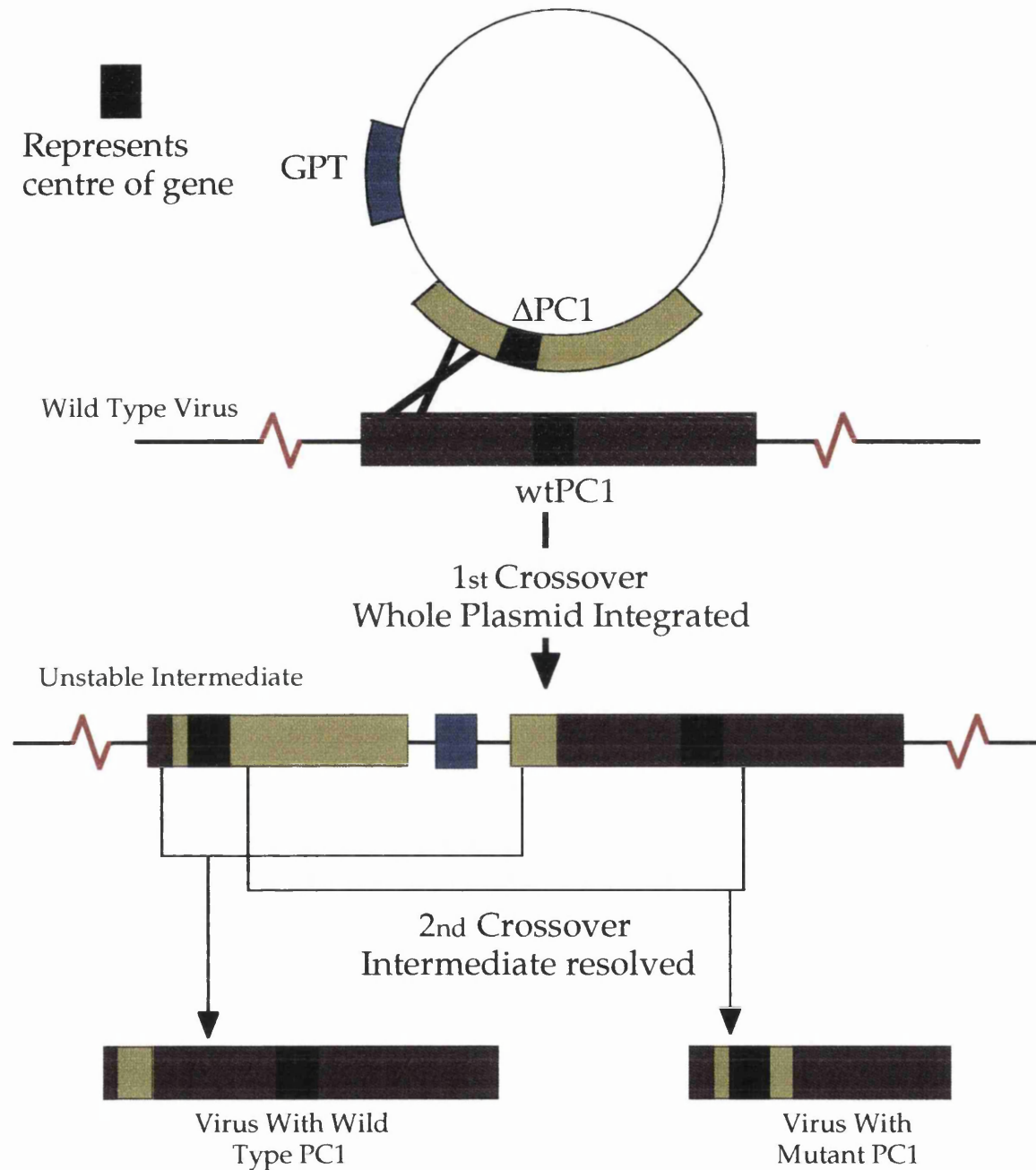
4.1 Introduction

In order to analyse the functional and phenotypic ramifications of removal of FP-PC1 from the FWPV genome, the transient dominant selection (TDS) method (Falkner & Moss, 1990) (fig. 4.1) was utilised to generate mutant viruses with specific deletions in the predicted coding sequence of FP-PC1 (see Chapter 3). Although this approach had not been previously used for FWPV, there was no obvious reason why similar studies in FWPV would not be just as effective, as deletions in VV genes have previously been constructed using the TDS technique (Falkner & Moss, 1990; Kettle *et al.*, 1995).

Briefly, the TDS method involves cloning of the full length FP-PC1 ORF in to a transfer vector containing the gene for the *E. coli* - xanthine-guanine phosphoribosyltransferase (EcoGPT) driven by the VV p7.5k early/late promoter. Various deletions were introduced into specific regions of the FP-PC1 ORF by utilising favourable restriction enzyme sites (fig. 4.2). Following transfection into FWPV infected CEFs, recombinant viruses were selected by growth in the presence of mycophenolic acid (MPA), which inhibits inosine monophosphate dehydrogenase, leading to arrest of the *de novo* purine metabolism pathway, and hence replication of wt FWPV. However, addition of exogenous xanthine and hypoxanthine allows the blockage to be overcome, as expression of EcoGPT from recombinant FWPV permits purine metabolism by the salvage pathway.

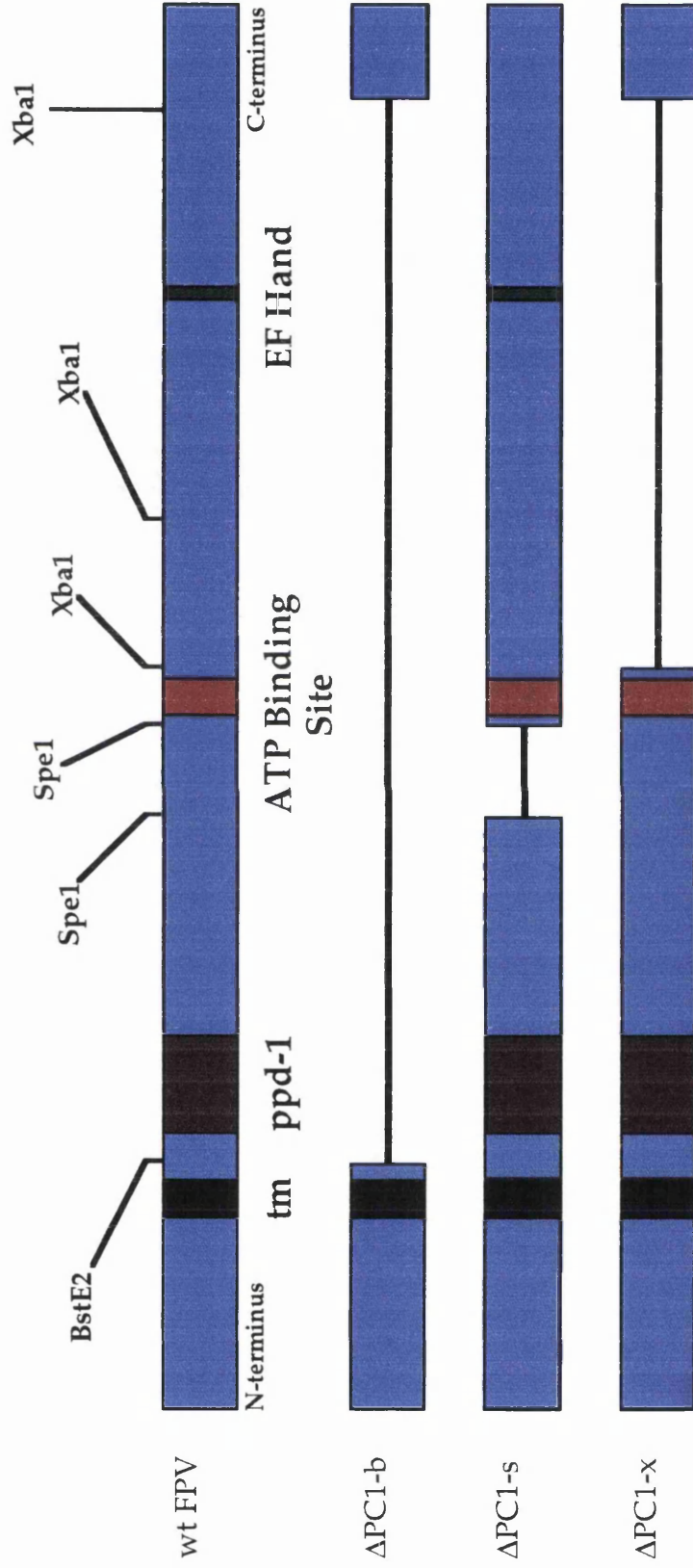
In this chapter the cloning of full length FP-PC1 from the FWPV genome, construction of deletions and the subsequent construction of mutant FWPV is discussed. The phenotypic analysis of the mutants is also reported.

Fig.4.1 Construction of a recombinant FWPV with a deleted copy of FP-PC1 by Transient Dominant Selection



CEF cells infected with FWPV strain FP9 were transfected with one of pFP-PC1b, pFP-PC1x or pFP-PC1s deletion plasmids. MPA based selection for GPT⁺ viruses allowed isolation of recombinant unstable intermediate virions which had incorporated the whole plasmid. As a consequence of removal of MPA selection recombinant virions resolved due to a second homologous recombination event leading to loss of the GPT gene as well as one of the copies of the FP-PC1 ORF, producing a recombinant virion with a full length or partially deleted ORF.

Fig. 4.2 Genotype of recombinant FP-PC1 deletion viruses



Representation of the genomic structure of FP-PC1 in recombinant FWPV. Deletion mutants were designed to allow study of deletion of each of the 3 functional domains. Δ PC1-b leads to deletion of all three domains, Δ PC1-s disrupts the reading frame and therefore leads to deletion of the ATP binding site and the EF-hand domain, and Δ PC1-x leads to deletion of only the EF-hand.

4.2 Construction of FP-PC1 deletion mutants by TDS

Full length FP-PC1 was assembled into transfer vector pNEB193-gpt by PCR directly from the FWPV genome using FP-PC1 gene specific flanking oligonucleotide primers, F1 and R1. Deletion mutant transfer vectors were constructed by making internal deletions in pFP-PC1 of sections between restriction enzyme sites (as shown in fig. 4.2), resulting in three vectors with deletions of 2184bp[Δ PC1-b], 1042bp[Δ PC1-x] and 169bp[Δ PC1-s] in the FP-PC1 gene (fig. 4.3). The B and X mutants maintained the correct reading frame of FP-PC1, whereas the S mutant disrupted the reading frame - due to a frameshift. The X, S and B mutants lead to deletion of the EF-hand, ATP binding site and PPD-1 functional domains from the FP-PC1 protein, as shown in fig. 4.2, with the last mutant deleted of all three domains.

Recombinant viruses with deletions in FP-PC1 were then constructed by TDS as shown in fig. 4.1. Briefly, transfer vectors pFP-PC1b, pFP-PC1x and pFP-PC1s were transfected into FWPV infected CEFs. Transfection medium was then replaced with GPT selective medium before 6 isolates of each mutant were plaque purified twice under GPT selective conditions. Only viruses which had undergone a crossover event incorporating the entire transfer vector were able to replicate due to the expression of GPT. These MPA resistant viruses were plaque purified three times for the S mutant and eight times for the B and X mutants in non-GPT selective conditions to allow a second crossover event, leading to resolution of viruses as indicated in fig. 4.1. Confirmation of the loss of the GPT gene was obtained by non growth of viruses in GPT selective medium. Only three plaque purifications in non GPT selective medium were required to resolve recombinants containing the S mutant. However,

despite eight plaque purifications in non GPT selective medium, no resolved recombinants containing the B or X mutant genotypes were isolated. Consequently, to isolate resolved B and X mutant viruses, twenty independent plaques were isolated from original transfection mixtures and plaque purified as described for the S mutant. Screening of these viruses resulted in resolved recombinant genotypes as represented in fig. 4.2.

Resolved viruses were amplified as outlined in Materials and Methods (chapter 2) before analysis and characterisation as discussed below.

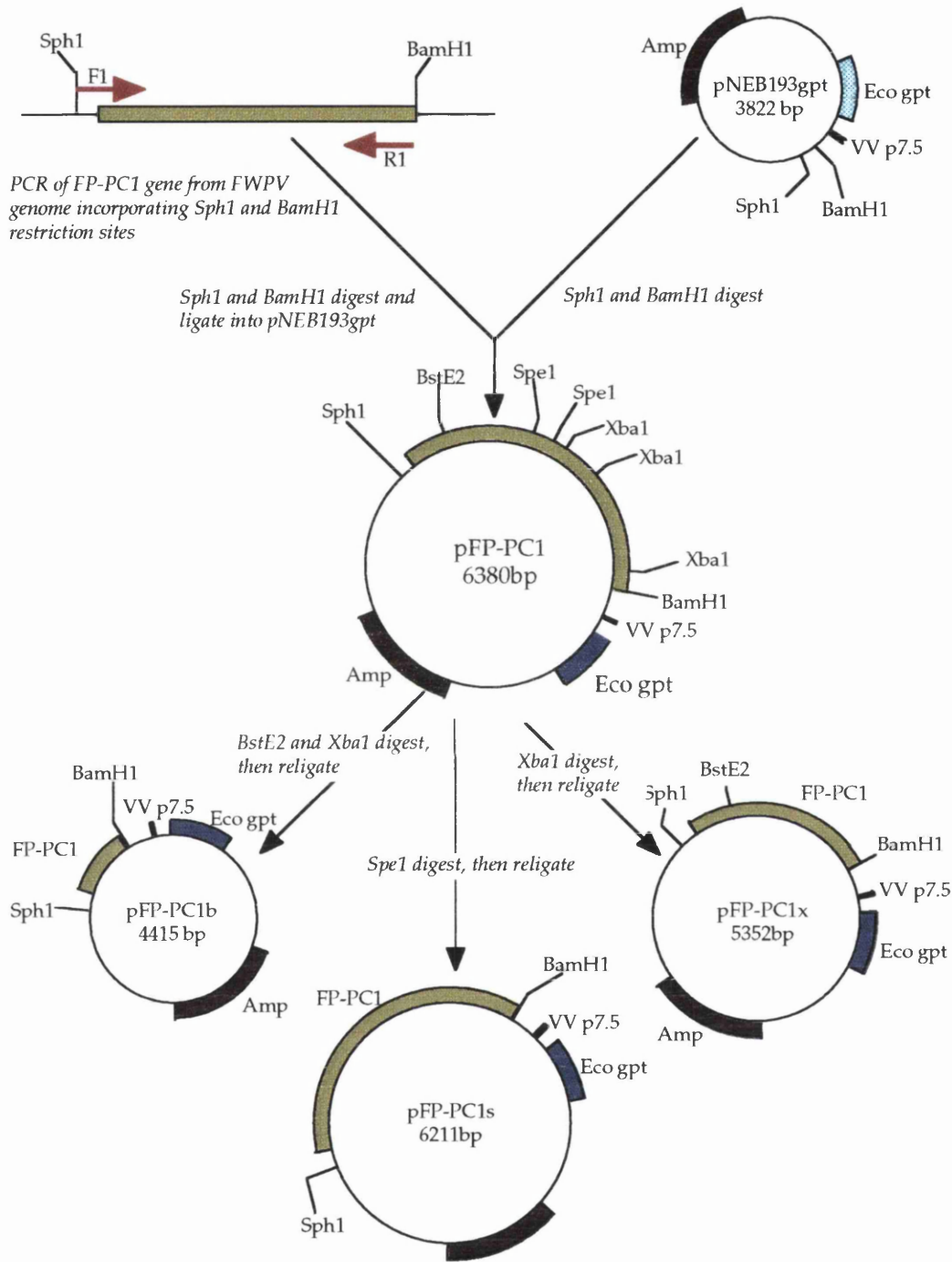
4.3 Confirmation of mutant FWPV viruses and transfer vectors

Verification of transfer vectors with wt and Δ PC1 inserts was obtained by restriction enzyme analysis (data not shown).

Confirmation of genotypes of mutant viruses and transfer vectors was performed by PCR analysis using template DNA extracted from wt FWPV and Δ PC1-b, Δ PC1-x and Δ PC1-s viruses as well as from plasmid DNA.

Flanking oligonucleotide primers for FP-PC1 (F1 and R1) amplified fragments representing the full length FP-PC1 gene (2611bp) from both wtFP9 and resolved viruses resulting from TDS (B5, X1, X3, X4, X10, X13, X19 and S1B), whereas viruses B3, B4, X17 and S1A gave fragment sizes consistent with the expected deletion (fig. 4.4) as shown in table 4.1. Fragments representing genotypes consistent with unresolved

Fig.4.3 Construction of FP-PC1 deletion transfer vectors pFP-PC1b, pFP-PC1s and pFP-PC1x.



The entire FP-PC1 ORF was amplified directly from the FWPV genome in 3 independent PCR reactions and cloned into the BamHI and SphI sites of pNEB193gpt. Various deletions were introduced into the FP-PC1 ORF by restriction enzyme digestion with Bam H1 and Bste2, Xba1 or Spe1 and religated. The resultant plasmids: pFP-PC1b, pFP-PC1s and pFP-PC1x were used as transfer vectors in TDS to introduce the same deletions into the FWPV genome.

intermediates were also observed where both mutant and wt copies of FP-PC1 are present (B9 and X20).

In order to exclude the possibility of presence of wt FP-PC1 sequences in viruses displaying mutant genotypes, PCR of virus genomic DNA was performed using a flanking primer and a primer internal to the deletion (R1 and Z respectively). Only viruses displaying wt FP-PC1 genotypes in fig 4.4 gave the expected product size of 1042bp in fig. 4.5.

4.4 Transcriptional characterisation of wt and mutant FP-PC1

(Northern blot courtesy of S. Laidlaw)

In order to characterise expression of wt and mutant FP-PC1 mRNA, probes were generated for Northern blotting by amplification of the sequence corresponding to the FP-PC1 internal oligonucleotide primer, F2. The resultant probe fragment was purified from a low melting point agarose gel, excised, and [$\alpha^{32}\text{P}$]-dCTP labelled probes were generated using a random priming kit. CEFs infected with either wtFWPV or $\Delta\text{PC1-b}$, $\Delta\text{PC1-x}$ or $\Delta\text{PC1-s}$ viruses were incubated for either 4 or 24 hours in the presence or absence of Ara-c, which acts as an inhibitor of DNA replication and therefore blocks intermediate and late gene expression, hence allowing exclusive analysis of early gene expression. Total RNA was then extracted and resolved by denaturing electrophoresis in a 1% formaldehyde agarose gel, before being blotted onto nitro-cellulose and hybridised with the F2 probe as described above. As shown in fig. 4.6 wt FWPV virus produced an abundant 3kb early transcript at 4h p.i., which is absent from non-infected cells. A strongly expressed transcript of heterogenous length ranging from

Fig.4.4 Confirmation of recombinant FWPV deletion mutants by PCR using flanking oligonucleotide primers

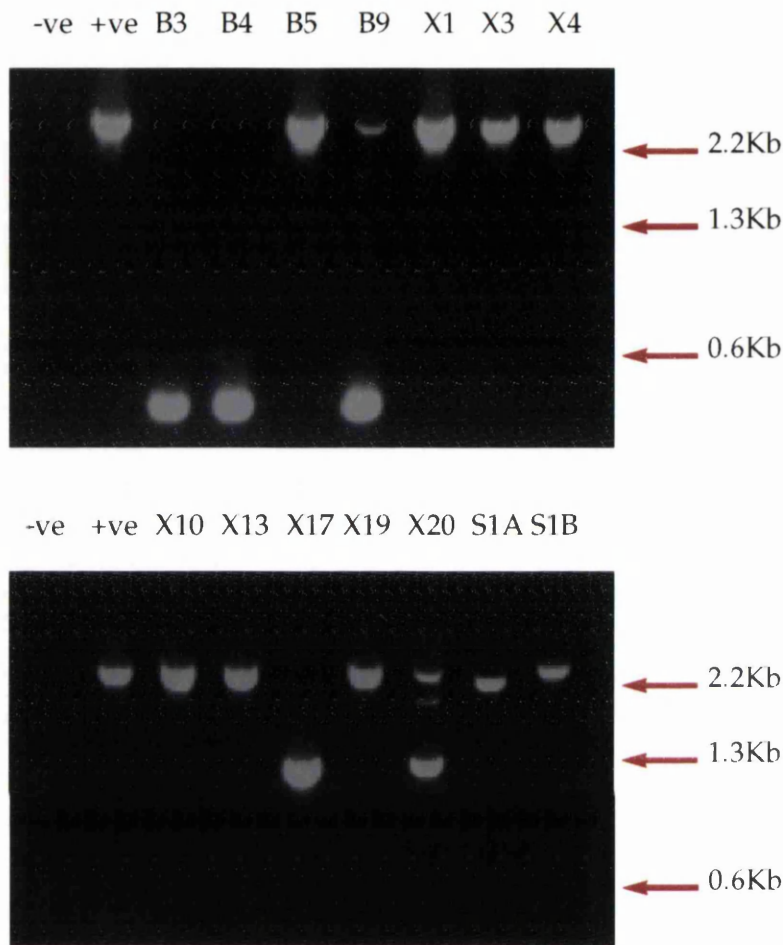
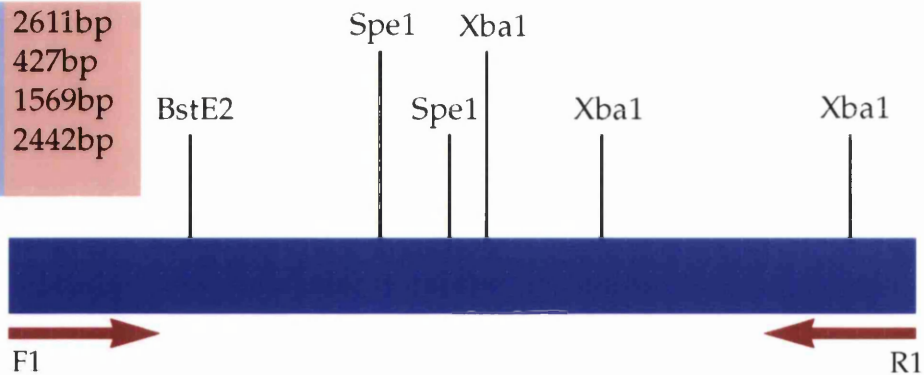


Table 4.1

wtFP9-FWPV	2611bp
Δ PC1-B	427bp
Δ PC1-X	1569bp
Δ PC1-S	2442bp



Following isolation of genomic viral DNA, FP-PC1 ORF flanking primers F1 and R1 were used to confirm the genotype. The position and orientation of the primers is shown above. The negative control was no DNA and the positive control was wt FWPV strain FP9. The sizes of the predicted products is shown in table 4.1. B3, B4, X17 and S1A show PCR products representative of mutant genotypes.

Fig.4.5 Confirmation of absence of wt FP-PC1 sequence in FWPV deletion mutants by PCR

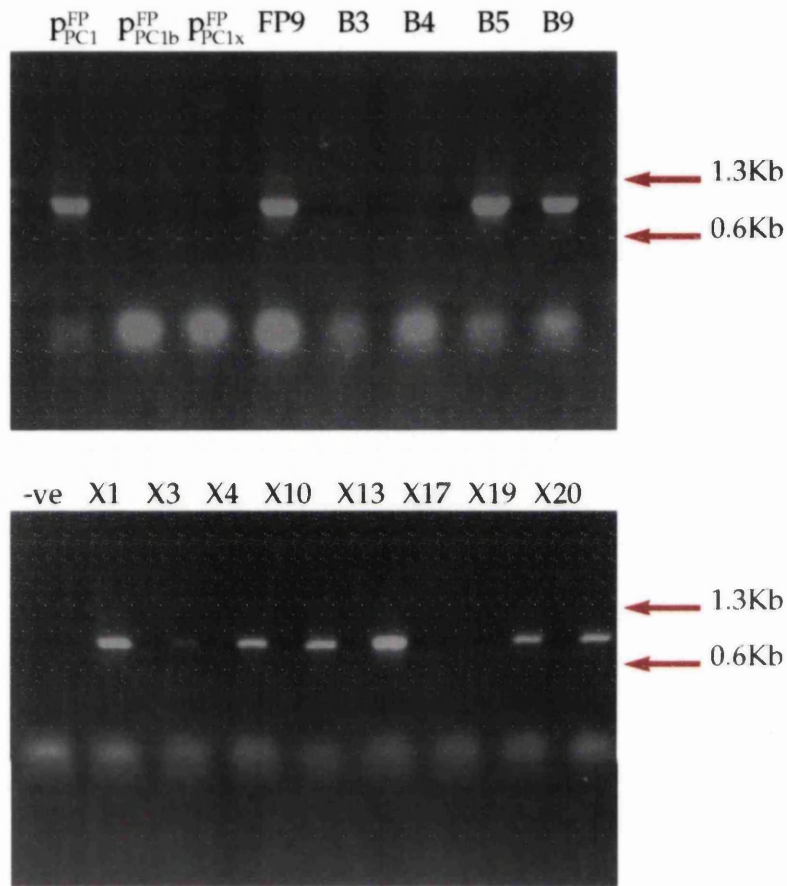
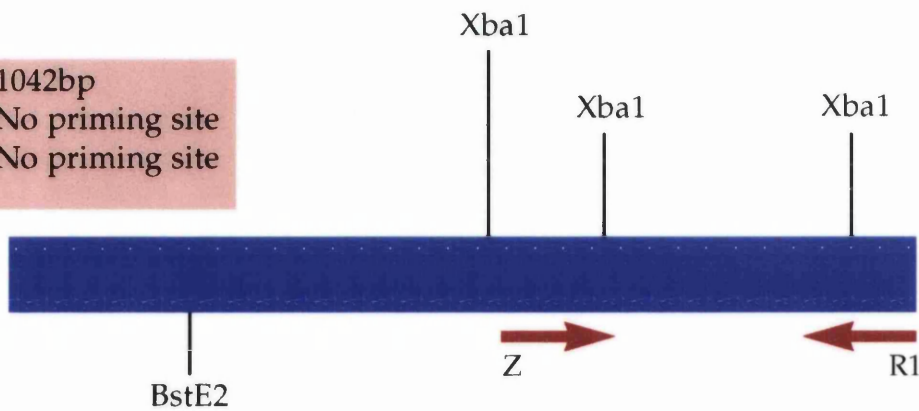


Table 4.2

wtFP9-FWPV	1042bp
Δ PC1-b	No priming site
Δ PC1-x	No priming site



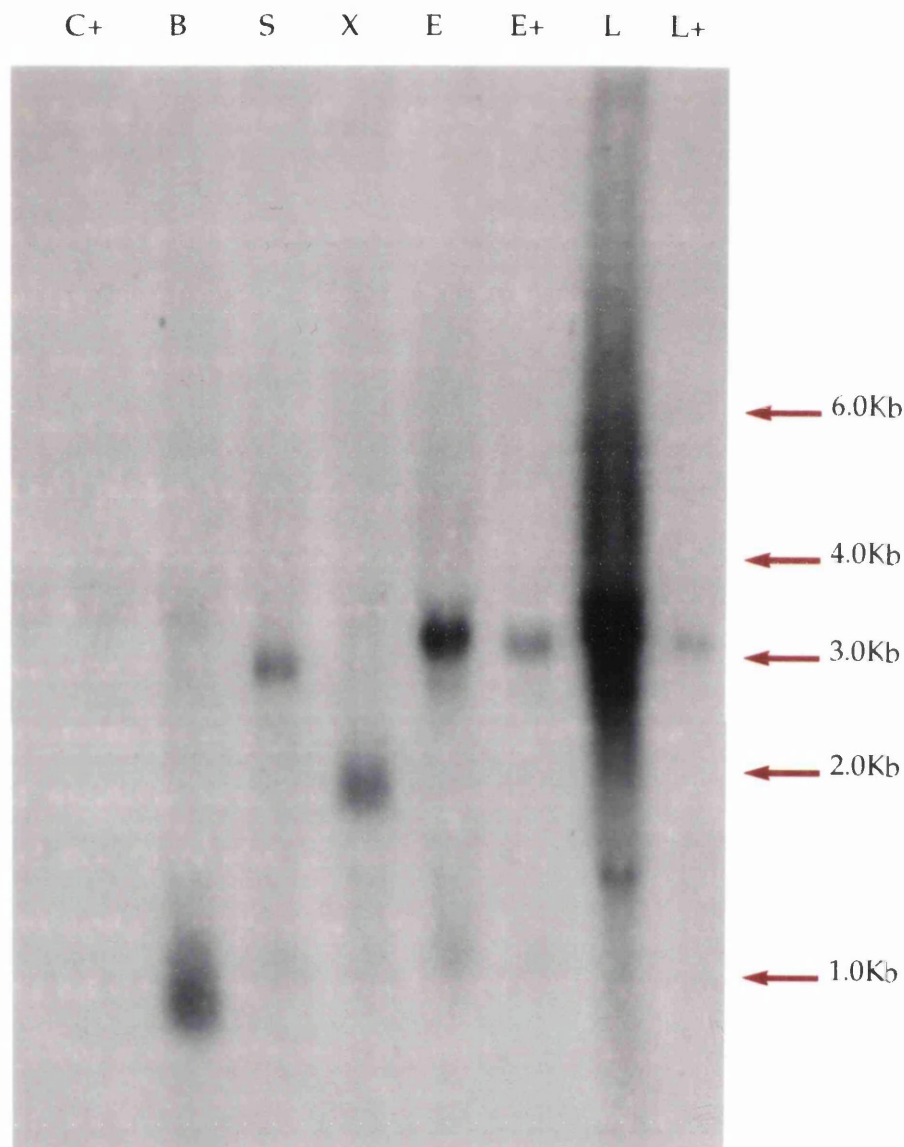
Following isolation of viral and plasmid DNA, FP-PC1 ORF internal and flanking primers Z and R1 were used to confirm the absence of wt FP-PC1 sequences in recombinant FWPV and transfer vectors indicating FP-PC1 deletions. The position and orientation of the primers is shown above. The negative control was no DNA. The sizes of the predicted products is shown in table 4.2. B3, B4, and X17, as well as their respective transfer vectors, indicate that only mutant genotype structures are present.

2.5 to 6.0kb was observed in the absence of araC at 24h p.i. but was absent in the presence of araC. Compared to cells infected with wt FWPV, the size of the early transcript from cells infected with the 169bp Δ PC1-s deletion mutant was reduced slightly, similarly the transcripts from cells infected with the 1042bp Δ PC1-x deletion mutant and the 2184bp Δ PC1-b deletion mutant were reduced to 2kb and 1kb respectively.

The isolation of FWPV viruses with deletions corresponding to the Δ PC1-b genotype confirmed that FP-PC1 is non-essential for FWPV replication *in vitro*. In order to assess if deletion of the FP-PC1 ORF has ramifications for FWPV replication *in vitro*, single and multi-step growth quantification and plaque size assays were carried out.

CEF monolayers were infected in triplicate with either wt FWPV or Δ PC1-b viruses at m.o.i. levels of 0.01 or 10. At various times post infection samples representing extracellular and intracellular virus as described in Materials and Methods were taken and quantified by plaque assay. The results showed that there was no significant difference in growth kinetics in single or multi-step growth models between wt FWPV and Δ PC1-b (figs. 4.7 and 4.8 respectively). This suggested that FP-PC1 does not play a major role in replication or dissemination of FWPV *in vitro*.

Plaque assay results had indicated a difference in plaque size between wt FWPV and Δ PC1-b viruses. To allow accurate visualisation of individual plaques, CEF monolayers were infected in duplicate with either wt FWPV or Δ PC1-b and incubated for 4 and 5 days. As shown in fig. 4.9, the average diameter of Δ PC1-b plaques was approximately 40% less than wt FWPV plaques. This was surprising as no difference in growth had been detected in growth analysis as discussed above. A possible explanation was that replication assays were performed in liquid Medium 199, whereas all



CEF monolayers were infected with wt FWPV strain FP9, Δ PC1b, Δ PC_x or Δ PC1s. Total RNA samples were taken at 4h p.i. (C+, B, S, X, E, and E+) or 24h p.i. (L and L+) with + indicating the presence of araC. RNA was blotted onto a nitrocellulose membrane and hybridised to α [32]P-dCTP labelled FP-PC1 probes generated using a random priming kit. Uninfected control cells (lane C+) show no labelling as expected, cells infected with wt FWPV (lanes E, E+, L, L+) indicate abundant defined early and heterogenous late transcripts. Cells infected with mutant FWPV as described above (lanes B, S and X) show transcripts that correlate in size with gene deletions (2184, 169 and 1042bp respectively).

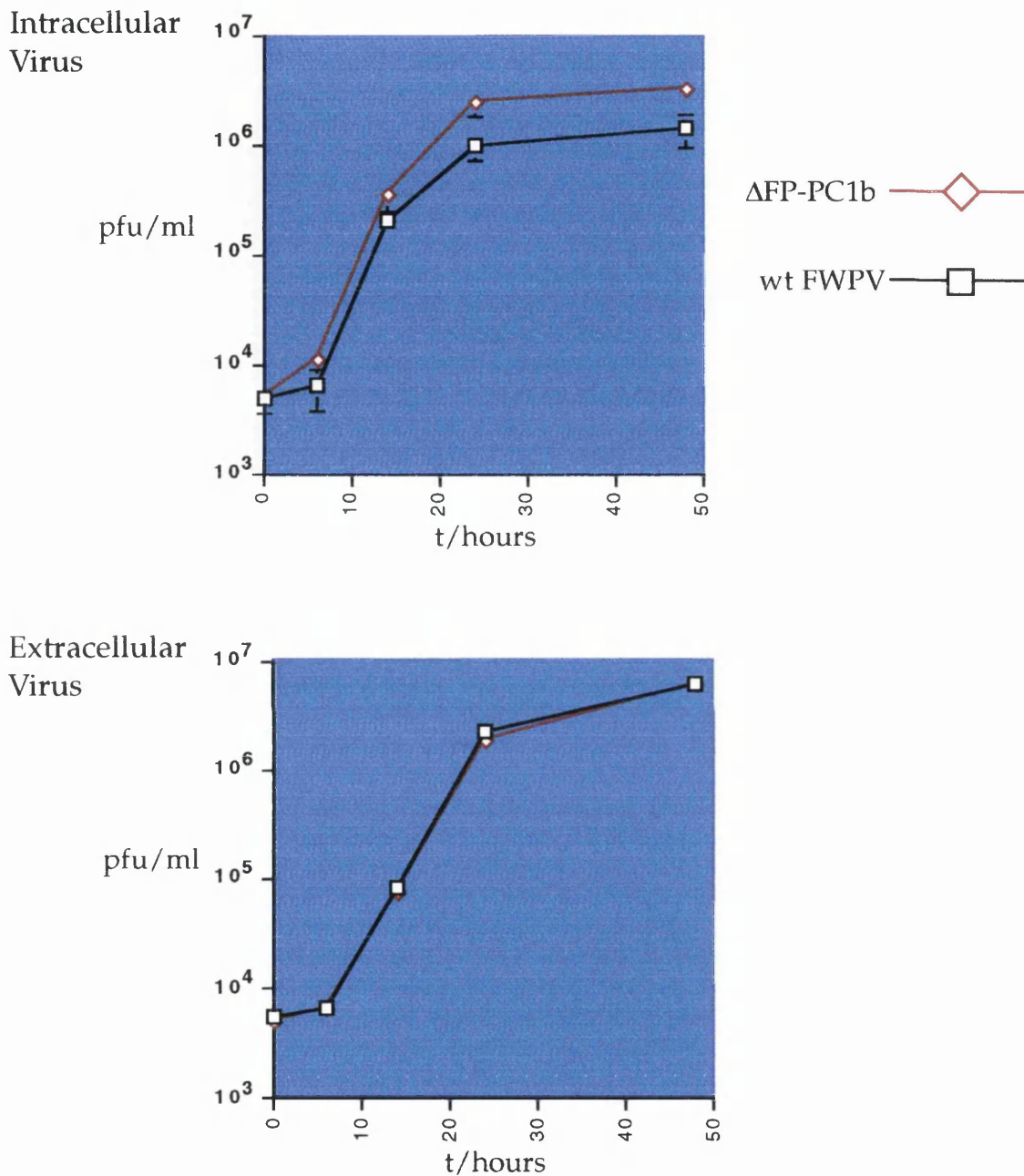
plaque assays were conducted using semi solid Medium MEM, the former medium being supplemented with nucleotides. This could account for the slower growth and hence smaller plaque size of Δ PC1-b virus compared to wt FWPV in semi solid medium. Therefore to investigate the hypothesis that FP-PC1 may be involved in nucleotide metabolism or more specifically the nucleotide salvage pathway, kinetics of Δ PC1-b and wt FWPV were investigated by repeating growth experiments as outlined above.

Virus samples were prepared as described in Materials and Methods, except that samples were grown in either Medium MEM or Medium 199. CEF monolayers were infected with either Δ PC1-b or wt FWPV at a m.o.i. of 50 and incubated in either Medium MEM or Medium 199 before quantification of total virus growth kinetics by plaque assay. As shown in fig. 4.10, no significant difference can be observed to suggest that FP-PC1 is involved in nucleotide metabolism. A similar result was also obtained in a multiple step growth kinetics experiment conducted at a m.o.i. of 0.01 quantifying extracellular virus (fig. 4.10). A single step growth curve carried out at an m.o.i. of 10 in differential media as described above also showed no difference between wt and FP-PC1 mutant viruses (data not shown).

4.6 Deletion of FP-PC1 does not affect virus growth in a chicken wing web scarification model

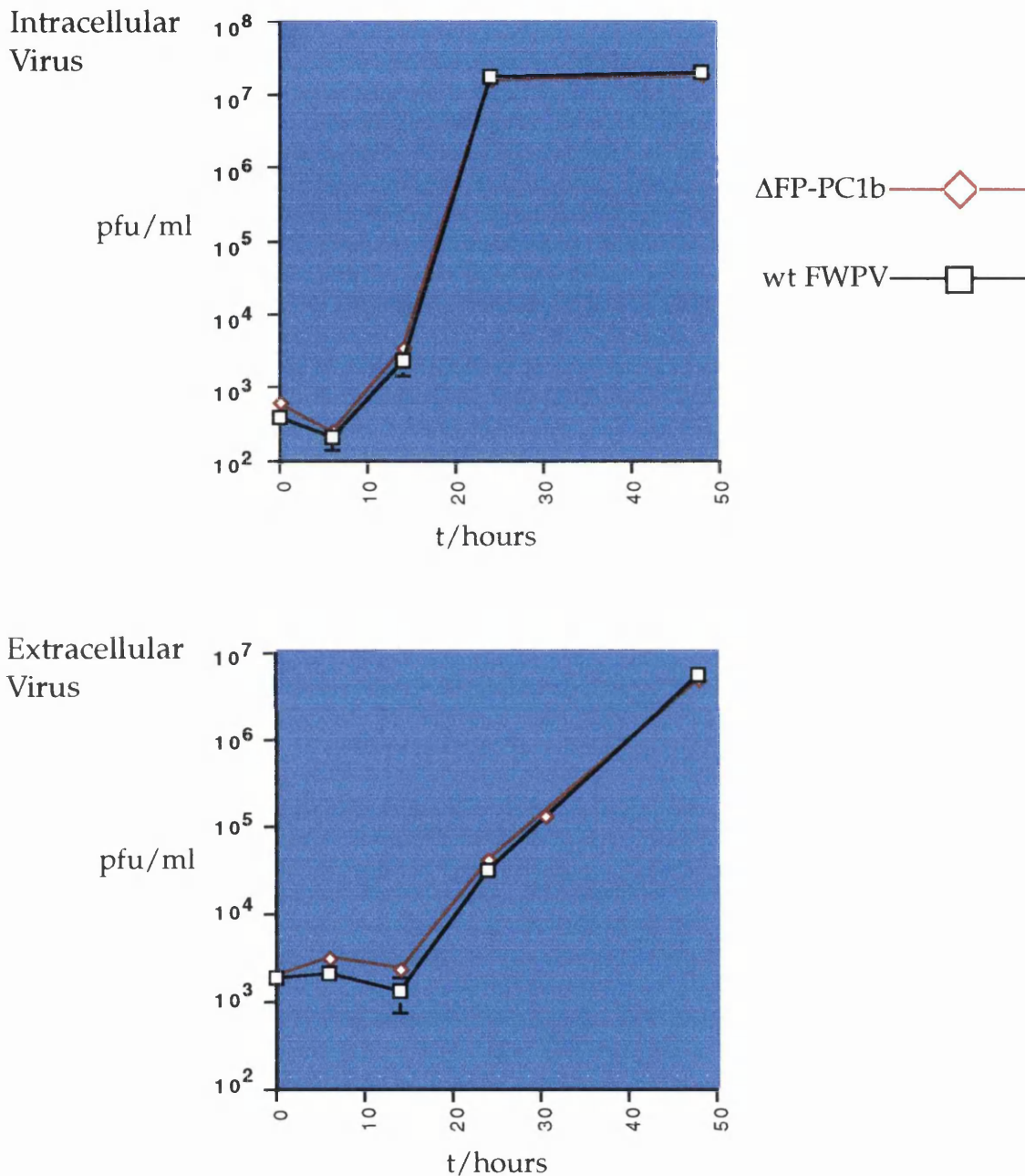
The results shown in section 4.3 confirm that the FP-PC1 gene is non-essential for FWPV replication *in vitro*. However in an attempt to evaluate if FP-PC1 plays a role *in vivo*, comparison between Rhode Island Red (RIR) and Light Sussex (LSx) chickens infected with either wt FP9

Fig.4.7 Single step growth kinetics comparison between wt FWPV and Δ FP-PC1b viruses

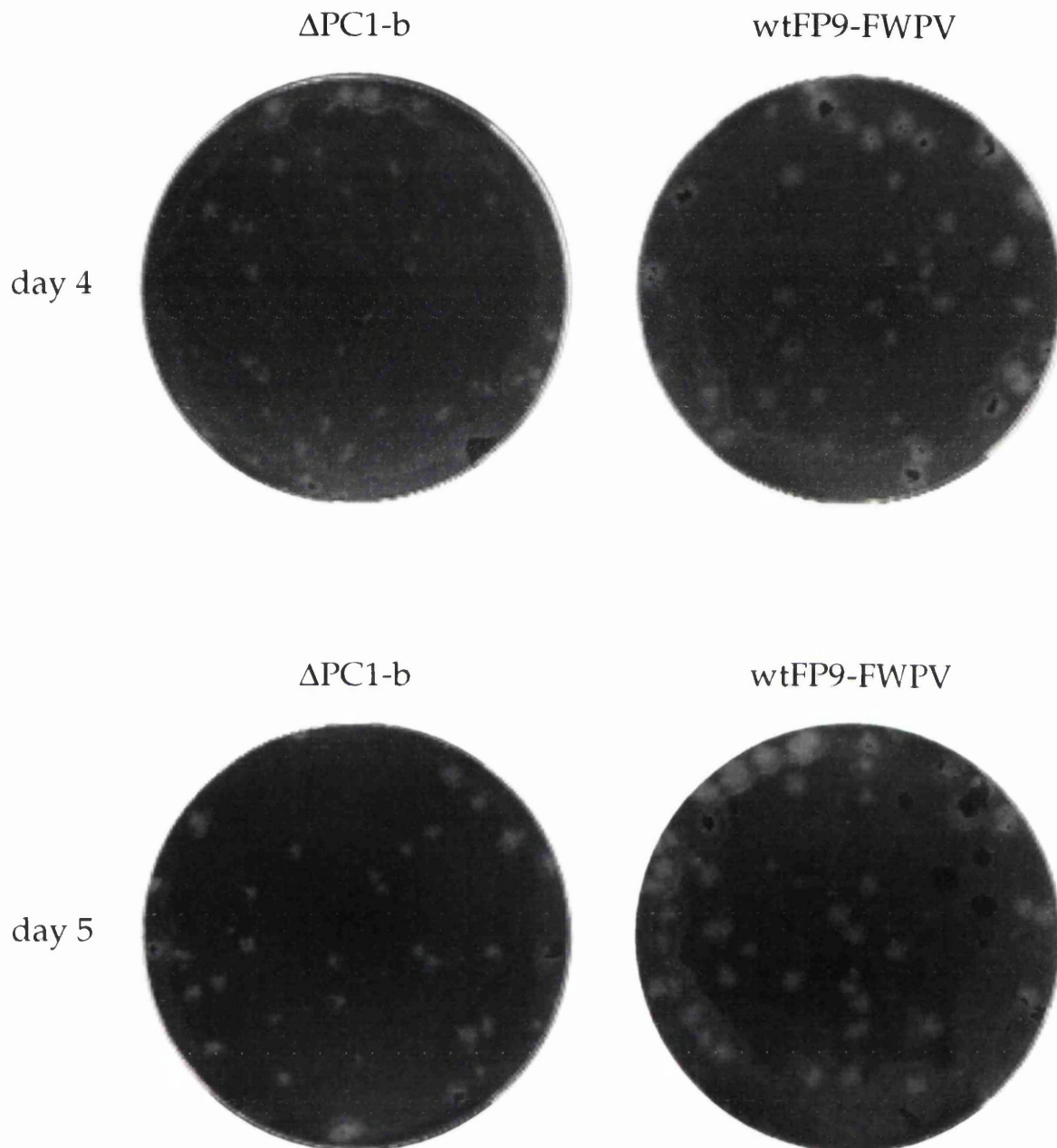


CEF monolayers were infected at a m.o.i of 10 with either wt FWPV or Δ FP-PC1b in triplicate for each time point. At each time interval, a 1ml aliquot of medium representing extracellular virus was removed and titrated, the remaining medium was discarded and cells washed before addition of 1ml of fresh medium. Cells were freeze thawed three times and the resultant samples representing intracellular virus were titrated. Data plotted is the mean from the triplicate sample, error bars are included but are largely masked by the symbols for the time points.

Fig.4.8 Multiple step growth kinetics comparison between wt FWPV and Δ FP-PC1b viruses



CEF monolayers were infected at a m.o.i of 0.01 with either wt FWPV or Δ FP-PC1b in triplicate for each time point. At each time interval, a 1ml aliquot of medium representing extracellular virus was removed and titrated, the remaining medium was discarded and cells washed before addition of 1ml of fresh medium. Cells were freeze thawed three times and the resultant samples representing intracellular virus were titrated. Data plotted is the mean from the triplicate sample, error bars are included but are largely masked by the symbols for the time points.



CEF monolayers were infected with wtFP9 or Δ PC1-B FWPV to m.o.i. levels between 10^{-3} and 10^{-7} , and overlaid in non GPT selective medium MEM. Virus plaques were visualised after 3 and 4 days p.i. by staining in crystal violet.

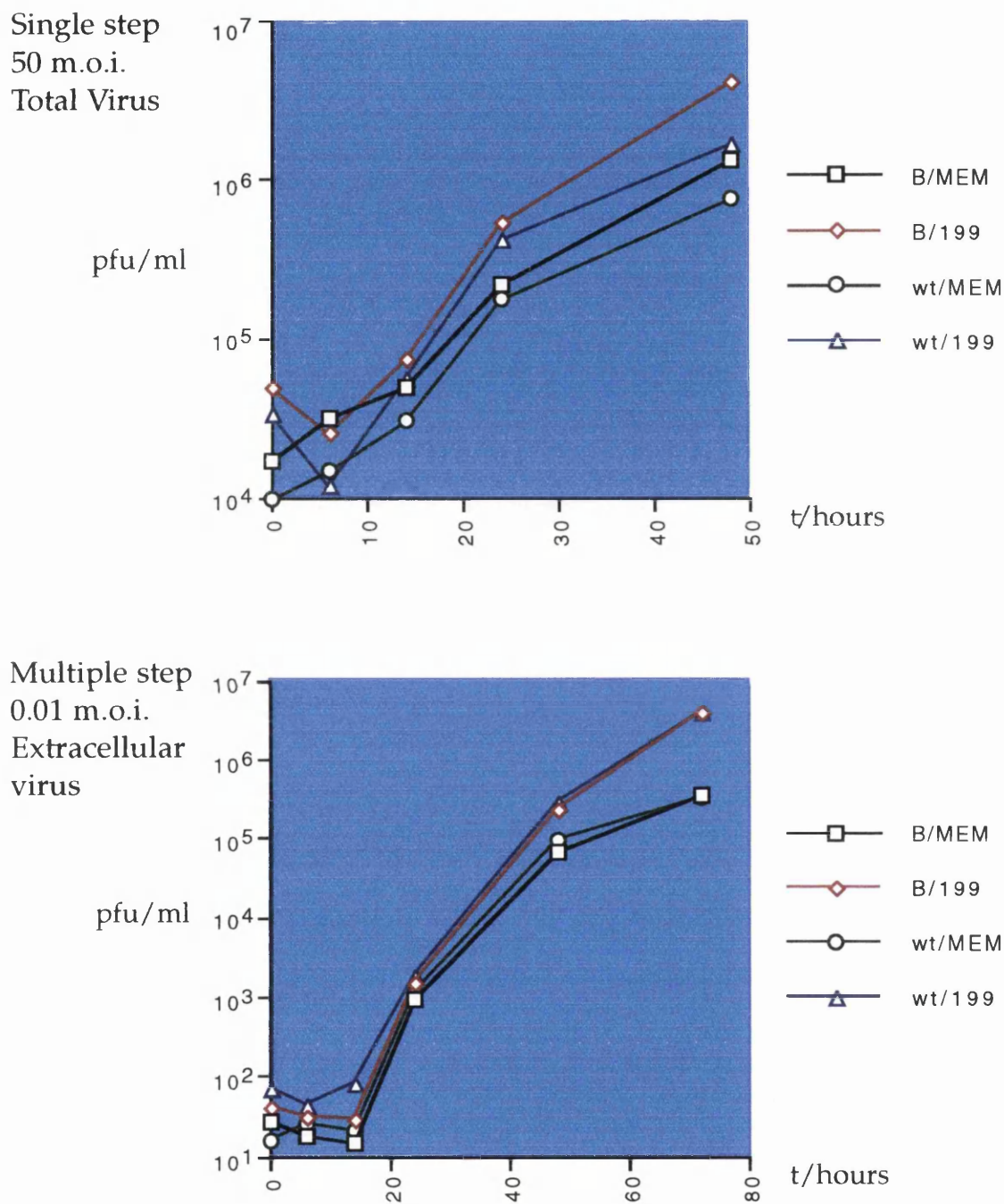
FWPV and Δ PC1-b FWPV was carried out. Three sets of six, five week old RIR and LSx chickens were inoculated by scarification at both wing webs, with either 50 μ l (approximately 5×10^7 p.f.u.) of purified wt FP9 FWPV, or Δ PC1-b or pBSA (negative control). Three further inoculations were carried out at 2 week intervals. Chickens were monitored for signs of illness and development of pocks. No difference was observed in any of these variables between chickens infected with wt FP9 FWPV or Δ PC1-b.

4.7 Analysis of Autophosphorylation and Alkaline phosphodiesterase 1 activity

Mammalian PC1 has been shown to have 5' nucleotide phosphodiesterase activity (Rebbe *et al.*, 1991) which is dependent upon the presence of divalent cations, as activity is diminished upon addition of chelating agents such as EDTA (Belli *et al.*, 1994) The enzymatic activity attributable to PC1 can be quantitatively assayed by measuring hydrolysis of p-nitrophenyl phenyl phosphonate. The presence of a phenyl group in this substrate renders the p-nitrophenol colourless. However, upon cleavage with phosphodiesterase, p-nitrophenol is released and a yellow colour can be observed.

In order to assess 5' nucleotide phosphodiesterase activity attributable to FP-PC1 expression; CEF, Vero and Ltk⁻ monolayers were infected in triplicate with wt FWPV and Δ PC1-b viruses, with or without Ara-c. At intervals up to 24h p.i. the inoculum was removed and an assay mixture comprising of calcium ions and p-nitrophenyl phenyl phosphonate was used to assay 5' nucleotide phosphodiesterase activity at the surface of infected cells. As shown in fig. 4.11 no difference in substrate

Fig.4.10 Single and multiple step growth kinetics analysis of wt FWPV and Δ FP-PC1b viruses in nucleotide deficient media.



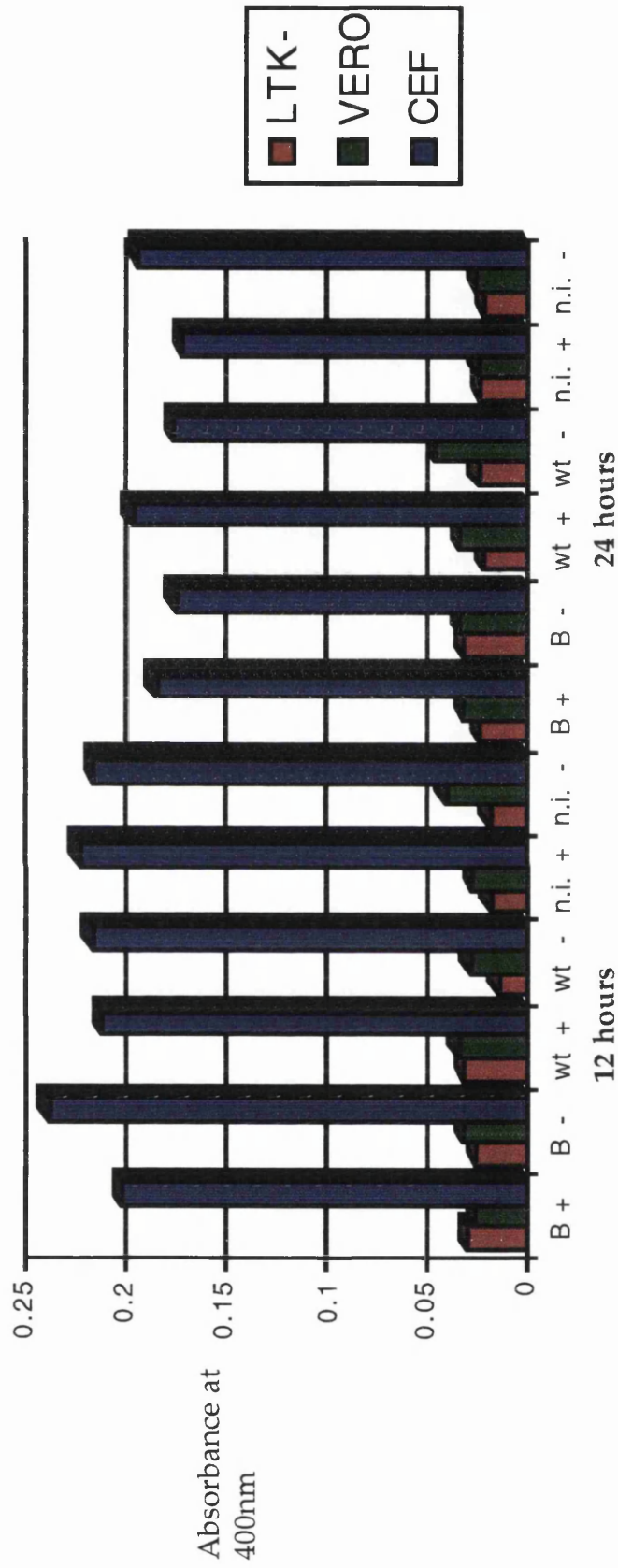
CEF monolayers were infected at a m.o.i of 50 or 0.01 with either wt FWPV or Δ FP-PC1b in triplicate for each time point. At each time interval, samples representing total virus or extracellular virus was taken and titrated on CEF monolayers. Data plotted is the mean from the triplicate sample, error bars are included, but are largely masked by the symbols for the time points.

hydrolysis as quantified by spectrophotometry was evident between wt FWPV, Δ PC1-b and uninfected control cells. CEFs showed 8-10 times greater 5' nucleotide phosphodiesterase activity than Veros, which in turn showed marginally higher activity than Ltk- cells. In summary no 5' nucleotide phosphodiesterase activity specific to FP-PC1 was detectable by the protocol utilised in this experiment.

The isolation of FWPV containing the Δ PC1-b deletion, which accounts for 84% of the FP-PC1 ORF, suggests that the FP-PC1 gene product is non-essential for FWPV replication *in vitro*. Earlier attempts to isolate Δ PC1-b and Δ PC1-x from 6 independent plaques had failed, probably as a result of bias for wt during 1st and 2nd crossover events in TDS (fig. 4.12). Assuming the likelihood of a recombination event is directly proportional to the length of homology between two sequences, a bias towards the wt can arise which is directly proportional to the degree of asymmetry in the deletion. This applies mostly for the Δ PC1-b and Δ PC1-x deletions and to a lesser extent to the near symmetrical Δ PC1-s deletion. As represented in fig. 4.12 the probability of obtaining a 1st crossover intermediate with orientation a or b is dependent on whether the region deleted is to the right or left respectively. But upon resolution after the 2nd crossover the wt is always favoured irrespective of whether the deletion is to the left or the right. In order to overcome this bias two possible approaches were used:

1. Selection of viruses after 1st crossover with orientation a (ori.a) for left handed asymmetric deletions or orientation b (ori.b) for right handed deletions. This approach would lead to selection of unresolved viruses which upon 2nd crossover would reverse the bias towards mutant viruses.

Fig.4.11 Assay to determine 5' Nucleotide Phosphodiesterase activity on intact cells using non-infected, wt FWPV and Δ FP-PC1-b



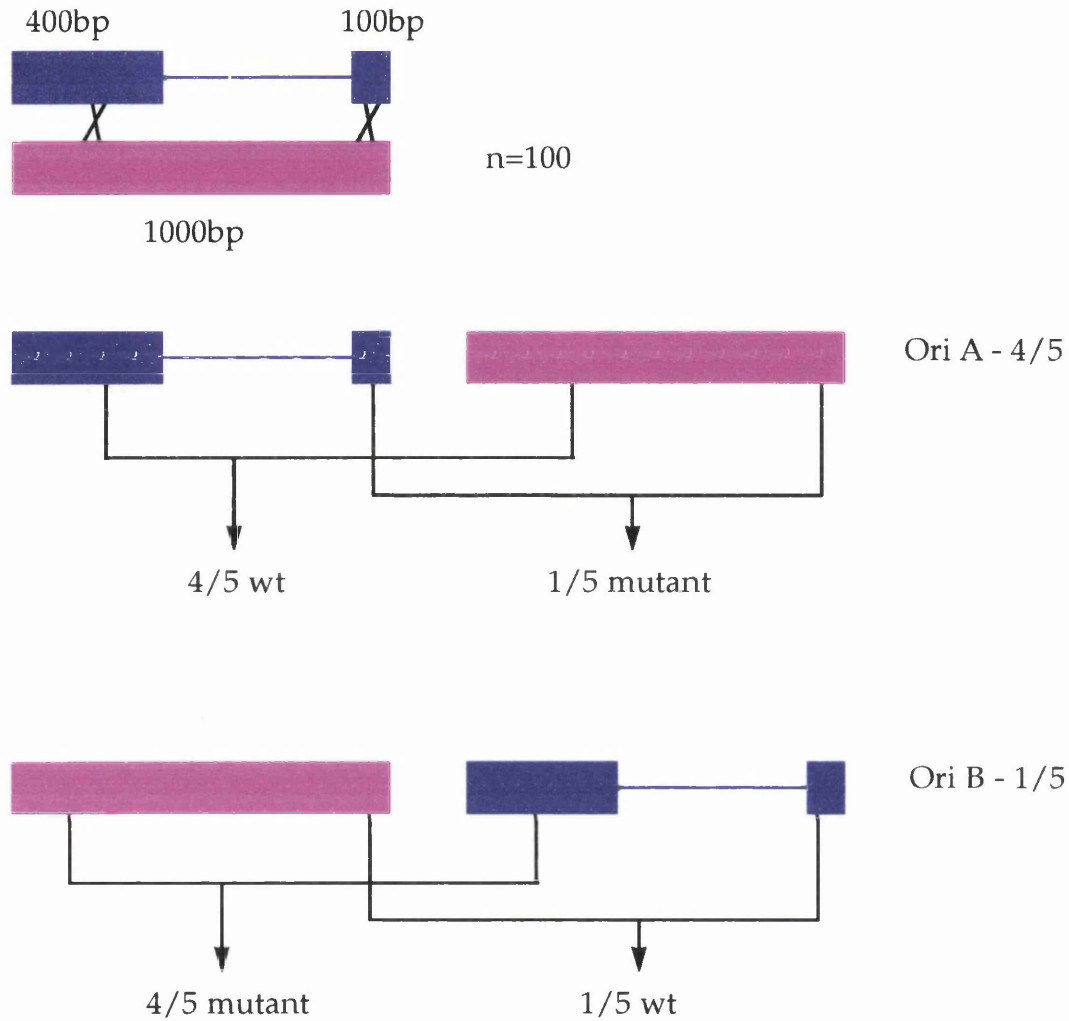
CEF, LTK⁻ and VERO monolayers were infected at an m.o.i. of 10 (in triplicate for each time point and set of conditions) +/- araC with either Δ FP-PC1b (B), wt FWPV strain FP9 (wt) or uninfected controls (n.i.). 12h and 24h p.i. medium was discarded and cells washed before addition of p-nitrophenyl phenyl phosphonate and calcium chloride to allow assay for possible 5' nucleotide phosphodiesterase activity of FP-PC1. Absorbance was quantified by use of a spectrophotometer at 400nm.

2. Screening of a larger number of individual recombinant viruses to overcome bias for wt.

Confirmation of the genomic structure of these mutant viruses was obtained by PCR using flanking primers. As shown in fig. 4.4 B9 and X20 viruses contained both mutant and wt FP-PC1 sequences, indicating that other virion genotypes (such as B4 and X17) which indicated mutant genotypes by PCR, may possibly contain wt sequence which has not been amplified or detected by this approach. In order to decrease this probability, PCR amplicons were overloaded to show up any full length wt sequence (2611bp) which would not be preferentially amplified or be as visible when compared to the amplicon representing the Δ PC1-b genotype (467bp). This is clearly evident by comparing the relative intensities of the wt and mutant amplicons in B9. Further confirmation of absence of wt sequences in mutant viruses, was obtained by use oligonucleotide primers that were internal to deleted regions, and would therefore lead to exclusive amplification of DNA from viruses containing wt FP-PC1 genotypes.

The finding that FP-PC1 is non-essential is not surprising as most essential poxvirus genes tend to be located in the central regions of the genome, whereas FP-PC1 is located towards the left hand side Inverted Terminal Repeat (ITR), and therefore likely to be non-essential. Other neighbouring FWPV genes (FWPV Cel-1 and FWPV SNAP) have also recently been shown to be non-essential (Laidlaw *et al.*, 1998). Further confirmation of the genotypes of mutant viruses was obtained by transcriptional analysis of early transcripts from cells infected with FP-PC1 mutants as described above, which correlated with the predicted size of deleted ORFs. In addition, transcriptional analysis confirmed that the

Fig.4.12 Wt bias in Transient Dominant Selection



Therefore the final number of wt and mutant virions from the given asymmetric right handed deletion where the total number of recombination events (n) =100 is:

1st Crossover Viruses with Ori A = $400\text{bp}/500\text{bp} \cdot 100 = 80$ virions

2nd Crossover resolved virions

wt virions = $4/5 \cdot 80 = 64$ virions

Mutant virions = $1/5 \cdot 80 = 16$ virions

Viruses with Ori B = $100\text{bp}/500\text{bp} \cdot 100 = 20$ virions

2nd Crossover resolved virions

wt virions = $1/5 \cdot 20 = 4$ virions

Mutant virions = $4/5 \cdot 20 = 16$ virions

Therefore the final ratio between wt and mutant virions = $68:32 = 17:8$

FP-PC1 ORF is strongly transcribed early and late during the infective cycle of FWPV, making it less likely that the FP-PC1 ORF was “junk DNA”.

Experiments designed to study single and multi-step growth kinetics of wt FWPV and Δ PC1-b viruses failed to show any difference between them, although a difference in plaque size was seen. The reason why growth kinetics experiments were repeated in nucleotide deficient media was to investigate if FP-PC1 was involved in the nucleotide salvage as has been recently demonstrated for mammalian PC1 (Deterre et al., 1996). Although results again showed no difference, suggesting that FP-PC1 is not involved in the nucleotide salvage pathway *in vitro*, a possible role in *in vivo* pathogenesis cannot be ruled out. Furthermore, as suggested from data from *in vivo* experiments (which also showed no difference between birds infected with wt or mutant FWPV), very subtle or transient roles, which may affect any aspect of replication, virulence, and/or dissemination, will probably not be detected by growth kinetics experiments *in vitro* or *in vivo*. Therefore the fact that none of the experiments studying various phenotypic ramifications of the deletion of the FP-PC1 ORF from the FWPV genome showed an effect, does not rule out a role for FP-PC1 in some aspect of replication or indeed the nucleotide salvage pathway.

There are several key factors which could explain why a difference in plaque size in nucleotide deficient was seen, and yet no difference in growth kinetics was observed in similar media. It is important to outline that plaque assays are carried out in semi-solid media, whereas growth kinetics analysis was carried out in liquid media. In the latter situation, there is a higher rate of diffusion of larger molecules compared to rates in semi-solid media. This factor may have had an affect on the virus

spread/growth in a semi-solid media, but no affect in liquid media. The failure to detect alkaline phosphodiesterase activity could be due to the sensitivity of the method used, or that FP-PC1 does have alkaline phosphodiesterase activity, but at very low levels. However, the possibility that FP-PC1 does not express an active phosphodiesterase cannot be ruled out. The contemporary FP-PC1 protein may be involved in a completely non-enzymatic role, or may be implicated in interactions with host or viral proteins. Further work could involve analysis of the FP-PC1 gene from the virulent precursor FWPV strain, HP1, to assess if similar data is attained. In addition techniques designed to characterise protein-protein interactions, such as cross-linking and yeast-two-hybrid analysis may be useful to show if FP-PC1 does have a role that does not rely on predicted enzymatic activities.

Chapter 5

Expression of FP-PC1 in Prokaryotic

Expression Systems

5.1 Introduction

In order to allow analysis of FP-PC1 by immunological techniques, large scale protein expression was necessary to enable production of antibodies. It was decided to express both the extracellular and alkaline phosphodiesterase 1 (PPD) domains in *E. coli*, using the FLAG expression system (IBI). The pFLAG.CTS vector from this system was chosen as it has two integral features which make it favourable for expression of potentially problematic proteins. Firstly, it contains an artificial, N-terminal, *E. coli*, outer membrane protein A (OmpA) signal sequence, permitting targeting of protein domains directly into the periplasmic space, therefore allowing easier purification. This signal sequence is cleaved from the FLAG protein by a signal peptidase as it crosses the inner cytoplasmic membrane into the periplasmic space. Secondly, use of the M2 anti-flag monoclonal antibody raised against the integral C-terminal flag octapeptide in pFLAG.CTS, should facilitate a simpler immuno-affinity based protein purification procedure compared to traditional protein purification methods based upon sequential chromatographic methods.

The cloning and expression of the two FP-PC1 domains referred to above are presented. In addition, protein fractionation and optimisation, as well as attempted purification are described.

5.2 Cloning strategy

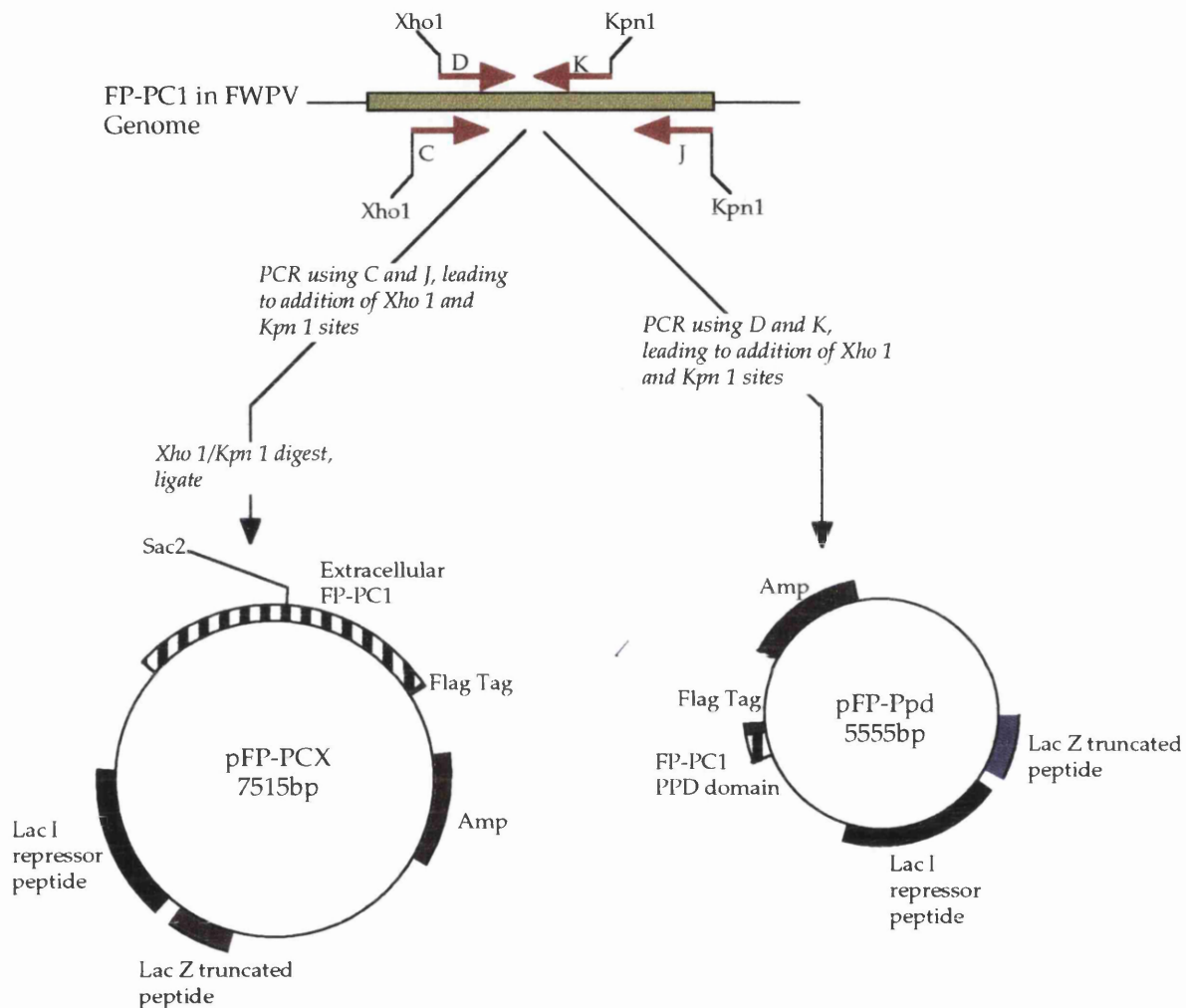
Both the extracellular and PPD FP-PC1 domains were amplified directly from the FWPV genome using gene specific primers, C/J and D/K respectively, which lead to addition of Xho 1 and Kpn 1 restriction enzyme

sites at 5' and 3' ends respectively (figs. 5.1 and 5.2). Resultant amplicons and pFLAG.CTS were sequentially double digested with Xho 1 and Kpn 1, purified from an agarose gel using "GeneClean", and ligated together using T4 DNA ligase. FP-PC1⁺ clones were identified by PCR and multiple restriction enzyme analysis. (The procedure of PCR amplification directly from the FWPV genome was adopted as a general strategy, whenever possible, in all cloning steps conducted for protein expression in both this chapter, and chapter 6, to minimise PCR related errors that may arise if following a strategy of sequential PCR cloning from other constructs).

5.3 Expression of extracellular and Alkaline Phosphodiesterase 1 active site domains using pFLAG.CTS

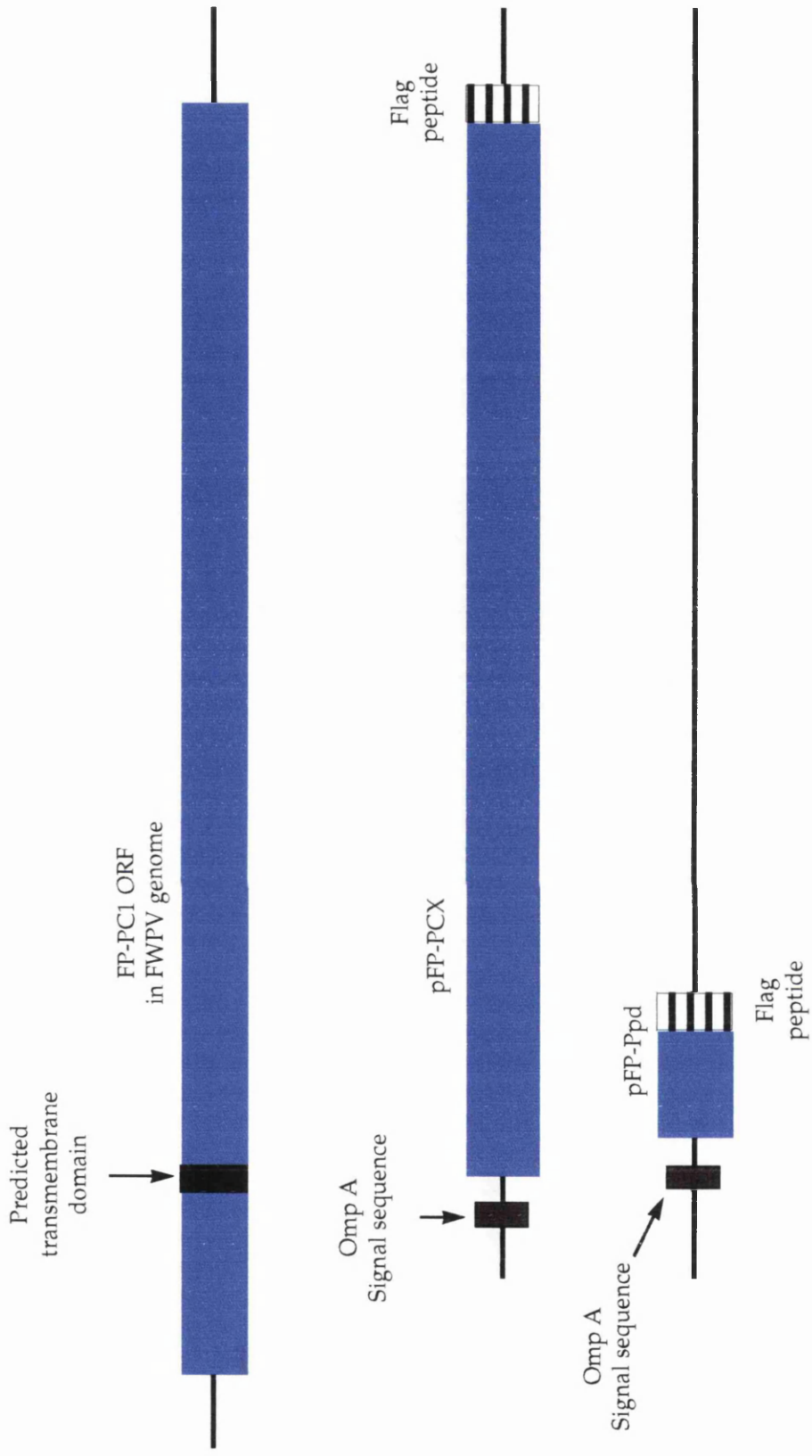
Bacterial cultures of clones identified as containing FP-PC1 inserts were grown up as 5ml overnight cultures. These were subsequently diluted into fresh LB broth and incubated until an OD₆₀₀ of 0.2 was reached, at which point expression was induced by addition of IPTG. After 2h, bacteria were pelleted by microcentrifugation and suspended directly in SDS-PAGE sample buffer. Flag-fusion proteins were screened by SDS-PAGE and detected by western blotting using the M2 anti-flag Mab. As shown in fig. 5.3, bands of sizes corresponding to both extracellular FP-PC1 (~90kDa band from clone SXL4) and PPD (~11kDa) domains were detected. However, there was evidence of significant protein degradation of the extracellular FP-PC1 protein, as indicated by the prominent degradation products at 30kDa and 14kDa (fig. 5.3), in contrast no such degradation was visible of the FP-PC1 PPD domain protein. No protein expression was detected upon screening of bacteria transformed only with pFLAG.CTS, suggesting that

Fig.5.1 Cloning strategy for expression of the extracellular and PPD domains of FP-PC1 in *E. coli*



The extracellular and alkaline phosphodiesterase 1 (Ppd 1) FP-PC1 domains were amplified directly from the FWPV genome using oligonucleotide primer pairs C/J and D/K respectively. Both amplicons were double digested with Xho 1 and Kpn 1 and ligated in to similarly the pre-digested flag vector - pFlag.CTS, leading to construction of pFP-PCX and PFP-Ppd expressing the extracellular and Ppd FP-PC1 domains respectively as flag fusion proteins.

Fig.5.2 Gene structure of FP-PC1 *E. coli* expression constructs



both the extracellular and PPD FP-PC1 domains had been successfully expressed in *E. coli*.

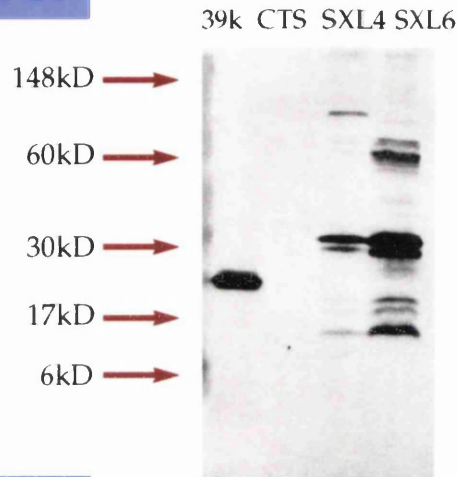
5.4 Analytical fractionation

In order to determine whether FP-PC1 flag-fusion domains fractionate to the periplasm, soluble whole cell fraction, insoluble whole cell fraction or the culture medium; overnight cultures were grown up and induced with IPTG as described previously. Samples representing each of the fractions described above were prepared as outlined in chapter 2. Initial analysis indicated that both the extracellular and PPD proteins fractionated only to the insoluble whole cell fraction or the whole cell fraction, with only small amounts of the latter protein in the periplasmic space (fig. 5.4 lower panel). The amount of protein fractionating to the periplasm was increased for both the extracellular FP-PC1 domain (fig. 5.4 upper panel) and the PPD domain (data not shown) by incubation and induction at 30°C. As the ease of purification from the periplasmic space was significantly easier, this fraction was chosen for further studies.

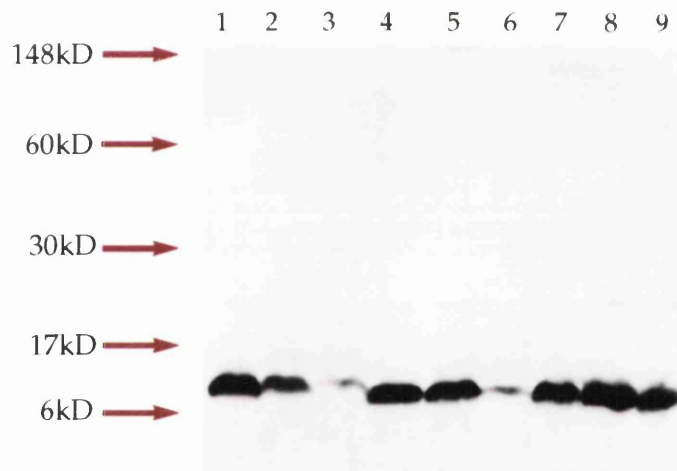
5.5 Optimisation of expression conditions

The yield of the FP-PC1 PPD domain protein in the periplasm appeared sufficient for purification, but conversely, the yield of the extracellular FP-PC1 domain protein appeared very low, probably as a consequence of low expression levels combined with extensive degradation

Extracellular FP-PC1

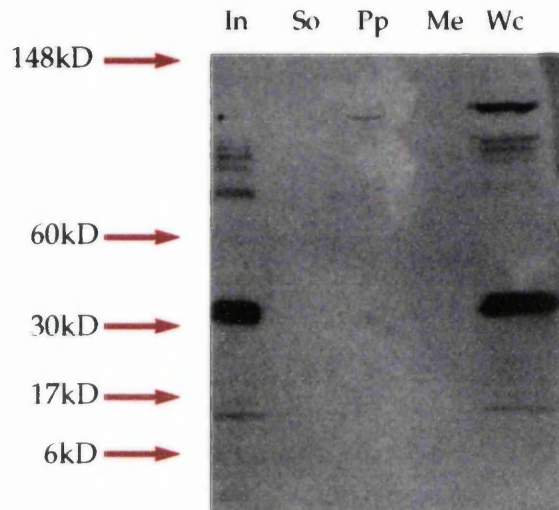


FP-PC1 PPD domain

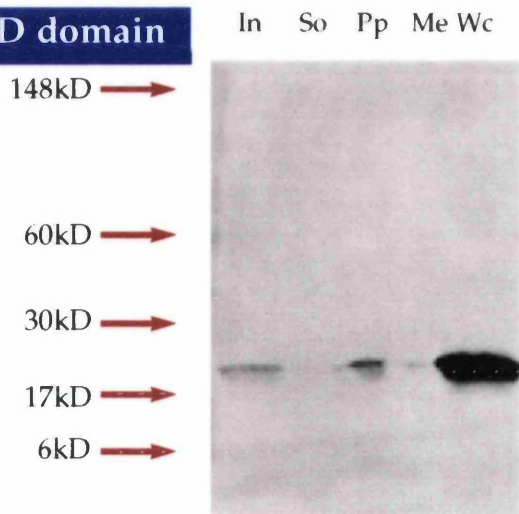


The FP-PC1 extracellular and PPD domains were cloned into p.flag.CTS to permit expression as flag fusion proteins. The resultant vectors, pFP-PCx and FP-PC.ppd, encoding the extracellular and PPD domains respectively, were transformed in to *E.coli* strain XL1blue and incubated overnight at 37°C in LB broth, supplemented with ampicillin at 100µg/ml. Bacteria were pelleted by microcentrifugation and pellets resuspended directly in SDS-PAGE sample buffer. Proteins were resolved by SDS-PAGE electrophoresis (10% gel) and immobilised on to a nitro-cellulose membrane by electrophoretic transfer. Epitope-antibody-conjugate complexes were detected by use of a western blot detection system based upon NBT/BCIP. The upper panel shows expression of the extracellular FP-PC1 domain (clones SLX4 and 6), the negative control was vector only (CTS) and the positive control was the central region of the FWPV-39kD protein. The lower panel shows expression of the FP-PC1 PPD domain from 9 independent clones.

Extracellular FP-PC1



FP-PC1 PPD domain



FP-PC1 extracellular and PPD domain flag fusion proteins were expressed in *E. coli* strain XL1 blue. In order to assess localisation of protein, analytical fractionation was performed as described previously (chapter 2). Samples from the insoluble (In), soluble (So), periplasmic space (Pp), culture medium (Me) and whole cell extracts (Wc) were prepared. Samples were then equivalently loaded and analysed by SDS-PAGE and western blotting as described in the legend to fig. 5.3.

of full length protein. In order to maximise the yield the following variables were used to determine the optimum conditions for expression:

Variable	Range
Temperature of incubation	25, 30 and 37°C
Length of induction in minutes	30, 60, 120 and 240
Final concentration of IPTG used during induction	0.005-10mM

5.6 Purification

Using the optimised conditions described above, immuno-affinity based purification of flag-fusion FP-PC1 proteins was attempted using “M2 affinity gel” (agarose bead slurry covalently linked to anti-flag M2 monoclonal antibody). Despite repeated purification attempts, neither the FP-PC1 PPD or extracellular domain proteins were detectable by immunoblotting.

5.7 Summary

The primary aim of using an *E. coli* based system for expression of the FP-PC1 extracellular and PPD domains was to attain large amounts of protein to be used for injection into rabbits for antibody production. It was clearly evident at an early stage that both the domains were being expressed at low levels, as even during initial fractionation analysis, neither protein was visible by coomassie blue staining. Despite optimisation of incubation and induction conditions, as well as attempts to express protein in different

strains of *E. coli* with varying protease deficient genotypes, no significant increase in yield was observed. The failure to attain high levels of expression of the FP-PC1 extracellular and PPD domains could have been improved by choosing a hydrophilic non-glycosylated FP-PC1 domain, which would have had a greater chance to get expressed at higher levels. Although different vectors could have been utilised to assess improvements in yield, prokaryotic expression was abandoned in favour of using eukaryotic expression systems to express various FP-PC1 domains as flag fusion proteins (chapter 6). By using this approach the need for FP-PC1 specific antibodies would be largely obviated as well as increasing the chances of expression of functional protein.

Chapter 6

Expression and analysis of FP-PC1 in Heterologous and Homologous Eukaryotic expression systems

6.1 Introduction

As described in detail in Chapter 3, the FP-PC1 ORF is predicted to express a N-linked transmembrane protein of approximately 94kDa, not including glycosylation. On the basis of hydrophobicity analysis, and comparison with related cellular PC1 genes, the protein is thought to traverse the plasma membrane only once, and has a type II orientation with a large extracellular catalytic domain and short cytoplasmic domain. No previous characterisation of the protein has been conducted.

In order to allow analysis and characterisation of FP-PC1, vectors were designed to allow expression in various eukaryotic expression systems. Initial key aims were based on confirmation of predicted protein attributes as described above, by expression of specific domains in a cell free coupled transcription/translation system (CTT) using rabbit reticulocyte lysates.

The second stage involved analysis of the FP-PC1 gene product in FWPV infected cells and COS-7 cells with the gene being expressed either from the FP-PC1 promoter or from the T7 bacteriophage promoter.

Finally, attempts were made to purify protein to enable functional analysis by means of enzymatic assays for activities associated with cellular PC1 gene family proteins – including autophosphorylation and 5' nucleotide phosphodiesterase activity.

6.2 Introductory summary of FP-PC1 expression constructs

A total of four different vectors were constructed for analysis and characterisation of FP-PC1 in eukaryotic systems. Cloning steps and constructs are described in detail in later sections and are shown in figs. 6.1 and 6.2. Briefly, pFP-PC1.fg and pT-PC1 contain full length FP-PC1 driven by the native FP-PC1 promoter or the T7 promoter respectively, and pSHT-PC1 and pb-PC1 contain FP-PC1 sequence corresponding to the extracellular domain, lacking both the short cytoplasmic and transmembrane domains. In addition pSHT-PC1 also contains a 5' artificial influenza haemagglutinin signal sequence to allow secretion of translated protein. All vectors were designed to incorporate a flag tag peptide at the 3' end to allow prospective immunoblotting, immunoprecipitation, localisation and purification studies.

6.3 Cloning Strategy

All three constructs used in the CTT system expressed FP-PC1 from the T7 promoter (fig. 6.2), which was chosen to allow high level expression in other homologous and heterologous systems, as well as the CTT system. The pFTM1 derived vector pFTM2, is primarily designed for high level expression of proteins from recombinant FWPV infected CEFs co-infected with rFWPV-T7. For secretion of extracellular PC1, the pSHT vector was used, which is specifically designed to allow expression and secretion of distinct protein domains (Madison & Bird, 1992) and is derived from a fusion between pSVT7 and pUC119 (Vieira & Messing, 1987). For expression of a non-secreted extracellular PC1 protein pBluescript KS⁻ was chosen.

The cloning strategy for all eukaryotic expression constructs relied on clones constructed for use in Transient Dominant Selection and prokaryotic expression studies (Chapter 4 and 5 respectively). The whole extracellular region of PC1 was amplified by PCR from pFP-PCX leading to addition of Bam H1 restriction enzyme sites at both the 5' and 3' ends. This PCR product and pFP-PC1 were digested with Bam H1 and Sac 2 and ligated to form pFP.PC1.fg, containing a full length FP-PC1 ORF with a 5' flag tag. This vector was used to generate a recombinant FWPV expressing a flag-tagged full length FP-PC1 gene. Full length tagged FP-PC1 was amplified by PCR from pFP.PC1.fg leading to addition of a Bam H1 site at the 5' end in addition to the existing Bam H1 site at the 3' end. This PCR product was cloned into pFTM2, by digestion of pFTM2 and the amplicon with Bam H1, and ligated using T4 DNA ligase. The resulting full length FP-PC1 construct was used for CTT reactions and over-expression of FP-PC1 in infected cells from the T7 promoter. In order to clone constructs containing extracellular FP-PC1, pFP-PCX was again used to generate an amplicon by PCR containing extracellular FP-PC1 with Bam H1 sites at both ends, this was cloned into pSHT by use of the Bam H1 cloning site to generate pSHT-PC1, to permit secretion of the extracellular domain of FP-PC1 in CTT studies and transient expression in COS-7 cells. In order to generate a T7 driven, non-secreted extracellular protein, extracellular FP-PC1 was amplified from pSHT-PC1, digested with Bam H1 and cloned into Bam H1-linearised pBluescript KS⁻, leading to the construction of pB-PC1.

6.4 Expression of secreted FP-PC1 in COS-7 cells

In order to express soluble protein primarily for analysis of enzymatic activity, but which would also be easy to purify, the extracellular

region of FP-PC1 lacking the cytoplasmic and transmembrane domains was cloned into pSHT, resulting in pSHT-PC1. The pSHT vector has been specifically designed for the expression and secretion of protein domains mediated through an artificial influenza haemagglutinin signal sequence (Madison & Bird, 1992).

For high level expression of secreted protein, pSHT-PC1 was transfected into COS-7 cells using DEAE-dextran and chloroquine. Three hours post transfection, the transfection mix was discarded and cells washed with DMSO before being incubated in serum plus MEM overnight, followed by two consecutive 72 hour incubations in serum free MEM. The supernatant from both these incubations was pooled and FP-PC1 expression analysed by immunoblotting and radioimmunoprecipitation as described previously. No protein was detectable by either of these methods. In order to confirm expression and to optimise transfection conditions, the vector pSV2-Cat was used as a reporter of transfection efficiency and protein expression by quantification of Chloramphenicolacetyltransferase (CAT). The following variables were used for optimisation:

Variable	Range
Length of time transfection mix left on cells	25 min. to 125 mins.
Amount of DNA used in transfection	0µg to 100µg
Concentration of DEAE-dextran in transfection mix	25µg/ml to 4000µg/ml

Fig.6.1 Cloning strategy for expression of FP-PC1

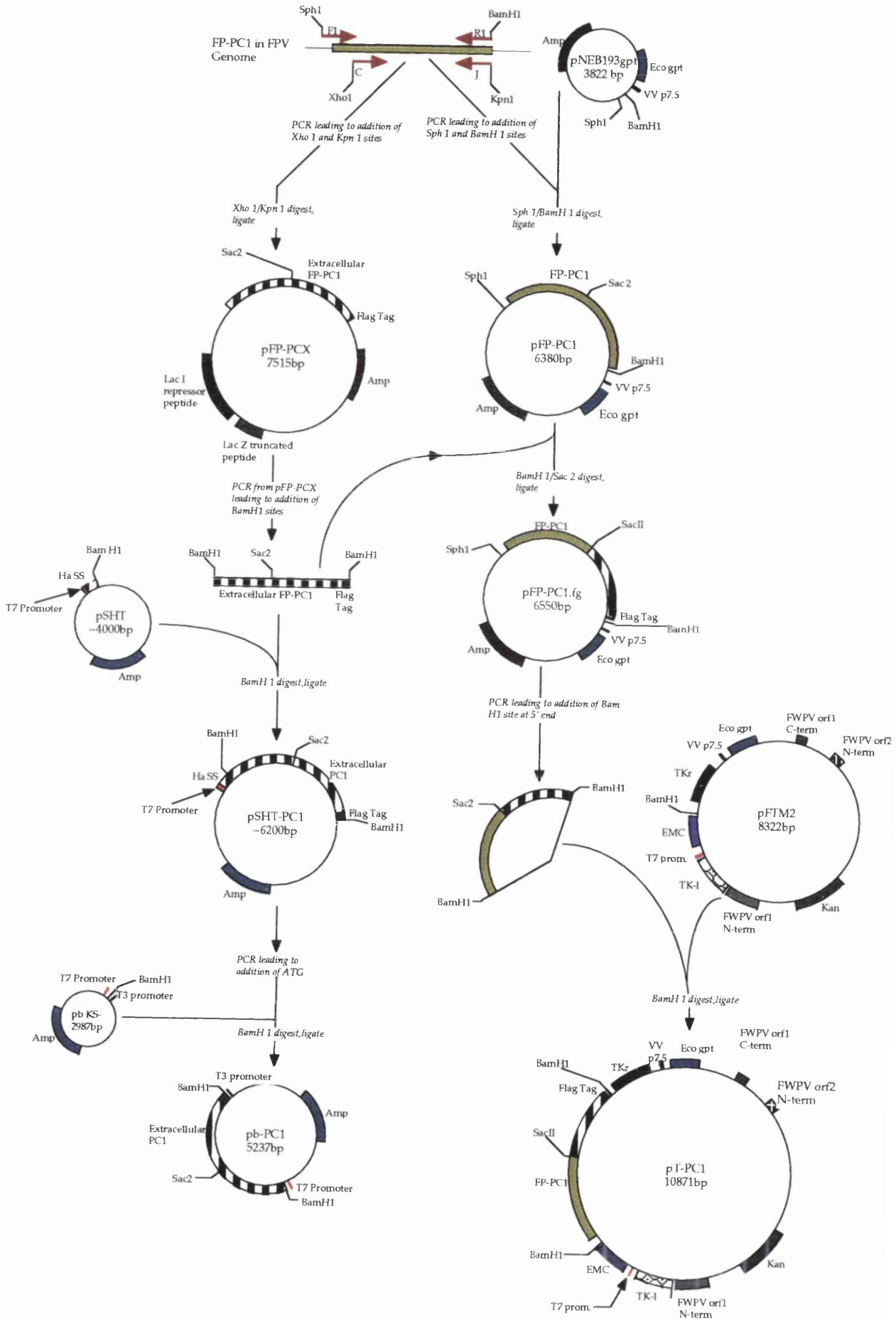
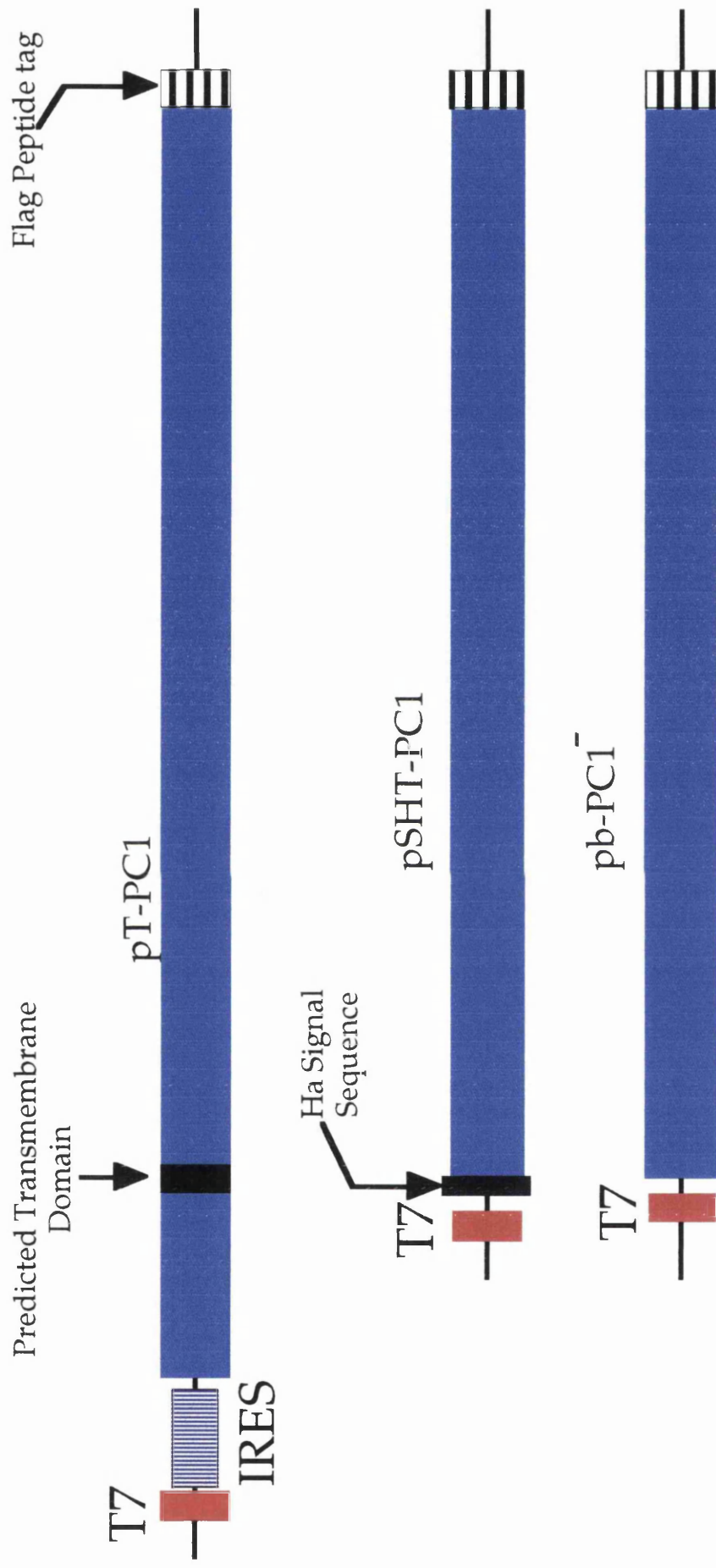


Fig.6.2 Gene structure of T7 promoter mediated FP-PC1 expression constructs



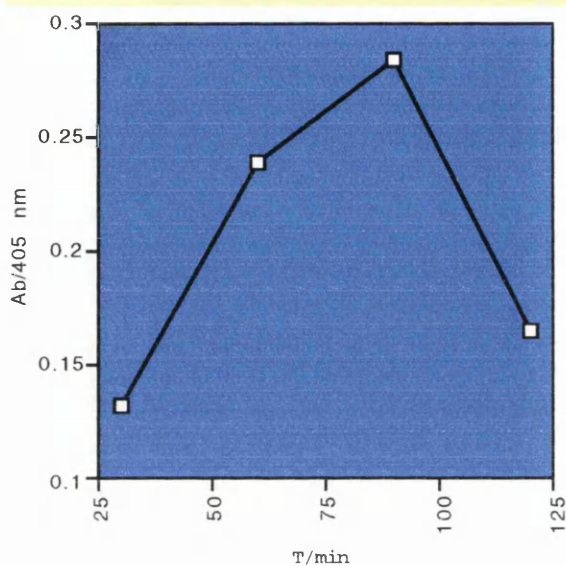
The combination of transfection conditions that gave the highest levels of CAT expression, were 25µg plasmid DNA, 300µg of DEAE-dextran in transfection mixtures left on cells for 75mins (fig. 6.3). Transfection with pSHT-PC1 was repeated alongside with pSV2-Cat using the optimised conditions described above. Although CAT expression was detected, no FP-PC1 protein was detectable by radioimmunoprecipitation or immunoblotting (data not shown).

6.5 Expression of full length and extracellular FP-PC1 in a cell free coupled transcription/translation system

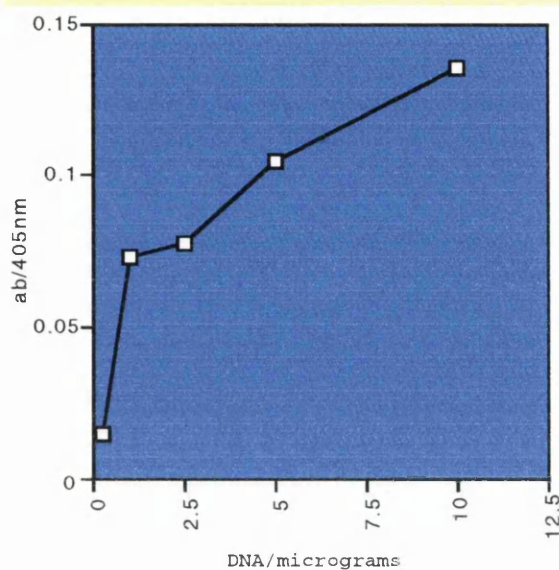
In order to confirm expression and reading frame integrity of the FP-PC1 ORF, both the extracellular and full length proteins were successfully expressed from pSHT-PC1 and pT-PC1 respectively, in a cell free coupled transcription/translation system utilising rabbit reticulocyte lysates and T7 RNA polymerase in the presence of [³⁵S]-methionine. Protein samples were visualised by SDS-PAGE followed by autoradiography. As shown in fig. 6.3, pT-PC1 and pSHT-PC1 each produced a major protein, of approximately 95kDa and 85kDa respectively (lanes 41-m and sht-m), representing good correlations with the predicted sizes of full length and extracellular FP-PC1. The positive control, which contained a copy of the FWPV-Cel 1 gene expressed from pFTM2, showed a band of 28kDa as expected, and no other proteins, therefore suggesting that the products in lanes 41-m and sht-m were FP-PC1 gene products.

Fig.6.3 Optimisation of DEAE-dextran based transfection conditions of COS-7 cells using CAT as a reporter

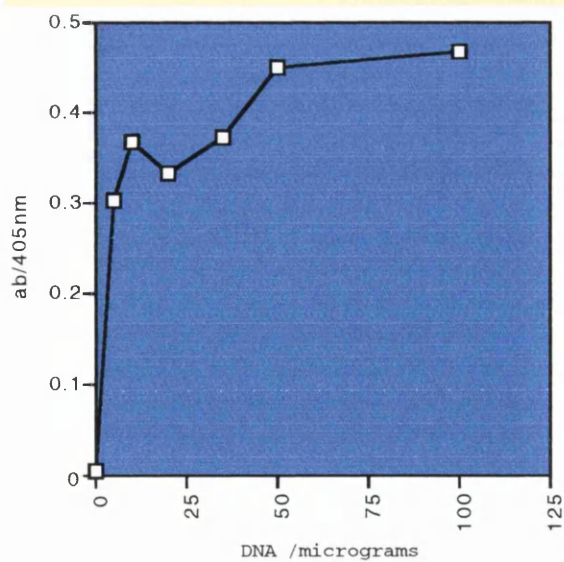
I. Effect of length of time on levels of CAT



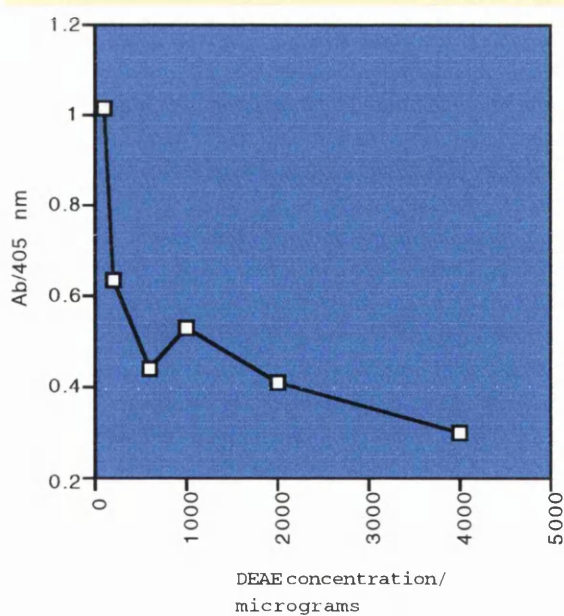
II. Effect of amount of DNA on levels of CAT



III. Effect of amount of DNA on levels of CAT



IV. Effect of DEAE conc. on levels of CAT



Sub-confluent COS-7 cells were transfected with the vector pSV2-Cat. In order to determine the combination of transfection conditions that gave the highest levels of CAT, the variables as shown above were evaluated.

6.6 Effect of presence of canine pancreatic membranes on gene products expressed in the CTT system

In order to investigate co-translational and post-translational processing events such as signal peptide cleavage, membrane insertion, translocation and core glycosylation, full length and extracellular FP-PC1 were expressed in the CTT system in the presence of canine pancreatic microsomal membrane vesicles – hereafter referred to as “membranes”. As evident from fig. 6.4 the size of both full length FP-PC1 and extracellular PC1 increases in the presence of membranes from approximately 95kDa and 85kDa, to 105kDa and 95kDa respectively (lanes 41+m and SHT+m compared with lanes 41-m and SHT-m). This indicated that both proteins are probably translocated and glycosylated. Further evidence supporting this is shown in fig. 6.5, where extracellular FP-PC1 expressed from pb-PC1, which lacks both the transmembrane domain and the influenza haemagglutinin signal sequence, showed no increase in size when expressed in the presence of membranes. This form of unmodified extracellular FP-PC1 has no signal sequence, and would therefore not be expected to traverse the microsome vesicle lipid bilayer. In addition glycosylation can only occur inside intact vesicles, therefore it is likely that the increase in size of modified extracellular and full length FP-PC1 represents core glycosylation occurring inside the lumen of membranes.

6.7 Radioimmunoprecipitation of flag and histidine tagged proteins expressed in the CTT system

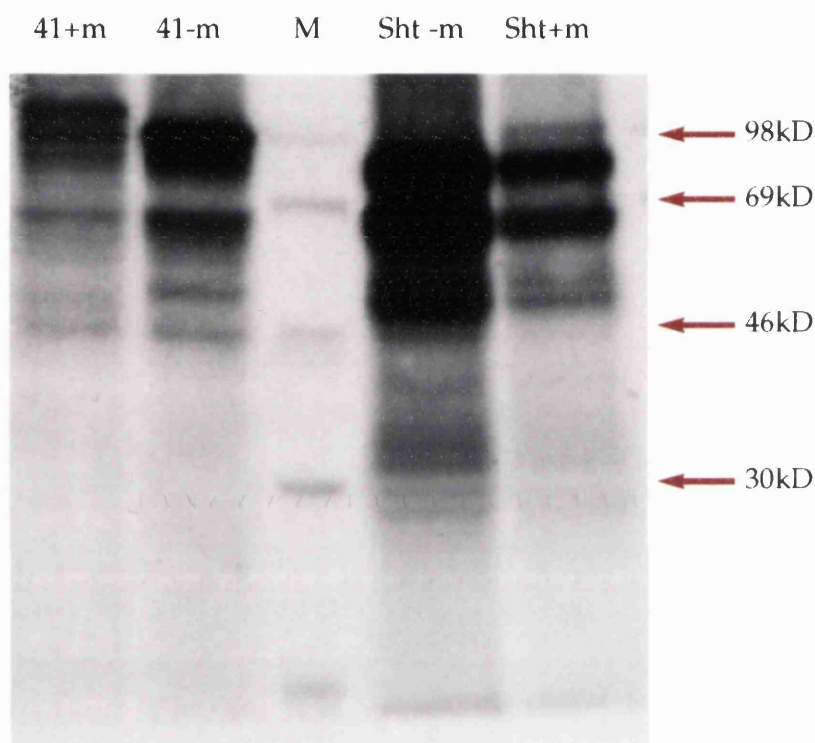
An important consideration was confirmation of expression of the 3' flag tag peptide in pb-PC1, pT.PC1 and pSHT-PC1. This was particularly

critical as the latter two constructs were to be used in expression in FWPV infected cells and COS-7 cells, as well as the CTT system. Histidine tagged IBDV protein VP1 and flag-tagged FWPV-Cel1, both expressed from pFTM2, were used as controls in radioimmunoprecipitation experiments. All five of the aforementioned proteins were synthesised in the CTT system and radioimmunoprecipitated using both flag and histidine monoclonal antibodies, unexpectedly all proteins were precipitated by both flag and histidine monoclonal antibodies (data not shown), indicating that specific immunoprecipitation was not occurring. This result was not surprising considering that unlike the situation in cellular expression systems, cell free expression from the T7 promoter results in almost exclusive expression of the target protein, hence antibody specificity is undermined due to the lack of any competitive binding from other proteins. In order to decrease this non-specific binding, radioimmunoprecipitation was repeated but antibody bound sepharose beads were preadsorbed with non-labelled CEF cell lysates, before addition of labelled CTT translated protein. As a consequence all proteins were preferentially precipitated by their respective tag specific monoclonal antibodies (fig. 6.6). For example, as seen in lanes VP1 F and H, and lanes 41 F and H; VP1 is preferentially immunoprecipitated by the histidine tag, and clone 41 of FP-PC1 by the flag tag. This indicated that the flag tag peptide was expressed and could be used for immunodetection.

6.8 Analysis of the translocation and topology of FP-PC1 by protease protection assay

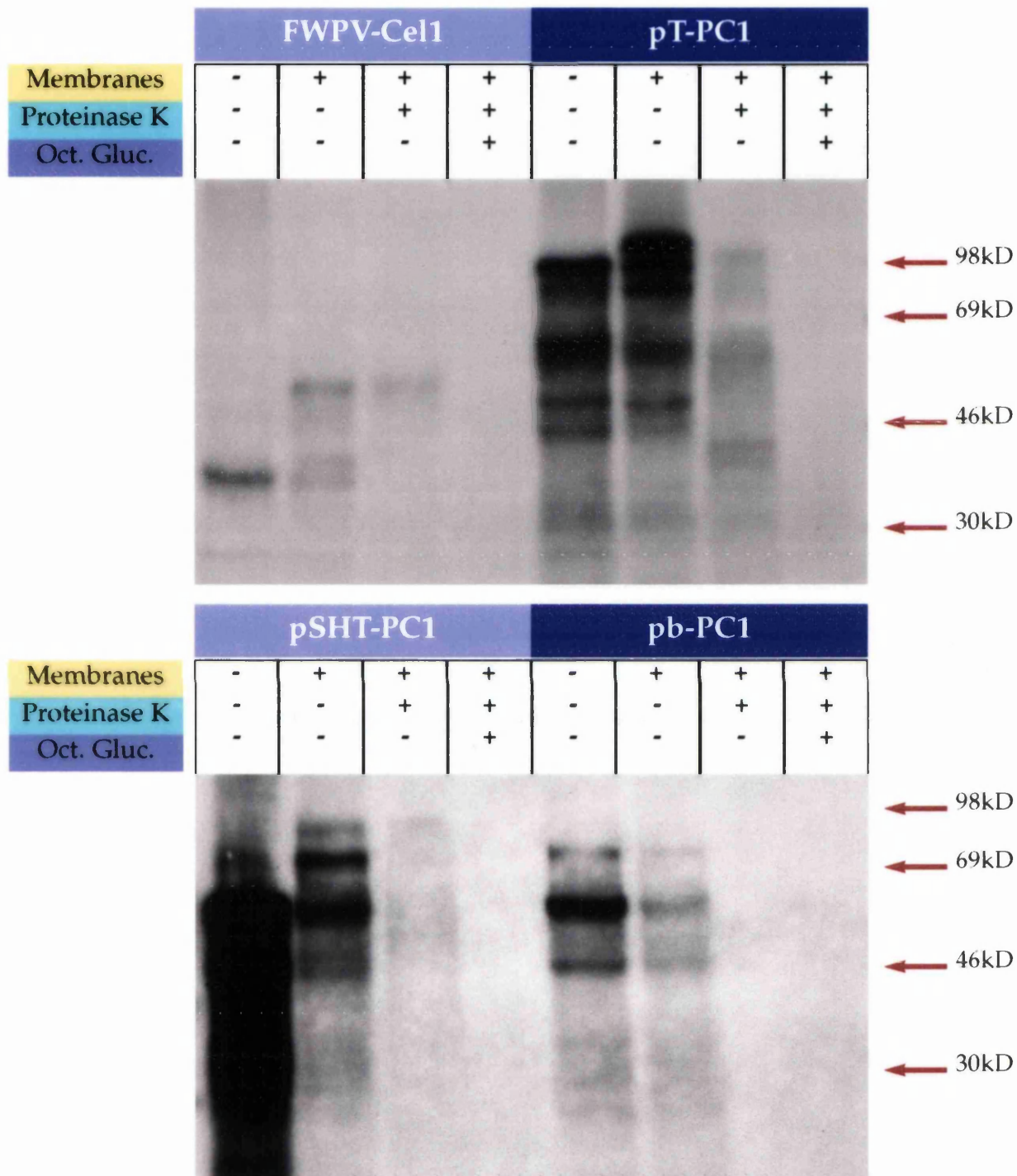
Protease protection assays of proteins expressed in the CTT system with and without the presence of membranes were performed broadly as

Fig.6.4 Effect of membranes on full length and extracellular FP-PC1 expressed in a cell free, *in vitro* coupled transcription/translation system



Full length FP-PC1 from pFTM2 clone 41 and extracellular FP-PC1 from pSHT were expressed in the CTT system (Promega) in a total volume of 25 μ l, by incubation for 1h at 30 degrees c with or without membranes (+m and -m respectively) in the presence of [³⁵S]-methionine. Protein samples were prepared by addition of 4 volumes of SDS-PAGE sample buffer to translation mixtures, followed by boiling for 2 minutes. Proteins were resolved by SDS-PAGE (12.5% gel) and detected by fluorography followed by autoradiography.

Fig.6.5 Protease protection assay of full length and extracellular FP-PC1 expressed in the CTT system



The genes described above were expressed in the CTT system in a total volume of 25 μ l, by incubation for 1h at 30 degrees c with or without membranes in the presence of [³⁵S]-methionine. Samples translated +m were split into three 8 μ l aliquots and treated as described above. Protein samples were prepared by addition of 4 volumes of SDS-PAGE sample buffer to translation mixtures, followed by boiling for 2 minutes. Proteins were resolved by SDS-PAGE (12.5% gel) and detected by fluorography followed by autoradiography.

described previously (Seal *et al.*, 1995). Briefly, each protein was translated in the CTT system with and without membranes, the latter sample being stored before analysis. The former sample was split into three aliquots, which were treated as described below:

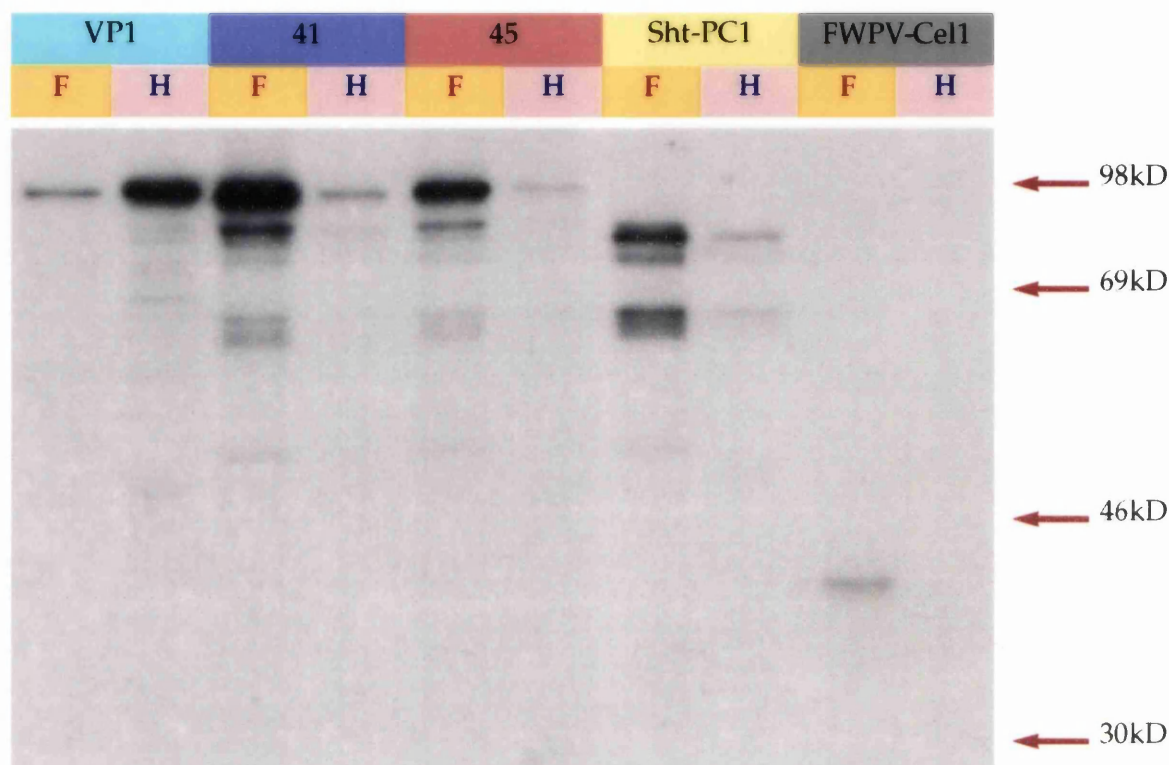
Sample 1 – Control, treated with 10mM CaCl₂ only

Sample 2 – Proteinase K (PK) only in 10mM CaCl₂

Sample 3 – PK and Octylβ-D-glucopyranoside in 10mM CaCl₂

As shown in figure 6.5, key structural information about FP-PC1 was revealed from the combination of the treatments outlined above. FWPV-Cel1 was shown to behave as a model secreted protein, as its size increases in the presence of membranes and a significant proportion of the full length protein is protected from PK digestion. Both full length and extracellular FP-PC1, expressed from pT-PC1 and pSHT-PC1 respectively, increased in size in the presence of membranes as shown previously, and like FWPV-Cel1, were partially protected from PK treatment. Compared to FWPV-Cel1, the key difference was that the size of the protected, full length FP-PC1 is reduced upon PK treatment, whereas no such reduction occurred with extracellular FP-PC1. This indicated that both full length and extracellular FP-PC1 (expressed from pSHT) were translocated across vesicular membranes, with full length FP-PC1 remaining anchored at the membrane with a only short portion of the protein remaining outside, whereas extracellular FP-PC1 is internalised into the lumen and hence

Fig.6.6 Radioimmunoprecipitation of CTT translation products using flag and histidine monoclonal antibodies



F Samples immunoprecipitated with M2 anti flag monoclonal antibody

H Samples immunoprecipitated with 6 x Histidine monoclonal antibody

Flag tagged full length FP-PC1 from pFTM2 clones 41 and 45, extracellular FP-PC1 from pSHT, and FWPV-Cel 1 from pFTM2; as well as histidine tagged IBDV VP1 from pFTM2 were expressed in the CTT system in a total volume of 25 μ l, by incubation for 1h at 30 degrees c without membranes, in the presence of [³⁵S]-methionine. Samples were divided into two equal aliquots and added to protein A sepharose CL4B beads, sequentially pre-adsorbed with unlabelled CEF cell lysates and either M2 flag or histidine monoclonal antibodies. Labelled CTT translated proteins were adsorbed to both sets of beads for 4h at 4 degrees c and washed 5 times in RIPA buffer 1 and two times in RIPA buffer 1. Proteins were eluted from beads by boiling for 2 mins., and samples prepared by addition of an equal volume of SDS-PAGE sample buffer to translation mixtures, followed by boiling for 2 minutes. Proteins were resolved by SDS-PAGE (12.5% gel) and detected by fluorography followed by autoradiography.

shows no change in size upon PK treatment. When extracellular FP-PC1 was expressed without a signal sequence from pB-PC1, there was no increase in size in the presence of membranes and the protein exhibited no protection upon PK treatment, indicative of the protein not being translocated or glycosylated, and therefore remaining outside vesicles and susceptible to PK mediated proteolysis.

In order to demonstrate that the protection from PK digestion was due to internalisation in membrane vesicles, full length and extracellular FP-PC1 were expressed in the CTT system in the presence of membranes, and treated with PK and Octyl β -D-glucopyranoside, which disrupts the lipid bilayer of microsomal membranes. In the presence of this detergent, proteins which were otherwise partially protected, were completely digested by PK. This confirmed that protease protection was as a result of translocation into membranes.

6.9 Expression of FP-PC1 in FWPV infected cells

6.9.1 Expression of full length FP-PC1 from the native promoter

In order to analyse expression and localisation of FP-PC1 in infected cells, a recombinant FWPV was constructed by TDS. The transfer vector pFP.PC1.fg which contains a full length copy of the FP-PC1 ORF with a 3' terminal flag tag peptide sequence was transfected into FWPV infected CEFs. Viruses incorporating the flag-tagged copy of FP-PC1 due to homologous recombination with pFP.PC1.fg were selected by growth in mycophenolic acid as described in Chapter 4, resulting in isolation of rFWPV-PC1.fg. Despite attempts to detect expression by western blotting

and radioimmunoprecipitation utilising the flag tag peptide monoclonal antibody M2, no protein was detected (data not shown). In order to proceed with analysis of FP-PC1 protein in infected cells expression was attempted by replacing the native promoter with the T7 bacteriophage promoter to facilitate higher level expression.

6.9.2 Expression and analysis of full length FP-PC1 expressed from the T7 promoter

In order to express FP-PC1 in FWPV infected cells, full length FP-PC1 with a flag tag peptide sequence at the 3' end was cloned as described in section 6.3 into the vector pFTM2, which contains an encephalomyocarditis virus 5' UTR sequence downstream of a T7 promoter sequence to allow cap independent translation.

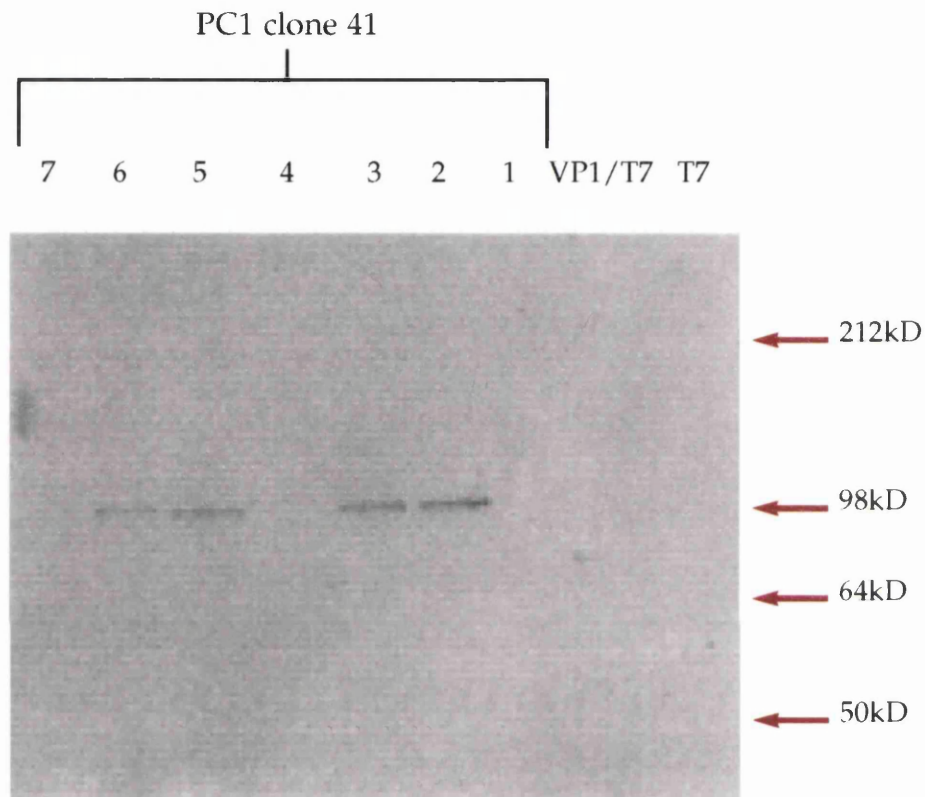
6.9.2.1 FP-PC1 is detected in FWPV infected cells when overexpressed from the T7 promoter

Initial analysis of T7 promoter mediated transient expression of FP-PC1 involved transfection of pT-PC1 into CEF monolayers infected with a recombinant FWPV expressing T7 RNA polymerase (rFWPV-T7). No expression of FP-PC1 was detected in these transient expression studies by immunoblotting or radioimmunoprecipitation. In order to get higher levels of stable expression, a recombinant FWPV, rFWPV-T7PC1, expressing a T7 promoter controlled full length FP-PC1 protein with a flag tag peptide at the 3' terminus. Construction was by TDS as described

previously, except that virions were maintained at 1st crossover stage by passage and amplification in the presence of mycophenolic acid to prevent resolution of intermediate GPT⁺ virions to GPT⁻. This strategy was employed as resolution could result in a FP-PC1 gene with a T7 promoter but no flag epitope, or a FP-PC1 gene with a flag epitope but an absent T7 promoter. Such an eventuality would result in either an undetectable FP-PC1 gene product or one expressed at low levels.

To take account of the possible complications described above, 20 individual clones of pT-PC1 were transfected into FWPV infected CEFs, and recombinants selected on the basis of GPT expression as described previously. After 3 plaque purifications in the presence of mycophenolic acid (MPA) each clonal recombinant virion was amplified in the presence of MPA. Harvesting of virus was modified to ensure no MPA contamination in subsequent co-infections involving the GPT⁻ virus rFWPV-T7, by washing of CEF monolayers twice in PBS before harvesting of virus in PBS by repeated freeze thaw cycles. CEF monolayers were then co-infected at an m.o.i. of 10 with rFWPV-T7 and rFWPV-T7PC1, 24h p.i. protein samples were harvested by discarding the existing supernatant, replacing with PBS and freeze thawing 3 times. Proteins were resolved by SDS-PAGE and visualised by immunoblotting using the M2 monoclonal antibody to the flag peptide. As shown in fig. 6.7 a single band of around 105kDa was present in samples prepared from cells infected with both rFWPV-T7 and rFWPV-T7PC1 (lanes 2,3,5 and 6) whereas no band was present when cells were infected with only rFWPV-T7 or rFWPV-T7 and rFWPV-T7VP1. This supported the conclusion that the 105kDa band, seen previously in expression experiments using the CTT system + membranes did represent full length FP-PC1.

Fig.6.7 Analysis of Flag tagged FP-PC1 expressed from the T7 promoter in FPV infected cells by immunoblotting

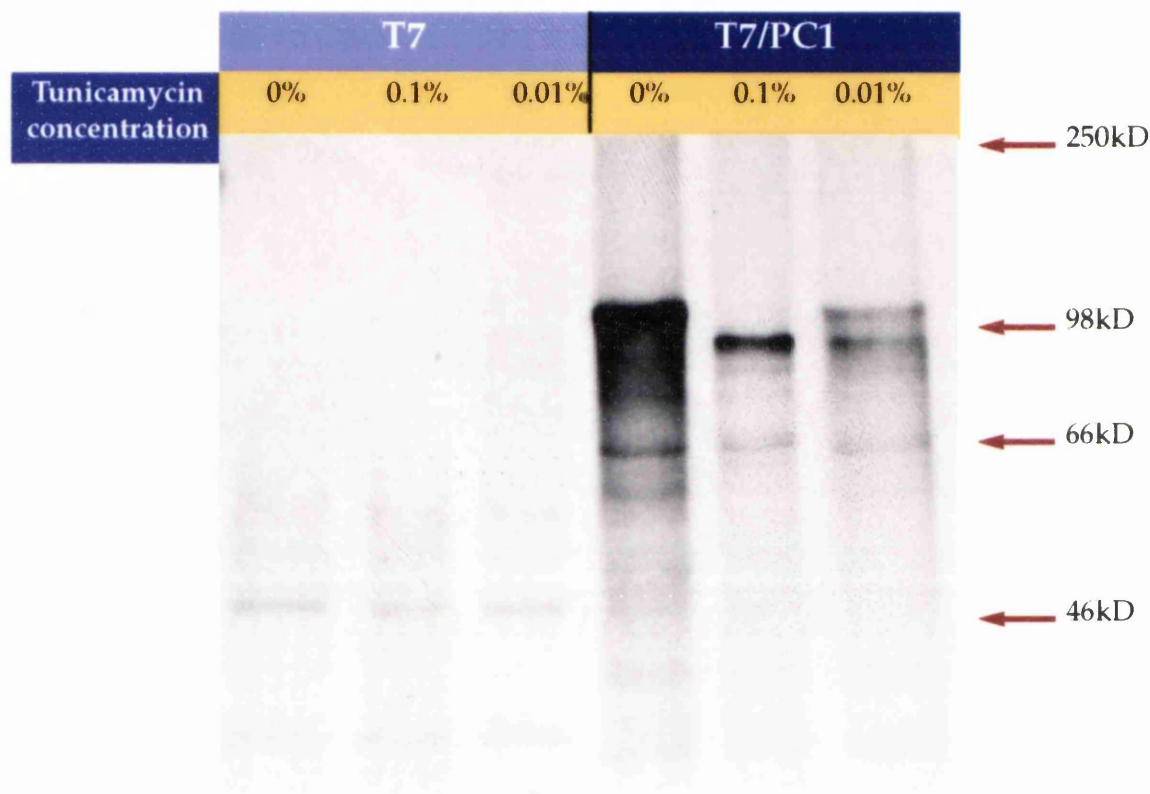


CEF monolayers were co-infected at a cumulative m.o.i of 20 with 7 different clonal viruses representing rFWPV-T7PC1, and rFWPV-T7 (lanes 1-7), or rFWPV-T7 and rFWPV-T7VP1 (lane VP1/T7) or rFPV-T7 only (lane T7) as a negative control. 24 h p.i., the growth medium/inoculum was discarded and monolayers washed in PBS, before preparation of protein samples by 2 freeze thaw cycles in PBSa. Proteins were resolved by SDS-PAGE electrophoresis (10% gel) and immobilised on to a nitro-cellulose membrane by electrophoretic transfer. Epitope-antibody-conjugate complexes were detected by use of a western blot detection system based upon NBT/BCIP. Only cells co-infected with rFWPV-T7PC1 and rFWPV-T7 were visible by immunoblotting.

6.9.2.2 Analysis of glycosylation by radioimmunoprecipitation from infected cells

As indicated by sequence analysis, FP-PC1 is predicted to have 6 possible N-linked glycosylation sites. In order to corroborate results suggesting glycosylation of FP-PC1 expressed in the CTT system, as well as to confirm the expression and detection based of the flag epitope, radioimmunoprecipitation from co-infected cells treated with different concentrations of tunicamycin was carried out. CEF monolayers were co-infected with either rFWPV-T7 and rFWPV-T7PC1, or with rFWPV-T7 and rFWPV-T7VP1, at an m.o.i. per virus. The viral inoculum was then removed and replaced with fresh medium. At 22h p.i., cells were washed and methionine-free medium containing tunicamycin at final concentrations of 0%, 0.1% or 0.01%, was added for 2h, before addition of fresh methionine free medium containing the same concentrations of tunicamycin and 100 μ Ci of [³⁵S]-methionine. After 2h of labelling, cells were washed and labelled proteins harvested by resuspending cells in RIPA 1 buffer and PMSF. Samples were then immunoprecipitated as described previously and visualised by SDS-PAGE followed by autoradiography. It is evident that full length FP-PC1 is glycosylated, as treatment with 0.1% tunicamycin leads to a reduction in size from 105kDa to 95kDa when compared to untreated protein (fig. 6.8). Treatment with 0.01% tunicamycin leads only to partial deglycosylation, as both the 95kDa and 105kDa proteins are immunoprecipitated.

Fig.6.8 Effect of Tunicamycin treatment of CEFs infected with FWPV expressing FP-PC1 from the T7 promoter



CEF monolayers were co-infected at a cumulative m.o.i of 20 with rFWPV-T7PC1 and rFWPV-T7 (lanes T7/PC1), or rFWPV-T7 only (lanes T7). 24 hours p.i. the growth medium was discarded and replaced with methionine free medium containing tunicamycin at a final concentration of 0%, 0.1% or 0.01% as shown above. After 2h, fresh medium containing the same concentrations of tunicamycin and 100 μ Ci of [³⁵S]-methionine was added and left on cells for 2h, after which samples were analysed by radioimmunoprecipitation followed by SDS-PAGE (10% gel) and autoradiography.

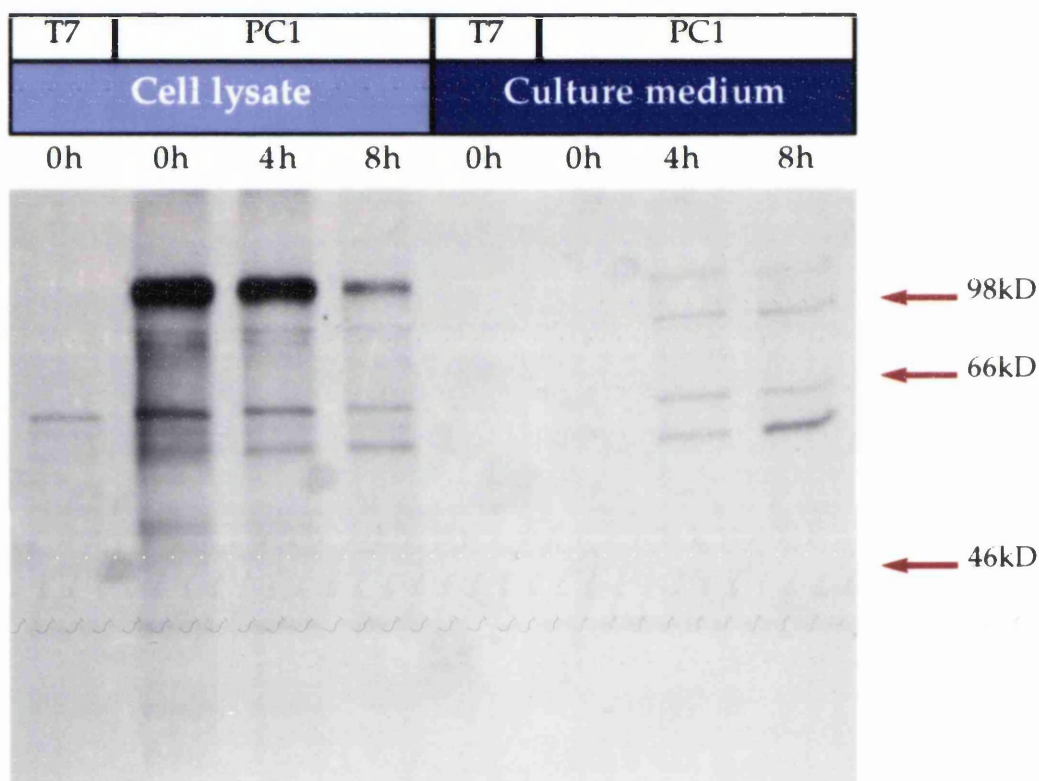
6.9.2.3 Localisation of FP-PC1 by Immunofluorescence

A key tool in the functional analysis of any novel protein is its sub-cellular localisation. In order to investigate the localisation of FP-PC1, CEF monolayers propagated on chamber slides were infected with either rFWPV-T7 and rFWPV-T7PC1, or only rFWPV-T7, with uninfected cells as a negative control. Twenty hours p.i., the inoculum was removed and monolayers washed with PBS and air dried. Cells were then fixed in methanol and incubated with either the M2 flag Mab, or the p65K FWPV Mab, with no antibody as a further control. After three washes in PBS a secondary antibody conjugated to fluorescein was added and incubated for 30mins before cells were dried and fluorescence detected by light microscopy. Initial analysis indicated that FP-PC1 localised to perinuclear regions and the plasma membrane (data not shown).

6.9.2.4 Biosynthesis and processing of FP-PC1 in infected cells

In order to assess biosynthesis and processing of FP-PC1 protein in infected cells, CEF monolayers were co-infected with either rFWPV-T7 and rFWPV-T7PC1 or rFWPV-T7 only, at a final cumulative m.o.i. of 20. After 24h cells were starved of methionine for 2h and followed by pulse labelling with [³⁵S]-methionine for 2h as described in section 6.9.2.2. Samples were then chased for 0, 4 or 8 hours with unlabelled methionine. Labelled protein was analysed by radioimmunoprecipitation from the culture medium and cell lysates. Analysis of intracellular and extracellular protein indicates that the vast majority of the protein is not secreted and remains cell associated (fig. 6.9) with the major translated product at around 105kDa probably representing full length glycosylated FP-PC1. Turnover of

Fig. 6.9 Analysis of biosynthesis of FP-PC1 by pulse-chase analysis of protein expressed in FWPV infected CEFs from the T7 promoter



CEF monolayers were co-infected at a cumulative m.o.i of 20 with rFWPV-T7PC1 and rFWPV-T7 (lanes PC1), or rFWPV-T7 only (lanes T7). 24 hours p.i. the growth medium was discarded and replaced with methionine free medium for 2h. This was in turn replaced with fresh medium containing 100 μ Ci of [35 S]-methionine for 2h, after which unlabelled methionine was added and left for a further 0, 4 and 8h. Samples were analysed from the culture medium and cell lysates by radioimmunoprecipitation followed by SDS-PAGE (10% gel) and autoradiography.

the labelled FP-PC1 protein can be observed as the amount of the 105kDa product decreases between 0 and 8 hours, and products at 50kDa and 95kDa disappear at 8 hours. In addition a band present at 60kDa becomes progressively sharper from 0 to 8 hours. Some minor products of lower mass are present but these probably represent either degradation, as they are not present at 0 hours, whereas minor bands that are present at 0h are probably as a result of internal initiation. No protein was detected in the extracellular sample until 4 and 8 hours, at both these time points the major product sizes were 105, 90, 65 and 60kDa, with the latter being the only product to exhibit an increase in abundance between 4 and 8 hours. This result suggests that some full length and other smaller processed protein products are being secreted, but the possibility that detected protein is due to cleavage of the extracellular C-terminal domain cannot be ruled out. The 60kDa FP-PC1 protein seen in the medium may be the only real secreted product. It is conceivable that the 60kDa protein, although only a minor product, may be cleaved by a specific mechanism and may play a specific role in virus biology, though it is clearly non-essential for replication *in vitro*.

6.10 Summary and Discussion

By utilisation of different expression systems with specific attributes, key structural and functional characteristics of FP-PC1 have been revealed. Earlier attempts to express the domains corresponding to the extracellular and 5' nucleotide phosphodiesterase domains in *E. coli* for production of protein to be used in antibody production were unsuccessful (chapter 5). Although protein was detected, the yield was insufficient to produce an effective response if injected into rabbits. In order to counter

this situation, all subsequent systems adopted incorporation of the octapeptide flag tag as part of the cloning strategy, therefore largely obviating the need for FP-PC1 specific antibodies.

Expression of full length FP-PC1 from the native promoter in rFWPV-PC1.fg was attempted but failed, possibly due to PCR-mediated cloning errors or mutations which may have lead to either non-expression of FP-PC1, or a frameshift error leading to a failure to express the flag peptide. Alternatively, the levels of expression might have been below the level of detection with the flag antibody.

In order to exclude the possibility of PCR related errors leading to problems in expression or detection of FP-PC1 from this, and all other constructs to be used, the reading frame and expression of the flag peptide was successfully confirmed, by expression of the predicted protein and specific radioimmunoprecipitation in the CTT system. This result suggested that problems encountered in detection of expression in COS-7 cells and from the native FP-PC1 promoter in FWPV were probably not as a result of an incorrect reading frame or absence of flag peptide expression. But rather due to the levels of expression being below detectable limits.

Aside from verification of vectors used in other expression systems, the CTT system also allowed analysis of both the extracellular and full length FP-PC1 proteins. The CTT system has been used in conjunction with canine pancreatic microsome vesicles to reveal membrane topology of many diverse membrane and secreted proteins including ionotropic glutamate receptor 1α , adenovirus E3 and human Jaw 1 (Behrens *et al.*, 1996; Krajcsi *et al.*, 1992; Seal *et al.*, 1995). In addition, core co-translational and post-translational glycosylation (Bulleid & Freedman, 1990), signal peptide cleavage, proteolytic processing, biosynthesis and oligomerisation

status have also been characterised (Chapdelaine *et al.*, 1985; Joseph *et al.*, 1997; Nash & Tate, 1984).

As described earlier, the FP-PC1 gene has only recently been identified (Laidlaw *et al.*, 1998) and the protein was previously uncharacterised. By use of the combination of FP-PC1 vectors encoding full length [tm+], extracellular [tm-] and extracellular [tm-, influenza haemagglutinin signal sequence+] RNA templates, many of the protein characteristics described above for other membrane proteins were successfully shown. In the presence of membranes both full length and extracellular FP-PC1 [tm-, influenza haemagglutinin signal sequence+] showed an increase in size of approximately 10kDa, whereas extracellular FP-PC1 [tm-] showed no increase. This data suggested that the increase in size is dependent on translocation and processing events in the luminal compartment of microsomal vesicles, as extracellular FP-PC1 without a signal sequence was not translocated or processed. The predicted extracellular domain of FP-PC1 has 6 N-linked glycosylation sites. In addition, it is known that protein glycosylation can only occur in the lumen of the golgi or rough endoplasmic reticulum, the latter being analogous to the lumen of microsomal vesicles. Therefore the observed increase in size probably does represent glycosylation.

The orientation and translocation status of FP-PC1 was analysed by PK protection assays of various domains expressed in the CTT system in the presence of membranes. This was facilitated as the luminal compartments of the endoplasmic reticulum, the golgi apparatus, microsomal and clathrin coated vesicles are topologically equivalent to the outside of the cell (Zubay, 1988). Indeed, during post-translational processing of proteins, clathrin coated vesicles preserve the orientation of

integral membrane proteins by fusing directly with target membranes. According to this rationale, any region of FP-PC1 protected from digestion from PK, represents the portion of the protein which is normally located outside the cell. In protease protection assays the three FP-PC1 constructs behaved as predicted by their respective domain structures; full length FP-PC1 showed a decrease in size upon treatment with PK approximately equivalent in size to the cytoplasmic domain, indicating that it was inserted into the membrane by the predicted dual function transmembrane domain/signal sequence, and that the large protected region represented the theoretical extracellular domain. This was further substantiated as extracellular FP-PC1 [tm-, influenza haemagglutinin signal sequence+], showed no change in size upon PK treatment, indicating that the influenza haemagglutinin signal sequence was in frame, leading to secretion of the entire protein into the lumen of vesicles. In contrast, the same region of FP-PC1 expressed without a signal sequence was not protected, as it was not translocated, remaining outside vesicles and susceptible to PK digestion. Taken together, these data confirm that the transmembrane sequence does have a dual function as a signal sequence and membrane anchor domain, characteristic of other type II membrane proteins, as its deletion leads to failure of translocation, membrane insertion and glycosylation.

A schematic representation of the likely topology of proteins expressed from pT-FP.Cel1, pT-PC1, pSHT-PC1 and pB-PC1 is shown in fig. 6.10.

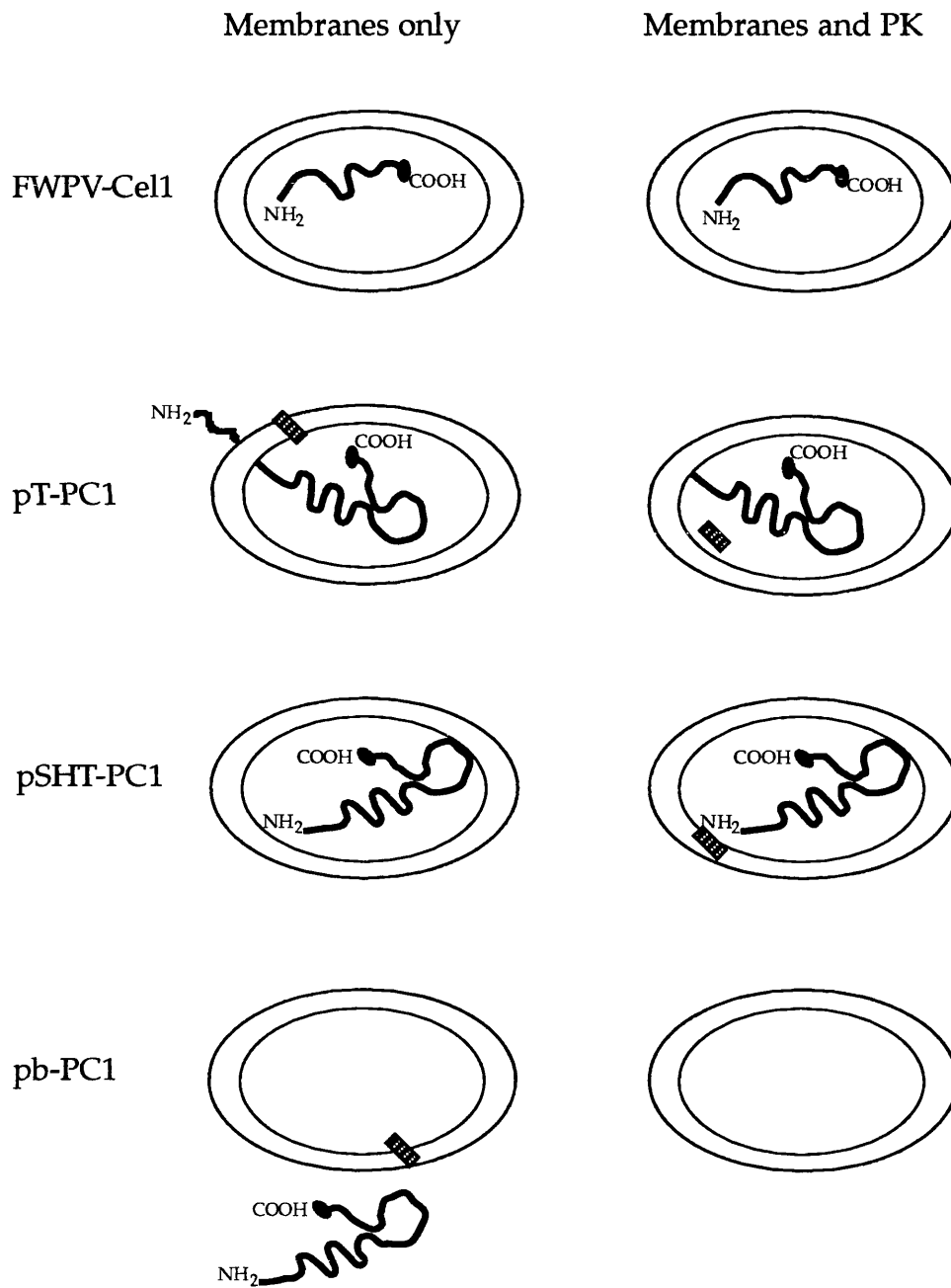
As well as expression in a cell free system as described above, analysis of FP-PC1 expressed in FWPV infected cells was a key aim in order to allow cellular localisation and further functional characterisation. Assessment of expression of FP-PC1 from FWPV infected cells was initially

hampered as no protein was detectable when expressed from the native promoter. This was despite the indication that FP-PC1 is strongly transcribed both early and late during the infective cycle (chapter 4). The failure to detect protein due to an error in the transfer vector was dismissed as the insert and the flag peptide were successfully expressed in the CTT system. Another explanation for non-detection is that mRNA levels do not necessarily have to correlate directly with protein levels, as a consequence possible differences between mRNA and protein levels may arise if FP-PC1 transcripts were translated with low efficiency, or if FP-PC1, in particular FP-PC1.fg, protein has a short half life inside the cell. Cleavage or recognition hindrance of the flag epitope when FP-PC1 is expressed at low levels may also explain why no protein was detected. The latter possibility is the least likely conjecture as FP-PC1 expressed from a recombinant FWPV using the T7 promoter was successfully immunoprecipitated and immunoblotted using a monoclonal antibody raised against the flag peptide.

Further analysis of FP-PC1 overexpressed in FWPV infected cells indicated that FP-PC1 undergoes N-linked glycosylation as tunicamycin treatment lead to a size reduction from 105kDa to 95kDa.

In summary, full length and extracellular FP-PC1 have been successfully expressed, therefore confirming the ORF of the PC1 gene. Full length FP-PC1 has been shown to be an N-linked glycosylated integral membrane protein with a short cytoplasmic amino terminal domain and a large extracellular carboxyl terminal domain, hence indicating a type II orientation. Analysis of FP-PC1 in FWPV infected cells has suggested that full length FP-PC1 is probably not secreted into the extracellular medium,

Fig.6.10 Schematic representation of the probable topological arrangements of FP-PC1 and FWPV-Cel 1 expressed in the CTT system in the presence of membranes and after PK treatment.



although a minor 60kDa product could represent a cleaved soluble form of FP-PC1.

Chapter 7

Conclusions and Discussion

7.1 Introduction

Apart from a few minor outbreaks in recent years (Fallavena *et al.*, 1993; Nyaga *et al.*, 1979; Tripathy *et al.*, 1974), FWPV disease has been largely eradicated for many decades, due to the use of readily available vaccines, using both live attenuated and killed immunogens (Jieyuan & Spradbrow, 1992; Mayr & Danner, 1976; Nagy *et al.*, 1990; Nishimura & Kawashima, 1968). Consequently, there is no significant research focus upon FWPV disease eradication. Recent research interest in FWPV has been dominated by its use for development of vaccines for other diseases, by insertion of foreign genes directly into the virus genome leading to expression of foreign proteins as antigens (Beard *et al.*, 1991; Boursnell *et al.*, 1990; Boyle & Heine, 1993; Ogawa *et al.*, 1990; Taylor *et al.*, 1988a; Wild *et al.*, 1990). The key advantage offered by FWPV in this respect, which has reinforced this attention, is that FWPV undergoes abortive replication in non-avian cells (Somogyi *et al.*, 1993). Hence, it has an integral safety feature applicable for use as a vaccine vector in non-avian targets. Despite this growing use of FWPV as a vaccine vector, FWPV, and avipoxviruses as a whole, remain largely uncharacterised, as the vast majority of poxvirus research has concentrated on orthopoxviruses, particularly vaccinia virus.

As part of the further investigation of FWPV, sequencing of a previously uncharacterised region of the genome revealed 5 major ORFs, none of which have been found in any other virus (Laidlaw *et al.*, 1998). This thesis describes detailed studies on one of these proteins - FP-PC1, which has been shown to have 38/39% amino acid identity with murine and human PC-1.

7.2 Summary of Results

The aims of this study can be divided into two main sections. Firstly, analysis of FP-PC1 by expression of full length and partial protein domains in eukaryotic protein expression systems. Secondly, use of the transient dominant selection system (Falkner & Moss, 1990) to determine whether the FP-PC1 gene is essential *in vitro*, leading to analysis of *in vivo* and *in vitro* ramifications of deletion of FP-PC1 from the FWPV genome.

7.2.1. Protein expression

The data presented in this thesis shows that three different FP-PC1 domains were expressed with varying success, in prokaryotic and eukaryotic expression systems.

The extracellular and alkaline phosphodiesterase FP-PC1 domains were expressed as flag fusion proteins in *E. coli*, in order to use purified protein for antibody production (chapter 5). Although proteins of the correct sizes were expressed, confirming the integrity of the partial ORF, the yield was substantially below that required to generate an effective response in rabbits for production of antibodies. Consequently, further optimisation and analysis in prokaryotic systems was abandoned in favour of expression of FP-PC1 flag fusion proteins in eukaryotic systems. This approach largely obviated the requirement for FP-PC1 antibodies, by utilisation of the M2 anti-flag monoclonal antibody.

Although no expression of extracellular FP-PC1 from pSHT-PC1 was detected in COS-7 cells, probably due to the low sensitivity of the

detection system, FP-PC1 from the same construct was successfully expressed, in an *in vitro*, cell free, coupled transcription/translation system (CTT). Further expression studies using the CTT system, using both extracellular (+/- artificial influenza haemagglutinin signal sequence) and full length FP-PC1, were used to investigate structural features predicted only by sequence analysis (chapter 3). All of these proteins were expressed as flag fusion proteins and were shown to be preferentially immunoprecipitated by the M2 flag monoclonal antibody. Although, weak non-specific radioimmunoprecipitation was observed with a hexa-histidine monoclonal antibody, this was expected due to the profile of proteins that are present in the CTT system. Unlike radioimmunoprecipitation studies using cellular systems, where a very large number of proteins apart from the gene of choice are being expressed, the protein profile in the CTT system is dominated by the protein being expressed under the T7 promoter. This leads to binding to non-specific antibodies due to the absence of competing proteins. This problem was partially solved by pre-adsorbing M2 sepharose beads with unlabelled CEF cell lysates, before addition of labelled CTT product.

Protease protection assays in the presence of canine pancreatic microsome vesicles (membranes) using all three constructs (as shown in fig. 6.2), confirmed that full length FP-PC1 is a type II transmembrane protein. By a process of elimination of results showing the region of full length and extracellular FP-PC1 that was protected, as well as analysis of regions likely to be glycosylated, a type II orientation was shown to be the most likely. Comparison of the three FP-PC1 constructs (fig. 6.2), expressed in the CTT system with and without membranes, showed that only full length FP-PC1 expressed from pT-PC1 and pSHT-PC1 increased in size in the presence of membranes. This indicates that both these proteins were

able to traverse the lipid bilayer using the native FP-PC1 signal sequence/anchor domain or the influenza haemagglutinin signal sequence respectively. Extracellular FP-PC1 expressed without an artificial signal sequence from pB-PC1 showed no increase in size. Similarly, expression of full length FP-PC1 overexpressed from the T7 promoter in CEFs co-infected with FWPV and rFWPV-T7, showed no increase in size in the presence of tunicamycin, indicating that N-linked glycosylation accounted for the increase in size in untreated cells.

Further analysis of FP-PC1 overexpressed in CEFs using T7 polymerase, indicated that full length FP-PC1 is probably not secreted, although a small amount of a 60kDa FP-PC1 protein domain was observed in the culture medium. It has been suggested that a functional, soluble form of mammalian PC1 arises by proteolytic cleavage (Belli *et al.*, 1993). Although further investigation needs to be conducted, it is tempting to propose that the 60kDa protein could serve a function distinct from the full length membrane protein.

Even though full length FP-PC1 was easily detectable in this system, the expression level was still below that observed with other FWPV and IBDV proteins overexpressed using the same system (personal communication, D. Hot and S. Laidlaw). In addition, analysis of FP-PC1 transcription has shown that the gene is transcribed both early and late. Full length FP-PC1 expressed as a flag-fusion protein from its own promoter, was not detectable by radioimmunoprecipitation or immunoblotting. Similarly, expression of extracellular FP-PC1 from COS-7 cells was not detected. A possible explanation could be that FP-PC1 mRNA and/or protein has a short half life. Comparison of FP-PC1 translation from the FWPV progenitor strain - HP1, may provide key data on this matter.

7.2.2. FP-PC1 deletion studies

The transient dominant selection technique was employed to introduce deletions directly into wt FP-PC1 in the FWPV genome. The largest deletion (Δ PC1-b) lead to deletion of all the functional domains identified by sequence comparison with mammalian PC1 genes, only the short cytoplasmic and transmembrane domains were left intact. The successful isolation of FWPV viruses with this deletion suggests that FP-PC1 is non-essential for FWPV replication *in vitro*. Analysis of ramifications of FP-PC1 deletion showed that there was no detectable effect on growth rate *in vitro*, however a difference in plaque size was observed. As discussed in chapter 4, this difference is likely to be as result of the difference in physical characteristics/kinetics between plaque formation in a semi-solid medium and virus growth in a liquid medium supplemented with nucleotides.

The finding that FP-PC1 is non-essential is not surprising as most essential poxvirus genes tend to be located in the central regions of the genome, whereas FP-PC1 is located towards the left hand side Inverted Terminal Repeat (ITR), and therefore likely to be non-essential. Other neighbouring FWPV genes (FWPV Cel-1 and FWPV SNAP) have also recently been shown to be non-essential (Laidlaw *et al.*, 1998). Further confirmation of the genotypes of mutant viruses was obtained by transcriptional analysis of early transcripts from cells infected with FP-PC1 mutants as described above, which correlated with the predicted size of deleted ORFs.

7.3 Possible role of FP-PC1 and future work

Since the discovery of murine PC1 as an antigen expressed on terminally differentiated B-cells in 1970 (Takahashi *et al.*, 1970), the precise physiological role of mammalian PC1 still remains unknown. However, mammalian PC1 has been shown to have alkaline phosphodiesterase/nucleotide pyrophosphatase activity (Rebbe *et al.*, 1991), as well as possible phosphatase and kinase activities (Oda *et al.*, 1991; Oda *et al.*, 1993; Uriarte *et al.*, 1993; Uriarte *et al.*, 1995). PC1 has also been implicated in inhibition of insulin receptor tyrosine kinase activity in insulin resistant individuals with non-insulin dependent (type II) diabetes mellitus (Maddux *et al.*, 1995) and has been linked to CPPD (Terkeltaub *et al.*, 1994). As FP-PC1 shows a high degree of amino acid identity in the key functional domains with mammalian PC1, it might have been expected that FP-PC1 would share the same enzymatic activities. In addition, the potential functions of the growing number of members of the PC1 gene family may also provide clues to the role of FP-PC1. Conversely, further analysis of FP-PC1 may also assist elucidation of the possible roles of mammalian PC1 proteins, as FWPV provides a good model for such studies. Screening extracts from CEFs infected with either wt FWPV or FWPV with a full gene FP-PC1 deletion failed to demonstrate alkaline phosphodiesterase activity using the substrate p-nitrophenol phenylphosphonate. A similar analysis of autophosphorylation also failed to show any activity. Although, this result suggested that FP-PC1 may not share the same enzymatic activities as mammalian PC1, further investigation with different substrates may have produced a different conclusion. It may also be possible for FP-PC1 to encode phosphodiesterase enzymatic activities that are not detectable with the substrate used in enzymatic assays. The likelihood that FP-PC1 encodes a non

phosphodiesterase enzymatic activity cannot be ruled out, but the high degree of sequence homology between FP-PC1 and mammalian proteins suggests that conclusive evidence needs to be obtained before FP-PC1 phosphodiesterase activity is ruled out. It is also possible that the role of FP-PC1 is non-enzymatic; it may function by interacting with host or viral proteins. Alternatively, it may have an enzymatic activity other than phosphodiesterase.

The fact that FP-PC1 does not affect FWPV replication *in vitro* suggests that FP-PC1 has a role in virus-host interactions (Laidlaw et al., 1998). Areas in which FP-PC1 could interact with host proteins and systems could include virus replication inside the cell, evasion of host immune systems and spread/infection to other hosts. Possible methods that could be utilised to detect such interactions include cross-linking experiments and use of the interaction trap method using yeast two-hybrid analysis. Further clues to ascertain if FP-PC1 causes up or down regulation of host genes could be assessed by using newly developed cDNA expression array technology. Once interacting proteins have been identified localisation of the interaction *in vitro* can be analysed by use of fluorescent resonance energy transfer (FRET), to allow detection based on energy transfer from interacting proteins expressed as fusion proteins with different variants of green fluorescent protein.

7.4 Origins of host gene acquisition and FP-PC1

The mechanism by which poxviruses acquire host genes is unknown. Gene acquisition and continued retention by viruses, indeed by any other organism, probably implies that there is a selective pressure related to direct functional benefits gained from possession of a particular gene (see chapter 4). The significance of this point becomes even greater for viruses, as they have a limited genome size and therefore little extra capacity. (Although poxviruses are able to accommodate large amounts of extraneous sequence, as well as tolerate large deletions without critical ramifications *in vitro* (Falkner & Moss, 1990; Kotwal & Moss, 1988a; Paez *et al.*, 1985; Panicali & Paoletti, 1982; Perkus *et al.*, 1991). Indeed many viruses use ingenious systems to maximise the number of proteins expressed from their genomes. Sendai virus and vesicular stomatis virus use multiple reading frames to express more than one protein from a single transcript by using different translation initiation points (Giorgi *et al.*, 1893; Herman, 1986), whereas influenza virus uses differential splicing of a single mRNA to express multiple proteins (Lamb & Choppin, 1983). It is therefore likely that any genes that are acquired, which are not utilised for important functions, will be readily lost through selective pressure.

To address how PC1 may have been acquired by FWPV, it is important to briefly address how DNA viruses may have originated and evolved. There are two main hypotheses for the origins of DNA viruses:

1. **The regressive theory** – which suggests that viruses are degenerate forms of intracellular parasites, which once inside a cell became dependent upon host biosynthetic machinery, and gradually lost their own

biosynthetic machinery (Strauss *et al.*, 1996) to resemble a functional ability profile resembling one of contemporary viruses .

2. Origin from host DNA/RNA – this hypothesis suggests that viruses arose from host cellular components (e.g. plasmids and transposons), that by an unknown mechanism became able to replicate autonomously of the cell, whilst continuing to use cellular biosynthetic machinery, and were then able to evolve independently (Strauss *et al.*, 1996).

The latter theory is perhaps the most attractive, as it would explain the origin of both DNA and RNA viruses. In addition, it would also explain the large numbers of host gene homologues that could be the remnant sequences of the genetic configuration of a previous cellular component. If the latter theory is to be considered, it is likely that the “parasite” or an ex-cellular component was able to acquire host genes by the possible mechanisms outlined below.

The process by which DNA viruses may acquire host genes, apart from the virus origin based mechanisms described above, is by homologous recombination in infected cells or by recombination with other viruses. Although there is no clear evidence of how poxviruses acquire host genes, and certainly no evidence of how FWPV acquired the PC1 homologue. By using all the theories presented above as a background, there are three possible hypotheses (by no means exclusive) that can be suggested as the origin of the FP-PC1 gene in FWPV:

1. FP-PC1 was present in the original unknown primogenitor of the FWPV virus, and was acquired directly from the host by either one of the theories presented above for the origin of viruses. Since then the FP-PC1 gene has diverged from the original host PC1 gene, by recombination and/or mutation.

2. The FP-PC1 gene was acquired by an avipoxvirus progenitor by homologous recombination from host cells and was retained due to a selective advantage. Since then the host gene sequence and/or function has diverged from the host gene due to mutations and/or further recombination events with *cis* or *trans* viral or host DNA.

3. The FP-PC1 gene may have been recently acquired by FWPV from the host or another virus, by homologous recombination with a currently unidentified member of the PC1 gene family.

It is relevant to point out that poxvirus genes have no introns, therefore host genes homologues were probably acquired as cDNA. A complication to this supposition is that poxviruses replicate exclusively in the cytoplasm. Therefore acquisition of genes as cDNA seems improbable. However, a possible mechanism was suggested by the recent discovery of field and vaccine FWPV isolates carrying sequences from reticuloendotheliosis virus - an avian retrovirus (Brunovskis & Velicer 1995). Hence, reverse transcriptase from endogenous and/or exogenous

retroviruses may facilitate the cytoplasmic incorporation of mRNA, as cDNA, into the FWPV genome.

A clearer indication of the true mechanism will only become available once there is more sequence information of both the possible host that FP-PC1 gene was acquired from (be it avian, mammalian or other), and also when the complete sequence of other avipoxviruses, as well as FWPV, becomes available. Upon this eventuality, both the origin and lineage of the FP-PC1 homologue may become clearer.

7.5 Conclusions

In summary, full length and extracellular FP-PC1 have been successfully expressed, therefore confirming the ORF of the PC1 gene, although the translation initiation codon has not been confirmed. Full length FP-PC1 has been shown to be an N-linked glycosylated integral membrane protein with a short cytoplasmic amino terminal domain and a large extracellular carboxyl terminal domain, hence indicating a type II orientation. Analysis of FP-PC1 in FWPV infected cells has suggested that full length FP-PC1 is probably not secreted into the extracellular medium, although a minor 60kDa product could represent a cleaved soluble form of FP-PC1.

Deletion studies using the transient dominant selection method were used for the first time in FWPV to generate 3 mutant FWPV viruses. A full FP-PC1 gene knockout FWPV was successfully isolated confirming that the FP-PC1 gene is non-essential for FWPV replication *in vitro*. No differences were observed in virulence or growth between wt FWPV and

Δ -PC1 FWPV *in vivo*. The only clear phenotypic difference was that Δ PC1 FWPV were 40% smaller than wt FWPV plaques.

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