CLONING AND EXPRESSION OF THE GLYCOPROTEINS OF PICHINDE VIRUS BY VACCINIA VIRUS

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By

Essam A. Wanas

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Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

Pichinde virus (Pic), like the other arenaviruses, possesses two glycoproteins, GP1 and GP2, that are derived by proteolytic cleavage from a precursor molecule, GPC. Within the arenaviruses, GP1 is the most heterogeneous protein, and GP1 of Pic differs from that of the other arenaviruses in having twice as many potential N-linked glycosylation sites, most of which appear to be utilized. In order to examine the effects of this heavy glycosylation on Pic GP1 structure and immunogenicity, GPC of Pic was cloned and expressed in vaccinia virus. The recombinant vaccinia (vvGPC) expresses authentic Pic GPC as demonstrated by immunoprecipitation with MAb and several polyclonal anti-Pic sera. GPC expressed in vaccinia is fully glycosylated as it comigrates with Pic GPC. At the same time, sequence analysis of cDNA shows both nucleotide and amino acid changes compared to published sequences for Pic GPC, indicating that variation in the same strain of this virus occurs as virus is passaged in separate laboratories. Experiments to assess the ability of Pic GPC expressed in vaccinia to elicit anti-Pic antibody show that rabbit anti-vvGPC detects authentic Pic GPC and GP1. Site-directed mutagenesis was employed to remove a potential N-linked glycosylation site (aa 181-183) in Pic GPC. Attempts were made to produce recombinant vaccinia, vvGPC-183, harbouring the mutant Pic GPC.

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DEDICATION

This thesis is dedicated to my wife Gillian and my parents

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LIST OF ABBREVIATIONS

A	Adenine base in a nucleotide sequence
aa	amino acid
Ab	Antibody
BHCl	Benzamidine-hydrochloride
Bis	N,N´-Bis-methylene-acrylamide
bp	base pair
BPB	Bromophenol blue
BRL	Bethesda Research Laboratories
BSA	Bovine serum albumen
°C	degree celsius
C	Cytosine base in a nucleotide sequence
cDNA	complementary DNA
cm	centimeter
CNS	central nervous system
CPE	cytopathic effect
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddDNA	double stranded DNA
ddGTP	dideoxyguanosine triphosphate
ddNTP	dideoxyribonucleotide triphosphate
ddTTP	dideoxythymidine triphosphate
dGTP	deoxyguanosine triphosphate

DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid sodium salt
ELISA	enzyme-liked immunosorbant assay
EtBr	ethidium bromide
FBS	fetal bovine serum
Fig	Figure
g	gram or gravity
G	Guanine base in a nucleotide sequence
h	hour
i.v.	intravenous
Kb	Kilobase
KD	kilo Dalton
LTD	Limited
MAb	Monoclonal Antibody
MEM	minimal essential medium
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MOI	Multiplicity Of Infection

mRNA	messenger ribonucleic acid
nm	nanometer
NENS	Na-acetate, EDTA, NaCl, and SDS
ng	nanogram
O.D.	Optic Density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
p.i.	post infection
PMSF	phenylmethylsulfonyl-fluoride
PPLO	pleuropneumonia-like organism
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
RNP	ribonucleoprotein
rRNA	ribosomal ribonucleic acid
sec	second
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
T	Thymine base in a nucleotide sequence
TBE	Tris borate, EDTA buffer
TE	Tris-EDTA buffer
TK	thymidine kinase
ts	temperature sensitive
uCi	microcurie
ug	microgram
ul	microliter

UV	ultraviolet
U	Uracil base in a nucleotide sequence
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
YT	yeast extract, tryptone, and NaCl

CHAPTER 1 GENERAL INTRODUCTION

1.) Pichinde Virus: Background

Origin, Family and Classification

Pichinde virus, (Pic) a member of the family Arenaviridae (Matthews, 1982), was first isolated in Colombia from its persistently infected host, Oryzomy albigularis (Trapido and Sanmartin, 1971). This family of viruses is one of the smallest families of RNA viruses, consisting of only 14 viruses. The name arenavirus is derived from *arenosos*, Latin for sandy, chosen because of the sandy appearance of virions when viewed by electron microscopy (Carter et al., 1973a; Farber and Rawls, 1975; Pederson and Konigshofer, 1976).

Arenaviruses, with the exception of Tacaribe, establish life long persistent infections in their rodent hosts in nature and in tissue culture (Rawls et al., 1981). Serological analysis and geographic distribution of arenaviruses and their natural hosts have led to a functional division of Arenaviridae into two groups: the Old World arenaviruses, which include Lassa, Mopeia, Mobala, Lymphocytic Choriomeningitis Virus (LCMV), and Callitrichid Hepatitis Virus (CHV), and the New World arenaviruses, Amapari, Tacaribe, Junin, Machupo, Tamiami, Parana, Pichinde, Latino and a newly described virus, Guanarito (Table 1).

Table 1*: Arenaviruses

Virus	Natural Host	Distribution	Human Disease
Old World			
LCMV	*Mus musculis*	Virtually worldwide	Undifferentiated febrile illness aseptic meningitis rarely serious
Lassa	*Mastomys natalensis*	West Africa	Lassa fever, mild to severe and fatal disease
Lassa-like viruses	*Mastomys, Praomys*	Mozambique, Cen.Afr.Rep.	Unknown
CHV**	*Callithrix jacchus* *Mus musculis*	America, Africa	None
New World			
Junin	*Calomys musuclinus*	Argentina	Argentine hemorrhagic fever rarely serious
Tacaribe	*Artibeus* bats	Trinadad, West Indies	None
Machupo	*Calomys musculinus*	Bolivia	Bolivian hemorrhagic fever
Amapari	*Oryzomys gaeldi* *Neacomys guianae*	Brazil	None
Parana	*Oryzomys buccinatus*	Paraguay	None
Tamiami	*Sigmodon hispidus*	USA (Florida)	Antibodies detected
Pichinde	*Oryzomys albigularis*	Colombia	None
Latino	*Calomys callosus*	Bolivia	Unknown
Flexal	*Oryzomys* species	Brazil	Unknown
Guanarito@	*Sigmodon hispidus*	Venezuela	Venezuelan hemorrhagic fever

* Modified from Wright et al., 1990a
** Stephensen et al., 1991 @ Salas et al., 1991

Morphology

Arenaviruses are enveloped, pleomorphic particles with a diameter ranging from 50-300 nm (Dalton et al., 1968; Gschwender et al., 1975). The envelope is formed from the host cell plasma membrane by budding and contains 5-10 nm viral glycoprotein surface projections, which are club shaped, sparse in number and irregular (Murphy et al., 1970; Murphy and Whitfield, 1975; Gard et al., 1977; Vezza et al., 1977). Variable numbers of host derived electron-dense granules resembling ribosomes (20-30 nm in diameter) are frequently observed in the virions and are responsible for the sandy appearance of the virions (Murphy and Whitfield, 1975; Farber and Rawls, 1975; Vezza et al., 1978).

Electron microscopic studies of arenaviruses have demonstrated the ribonucleocapsid (RNP) to be a filamentous structure consisting of viral RNA and nucleocapsid protein (NP). The nucleocapsid has been visualized as beaded, loosely helical (9-15 nm in diameter) and circular. The beads, which represent the sites of nucleoprotein deposition, have been estimated to be 4 nm in diameter (Young et al., 1981; Young and Howard, 1986). Another protein, L, which has an RNA dependent RNA polymerase activity, has been identified in arenaviruses, including Pichinde virus particles (Leung et al., 1977; Ramsingh et al., 1980; Harnish et al., 1983). Like other members of Arenaviridae, Pichinde virus has a segmented single stranded RNA genome consisting of two unique species

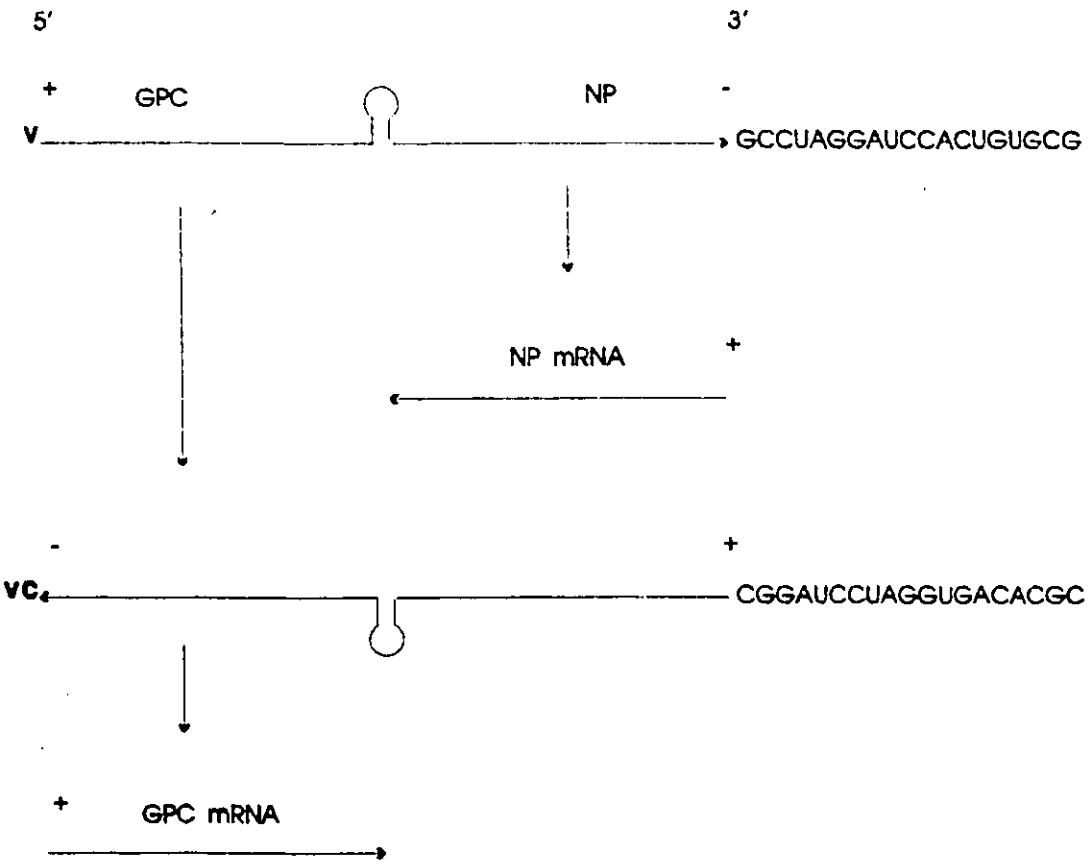
large (L) and small (S). Other species of RNA (28S, 18S, 4-6S) which are of host cell origin are also found in the virion, (Carter et al., 1973b; Ramsingh et al., 1980).

2.) Genome Organization

Pichinde virus, as with the other arenaviruses, possesses an RNA genome that is linear, single stranded and of two species, large (L) (2.8×10^6 daltons) and small (S) (1.16×10^6) (Leung et al., 1977; Ramsingh et al., 1980; Auperin et al., 1986). Analysis of several arenaviruses has established that L and S RNA species have common consensus 3'end sequences that are complementary to their 5'end sequences (Auperin et al., 1982a, 1982b, 1984a, 1986; Clegg and Oram, 1985; Franze-Fernandez et al., 1987). The full nucleotide sequence of Pichinde virus S RNA has been determined (3,419b) (Auperin et al., 1984a). S RNA possesses 2 non-overlapping reading frames separated by a short, noncoding, intergenic region of 87 nucleotides, which forms a 14 G-C, 4 A-U stabilizing hairpin structure. This sequence is believed to act as a transcription terminator. Similar intergenic regions have been identified for LCMV, Lassa and Tacaribe viruses (Auperin et al., 1986; Bishop and Auperin 1987; Iapalucci et al., 1991), while two hairpin loops instead of one were identified for Junin (Ghiringhelli et al., 1991), Mopeia and Tacaribe viruses (Wilson and Clegg, 1991). The two hairpins are

similar in sequence and may have been formed in a duplication event during RNA replication. The S RNA encodes the viral nucleoprotein (NP) (64KD) in the negative sense at the 3'end and the viral glycoprotein precursor (GPC) (79KD) in the positive sense at the 5'end (Harnish et al., 1981) (Figure 1). The nucleotide sequence for the L segment of Pic is not known although it is estimated to be 7.2 Kb, the same as the L RNAs of LCMV and of Tacaribe which have been sequenced (Singh et al., 1987; Salvato and Shimomaye, 1989; Salvato et al., 1989). In LCMV and Tacaribe viruses the L segment also has two open reading frames separated by a hairpin structure (Auperin et al., 1984b; Bishop and Auperin, 1987; Franze-Fernandez et al., 1987; Salvato and Shimomaye, 1989; Iapalucci et al., 1991). The L RNA of all arenaviruses encodes a protein of 200 KD in the negative sense at the 3'end (Harnish et al., 1983; Buchmeier and Parekh, 1987). This is believed to be the viral RNA polymerase (Harnish et al., 1983). In LCMV and Tacaribe virus, a second reading frame is in the positive sense at the 5'end and this gene has the capacity to encode a zinc binding protein (P11) (10KD), possibly involved in transcription regulation through a zinc finger domain (Iapalucci et al., 1988, 1989, 1991; Salvato et al., 1989; Salvato and Shimomaye, 1989). Although not yet determined for Pic or other arenaviruses, this second reading frame is assumed to be present.

Figure 1: Diagramatic representation of genome organization and ambisense coding strategy of Pic S RNA. The intergenic region is represented as a hairpin stem-loop structure. The sequence shown at the 3′ end of genome RNA represents the consensus sequence among arenaviruses. Sequence at the 5′ end of the vcRNA is complementary to the 3′ end sequence of the vRNA strand. The viral RNA is indicated by (v). The viral complementary RNA is indicated by (vc).


5'
3'
+
GPC
NP
-
V
GCCUAGGAUCCACUGUGCG
NP mRNA
+
-
+
VC
CGGAUCCUAGGUGACACGC
+
GPC mRNA

3.) Replication

The replication cycle of arenaviruses is slow in comparison to other negative-stranded RNA viruses, often taking four days for maximum level of virus production (Tishon and Oldstone, 1987). Generally arenaviruses replicate without significantly disturbing host-cell macromolecular synthesis and without producing a cytopathic effect (Gimenez and Compans, 1980; Dimock et al., 1982). It has been reported that Pichinde virus requires a cell nucleus at least for the first 8h of infection (Banerjee et al., 1976). Attachment to the surface of the host cell occurs by an interaction between the viral glycoprotein GP1 (Burns and Buchmeier, 1991) and a receptor protruding from the cell membrane. Borrow and Oldstone (1992) have shown that LCMV binds to a protein(s) with a molecular mass of 120-140 KD in membranes from cell lines permissive for the virus. This protein(s) has complex N-linked sugars that are not involved in virus binding. Entry of the ribonucleoprotein (RNP) complex into the cell might occur by either of two mechanisms. Immediately following attachment, GP1 may mediate the fusion of the viral envelope with the plasma membrane, thereby depositing the RNP into the host-cell cytoplasm (Bishop, 1990). Other authors have shown that entry of RNP into the cell occurs by adsorptive endocytosis with the participation of acidic intracellular vesicles (Glushakova and Lukashevich, 1989).

Auperin et al., (1984a) and Bishop and Auperin, (1987) have proposed an ambisense model for the regulation of S RNA transcription and replication in which the nucleoprotein (NP) mRNA could be synthesized directly using the viral genome as template, while the synthesis of the glycoprotein (GPC) mRNA must await the formation of full length genomic complementary RNA to serve as a template (Figure 1). Thus the first event after viral infection would involve the synthesis of NP mRNA which may be concomitant with, or followed by, the production of full-length genomic complementary RNA (i.e., the replication intermediate). The genomic complementary RNA could then be used as a template for either transcription of GPC mRNA or replication of genomic sense RNA. The replication of LCMV L segment has been shown to follow the same pattern as that of the S segment, although there is a preferential accumulation of genomic S RNA over genomic L RNA during acute infection. Studies of the accumulation of LCMV viral genomic and messenger RNAs during the first 24h of acute infection of tissue culture cells have supported this model (Fuller-Pace and Southern, 1988). NP mRNA was detected simultaneously with genomic S RNA at 6-9h post infection, but the amount of NP mRNA accumulating during this period of infection was higher than that of GPC mRNA. Genomic L RNA was first detected at approximately the same time as genomic S RNA although the amount of L RNA, particularly at earlier times, was lower.

There was no significant accumulation of L RNA until 15h post infection, as compared with 9h for S RNA.

Lassa, Pic (Raju et al., 1990) and Tacaribe (Garcin and Kolakofsky, 1990) mRNAs have been found to contain from 1 to 5 extra nontemplated nucleotides at the 5′ ends which are capped and heterogenous in sequence. It is possible that the 5′ extensions on arenavirus mRNAs are derived from host mRNAs via cap snatching (Garcin and Kolakofsky, 1990; Raju et al., 1990). The manner in which arenavirus RNA synthesis is initiated has recently been investigated. Genome replication is primer dependent, where pppGpC is first made on the template at position +2 and +3 then slips backwards on the template so that the 5′ end is at position -1 before elongation continues (Garcin and Kolakofsky, 1990, 1992; Raju et al., 1990). Iapalucci and colleagues (1991) have shown that the 3′ end sequences of the four putative mRNAs of Tacaribe virus S and L RNAs form GC-rich stable hairpins. These hairpins might be the signals for termination of arenavirus transcription. They also suggested that viral NP might function as an antiterminator, and thus regulate the shift from transcription to replication.

Protein synthesis during acute infection has been studied in LCMV, Pichinde and Tacaribe virus-infected cells. In the leader sequence of S and L RNA species, there is a conserved six nucleotide sequence that is complementary to a 3′ terminal sequence present in 18S rRNA. This complementarity may be

important in arenavirus mRNA-ribosome interaction and subsequent translation of mRNA (Bishop and Auperin, 1987). Synthesis of the nucleoprotein (NP), the most abundant protein made during infection, can be detected 6-12h post infection with LCMV and 12-24h post infection with Pic and Tacaribe depending on the multiplicity of infection. L protein can be detected 12-24h post infection depending also on the multiplicity of infection (Buchmeier et al., 1978; Saleh et al., 1979; Harnish et al., 1981; Dimock et al., 1982; Clegg and Lloyd, 1983). Arenavirus glycoprotein synthesis is not detected until sometime after NP expression, 24-48h post infection (Buchmeier et al., 1978; Saleh et al., 1979; Harnish et al., 1981; Dimock et al., 1982; Clegg and Lloyd, 1983). The synthesis of GPC mRNA from an intermediate of RNA replication, rather than from virion RNA, may be an advantage in regulating virus infection since GPC synthesis can occur independently of NP mRNA synthesis. Also the segregation of the synthesis of the two S-coded mRNAs may be important in the establishment of arenavirus persistent infection (Bishop, 1990). Other than the observation that arenaviruses bud at the cellular membrane, nothing is understood about the morphogenetic process involved in virus maturation, although a recent report indicates that an interaction between GP2 and NP may direct the maturation and budding of LCM virions (Burns and Buchmeier, 1991).

Replication in LCMV persistently infected cell cultures has also been studied where few infectious virus particles were produced. S RNA and genomic-sized viral complementary S RNA were detected and, in addition, considerable quantities of shortened molecules of either species. The cells' content of NP was high but they contained little GPC; instead, a viral glycoprotein with molecular weight of 65 KD was present. It is believed that along the viral complementary S RNA (vc-S RNA) there is more than one recognition site for the viral RNA-dependent RNA polymerase, which leads to the generation of truncated forms of S RNA, vc-S RNA and mRNA for GPC; this, in turn, results in relative overproduction of NP and relative underproduction of GPC as well as the emergence of new forms of viral glycoproteins (Gimenez and Compans, 1980; Oldstone and Buchmeier, 1982; Zeller et al., 1986; 1988; Bruns et al., 1990).

4.) Glycoproteins

Arenaviruses contain either one surface glycoprotein (G), as reported for Tacaribe and Tamiami viruses (Gard et al., 1977; Howard et al., 1985) or two (GP1 and GP2) for the other arenaviruses (Vezza et al., 1977; Harnish et al., 1981; 1983; Buchmeier et al., 1987). The surface glycoproteins are encoded by a single gene (GPC), which encodes a precursor polypeptide (GPC) of 79 KD for Pichinde virus (Harnish et al.,

1981) and 75-76 KD for LCMV (Buchmeier and Oldstone, 1979). GPCs for Machupo, Tacaribe and Lassa viruses are of similar sizes (Saleh et al., 1979; Clegg and Lloyd, 1983; 1984; Lukashevich and Lemeshko, 1985). GPC is an oligomannosyl rich glycopeptide which is processed post translationally by carbohydrate trimming and proteolytic cleavage prior to virus release. After initial folding, processing and oligomerization in the endoplasmic reticulum (ER), GPC of LCMV is transported to the Golgi where it is cleaved in either the trans-Golgi compartment or the trans-Golgi network to yield GP1 and GP2 (Buchmeier and Oldstone, 1979; Wright et al., 1990b). Wright et al. (1990b) have shown that maturation of GPC oligosaccharide from high mannose carbohydrate to complex carbohydrate occurs before cleavage. Also glycosylation of GPC is essential for transport and cleavage, but it is not clear whether cleavage itself is necessary for transport to the cell surface.

The gene order on the GPC message is NH_2-(GP1)-(GP2)-COOH. The cleavage site utilized on GPC to yield GP1 and GP2 is defined by a conserved double basic amino acid sequence flanked by hydrophobic amino acids (Figure 2). This sequence is (Arg-Arg) for LCMV, Lassa fever and Mopeia viruses (Buchmeier and Parekh, 1987; Wilson and Clegg, 1991) and (Arg-Lys) for Pichinde and Tacaribe viruses (Buchmeier and Parekh, 1987; Allison et al., 1991). Hydrophobicity profiles of the glycoproteins of LCMV and Pichinde virus have been determined

Figure 2: Multiple sequence alignment of arenavirus glycoproteins (Southern and Bishop, 1987). Gaps, indicated by dots (...), are introduced to maximize the alignment of amino acids. Residues in common are indicated in the line below the individual amino acid sequences. Potential N-linked glycosylation sites are indicated in bold italic type. The cleavage site is indicated by (**). ARM; LCMV ARM strain: WE; LCMV WE strain: LAS; Lassa virus: Pic; Pichinde virus.

```
ARM        MGQIVTMFEA LPHIIDEVIN IVIIVLIVIT GIKAVYNFAT CGIFALISFL
 WE        MGQIVTMFEA LPHIIDEVIN IVIIVLIIIT SIKAVYNFAT CGILALVSFL
LAS        MGQIVTFFQE VPHVLEEVMN IVLIALSVLA VLKGLYNFAT CGLVGLVTFL
PIC        MGQIVTLIQS IPEVLQEVFN VALIIVSVLC IVKGFVNLMR CGLFQLVTFL
CONSERVED  MGQIVT      P    EV N    I         K   N    CG   L  FL

           LLAGRSCGMY GLKGPDIYKG VYQFKSVEFD MSHLNLTMPN ACSANNSHHY
           FLAGRSCGMY GLNGPDIYKG VYQFKSVEFD MSHLNLTMPN ACSVNNSHHY
           LLCGRSCT.. ....TSLYKG VYELQTLELN METLNMTMPL SCTKNNSHHY
           ILSGRSCDSM MIDRRHNLTH VEFNLTRMFD NL......PQ SCSKNNTHHY
            L GRSC                V                P   C  NN HHY

           ISMGTS...G LELTFTNDSI ISHNFCNLTS AFNKKTFDHT LMSIVSSLHL
           ISMGSS...G LEPTFTNDSI LNHNFCNLTS ALNKKSFDHT LMSIVSSLHL
           IMVGNET..G LELTLTNTSI INHKFCNLSD AHKKNLYDHA LMSIISTFHL
           YKGPSNITWG IELTLTNTSI ANETSGNFSN IGSLGYGNIS NCDRTREAGH
                     G  E T TN SI       N

           SIRGNSNYKA VSCDFNNG.. .......... .ITIQYNLTF SDAQSAQSQC
           SIRGNSNYKA VSCDFNNG.. .......... .ITIQYNLSS SDPQSAMSQC
           SIPNFNQYEA MSCDFNGG.. ....K..... .ISVQYNLSH SYAGDAANHC
           TLKWLLNELH FNVLHVTRHI GARCKTVEGA GVLIQYNLTV GDRGGEVGRH
                                                QYNL

           RTFRGRVLDM F.RTAFGGKY MRSGWGWTGS DGKTTW.CSQ TSYQYLIIQN
           RTFRGRVLDM F.RTAFGGKY MRSGWGWTGS DGYTTW.CSQ TSYQYLIIQN
           GTVANGVLQT FMRMAWGGSY I......ALD SGRGNWDCIM TSYQYLIIQN
           LIASLAQIIG DPKIAWVGKC FNNCSGDTCR LTNCEGGTH. ..YNFLIIQN
                       A  G                            Y  LIIQN
                                                  **
           RTWENHCTYA ..GPFGMSRI LLSQEKTKFF TRRLAGTFTW TLSDSSGVEN
           RTWENHCRYA ..GPFGMSRI LFAQEKYKFL TRRLSGTFTW TLSDSSGVEN
           TTWEDHCQFS RPSPIGYLGL LSQRTRDIYI SRRLLGTFTW TLSDSEGKDT
           TTWENHCTYT ...PMATIRM ALQRTAYSSV SRKLLGFFTW DLSDSSGQHV
            TWE HC       P                   R L G FTW  LSDS G

           PGGYCLTKWM ILAAELKCFG NTAVAKCNVN HDAEFCDMLR LIDYNKAALS
           PGGYCLTKWM ILAAELKCFG NTAVAKCNVN HDEEFCDMLR LIDYNKAALS
           PGGYCLTRWM LIEAELKCFG NTAVAKCNEK HDEEFCDMLR LFDFNKQAIQ
           PGGYCLEQWA IIWAGIKCFD NTVMAKCNKD HNEEFCDTMR LFDFNQNAIK
           PGGYCL  W     A  KCF  NT  AKCN   H  EFCD  R L D N  A

           KFKEDVESAL HLFKTTVNSL ISDQLLMRNH LRDLMGVPYC NYSKFWYLEH
           KFKQDVESAL HVFKTTLNSL ISDQLLMRNH LRDLMGVPYC NYSKFWYLEH
           RLKAEAQMSI QLINKAVNAL INDQLIMKNH LRDIMGIPYC NYSKYWYLNH
           TLQLNVENSL NLFKKTINGL ISDSLVIRNS LKQLAKIPYC NYTKFWYIND
                        N L I D L   N  L      PYC NY K WY

           AKTGETSVPK CWLVTNGSYL NETHFSDQID QEADNMITEM LRKDYIKRQG
           AKTGETSVPK CWLVTNGSYL NEIHFSQIEM QEADNMITEM LRKDYIKRQG
           TTTGRTSLPK CWLVSNGSYL NETHFSDDIE QQADNMITEM LQKEYMERQG
           TITGRHSLPQ CWLVHNGSYL NETHFKNDWL WESQNLYNEM LMKEYEERQG
             TG  S P  CWLV NGSYL NE HF        .  N   EM L K Y  RQG

           STPLALMDLL MFSTSAYLVS IFLHLVKIPT HRHIKGGSCP KPHRLTNKGI
           STPLALMDLL MFSTSAYLIS IFLHFVRIPT HRHIKGGSCP KPHRLTNKGI
           KTPLGLVDLF VFSTSFYLIS IFLHLVKIPT HRHIVGKSCP KPHRLNHMGI
           KTPLALTDIC FWSLVFYTIT VFLHIVGIPT HRHIIGDGCP KPHRITRNSL
            TPL L D     S   Y     FLH V IPT HRHI G  CP KPHR

           CSCGAFKVPG VKTVWKRR
           CSCGAFKVPG VKTIWKRR
           CSCGLYKQPG VPVKWKR
           CSCGYYKYQR NLTNG
           CSCG  K
```

(Auperin et al., 1984a; Bishop and Auperin, 1987; Southern and Bishop, 1987). The GPC precursors of two strains of LCMV, WE and ARM, consist of 498 amino acids. The protein contains two significant hydrophobic domains, one at the amino terminus of GP1, which is thought to represent the signal sequence, while the other one, the carboxyl domain of of GP2, represents the membrane anchor (Burns and Buchmeier, 1991). Amino acid sequence comparison with the corresponding GPC gene of Pichinde virus has revealed that the gene contains two similar domains (Auperin et al., 1984a).

The macromolecular arrangement of glycoproteins of LCMV in the membrane has been studied by Burns and Buchmeier (1991) and Wright et al. (1989). They suggested that GP1 is a peripheral protein, whose native structure is a disulphide-linked homotetramer, while GP2 is an integral membrane protein, also assembled as a tetramer, that spans the lipid bilayer and may integrate with the ribonucleoprotein (RNP) via a carboxy-terminal cytoplasmic tail. The interaction of GP2 and RNP may be important in directing the maturation and budding of LCM virions (Morrison et al., 1990).

Sequence comparisons of the GPC precursors of arenaviruses have shown extensive conservation of deduced amino acid sequences in GP2 proteins, on the other hand GP1s exhibit the least amino acid sequence similarity. This has been reported for LCMV, Pichinde, Lassa, Tacaribe and Junin viruses (Buchmeier and Parekh, 1987; Franze-Fernandez et al.,

1987; Weber and Buchmeier, 1988; Auperin and McCormick, 1989; Clegg et al., 1990; Ruo et al., 1991; Ghiringhelli et al., 1991). Many of the conserved residues in GP1 are located within sixty residues of the N-terminus in the hydrophobic region which appears to be removed from the mature virion (Burns et al., 1990).

GP1 and GP2 of LCMV ARM and Lassa bear five and two potential N-linked glycosylation sites respectively (Wright et al., 1990b; Clegg et al., 1990) as identified by the consensus sequence Asn-X-Ser or Thr where X is any amino acid except proline and possibly aspartic acid (Kornfeld and Kornfeld, 1985). Also the N-linked glycosylation sites are conserved across all arenaviruses at one position in GP1 and three positions in GP2, although in GP1 this number rises to four if non-exact (within 5 residues) alignment is applied (Clegg et al., 1990) (Figure 2). Pic is unusual in the number of glycosylation sites where its GP1 and GP2 virus bear eleven and five sites respectively (Auperin et al., 1984b; Southern and Bishop, 1987) (Figure 2). The unglycosylated form of Pic GPC was shown to migrate on SDS-PAGE at 42 KD, compared to 79 KD for the mature form, suggesting that most if not all the glycosylation sites are utilized (Harnish et al., 1981).

As discussed above, GP1 of arenaviruses appears to be more accessible to antibody binding at virion and cell surfaces than is GP2, and GP1 is presumably involved in receptor binding to the cell surface (Buchmeier and Oldstone,

1979; Bruns et al., 1983; Burns and Buchmeier, 1991; Borrow and Oldstone, 1992). Monoclonal antibodies (Mabs) against GP1 are either virus specific or cross-react only with very closely related heterologous viruses. This is certainly a reflection of the sequence diversity of arenavirus GP1s (Buchmeier et al., 1981; Parekh and Buchmeier, 1986). The sequence conservation in GP2 is reflected by group-specific determinants on GP2 of LCMV which are conserved across a wide range of arenaviruses representing both Old and New subgroups (Parekh and Buchmeier, 1986; Weber and Buchmeier, 1988).

Parekh and Buchmeier (1986) have described the antigenic topography of GP1 and GP2 of LCMV. GP2 contains three overlapping B-cell epitopes of which two are conserved among arenaviruses (Weber and Buchmeier, 1988) while GP1 contains four B cell epitopes that are virus specific. Two of the GP1 epitopes, A and D are partially overlapping and are targets for neutralizing MAbs. The neutralization-associated epitopes on GP1 of LCMV are conformational and dependant both on the presence of disulfide bridges and on N-linked glycosylation (Wright et al., 1989). However epitope D is unique to certain isolates of the Armstrong strain of LCMV and is absent from LCMV WE strain. The only sequence difference in GP1 of these viruses results in the loss of a potential glycosylation site (aa 171-173) on the variant with the extra epitope, and it has been shown that this variant has lost one carbohydrate molecule (Wright et al., 1989).

Competitive binding and neutralization kinetic studies of Tacaribe virus indicated the existence of 2 neutralization epitopes on the single glycoprotein (G) of the virus (Howard et al., 1985). For the Josiah strain of Lassa virus, 2 antigenic sites on GP1 and 6 on GP2 were detected, however MAbs specific to antigenic sites on GP1 and GP2 showed neutralizing ability at low titres (Ruo et al., 1991). Such mapping has not been done for Pic because to date it has been difficult to generate Mabs to the glycoproteins (Buchmeier, personal communication).

5.) Immune responses to arenaviruses

Arenaviruses persist in nature by establishing persistent infection in rodent hosts (Johnson, et al., 1965; 1967; Justines and Johnson, 1969; Webb et al., 1970). The immune response to arenaviruses depends on the route of infection, the age and the genetic background and the immunocompetency of the hosts. The study of LCMV infection of mice has provided comprehensive data on immune response to arenaviruses. Acute infection of adult immunocompetent mice by a peripheral route results in a full array of responses such as interferon, natural killer cells (NK) cells (Welsh, 1987), antiviral Abs and cytotoxic T lymphocytes (CTL) (Oldstone and Dixon, 1969; Buchmeier al. 1980a; Oldstone, 1987).

T-cell responses play an important role in the control and elimination of arenaviral infections (Zinkernagel et al., 1979; Byrne and Oldstone, 1984; Oldstone et al., 1986; Grossmann et al., 1991). LCMV infection of adult mice leads to both delayed type-hypersensitivity (DTH), as demonstrated by footpad swelling (Tosolini and Mims, 1971; Zinkernagel et al., 1985), and cytotoxic T cell responses (CTL) observed by the fifth day and peaking at day 7 to 9 post infection (Zinkernagel and Doherty, 1974; Buchmeier et al., 1980a; Allen et al., 1987; Battegay et al., 1992). CTL epitopes on LCMV NP and GPC are multiple. Although all five strains of LCMV share common T cell epitopes, they also have unique determinants and their recognition varies with the MHC class I background of the host (Ahmed et al., 1984a; 1984b; Byrne et al., 1984; Riviere et al., 1986; Whitton et al., 1988a; 1988b; Oldstone and Whitton, 1989). CTL responses are essential for clearance of LCMV, and lack of CTL responses results in persistent infection (Marker and Volkert, 1973; Byrne and Oldstone, 1984; Moskophidis et al., 1987; Joly et al., 1991). CTLs are also the cause of immunopathology when LCMV is inoculated into immunocompetent adult mice by the intracerebral route to result in lymphocytic choriomeningitis (Allen et al., 1987).

Acute infection of mice with LCMV by the peripheral route results in the development of Abs 5 days p.i. that remain high for several months (Buchmeier et al., 1980b). Neutralizing Abs appear at 1-3 weeks p.i. depending on the virus dose and the

mouse strain and persist for at least one year (Kimmig and Lehmann-Grube, 1979). Neutralization epitopes as discussed above, are conformational and restricted to GP1. The mechanism of neutralization is unknown but presumably binding of neutralizing Abs prevents either the attachment of virus to a particular host cell by steric hinderance, the entry of virus into the cell, or the uncoating of the virus particle (Della-Porta and Westaway, 1978; Mandel, 1979). Neutralizing Abs play no role in clearing LCMV infection, and LCMV can persist in the presence of neutralizing Abs (Buchmeier and Oldstone, 1978). However, Wright and Buchmeier (1991) have defined B-cell epitopes that attenuate T-cell-mediated choriomeningitis in adult LCMV infected mice. Pre-existence of Ab or rapid induction of appropriate B cell responses can protect against induction of lymphocytic choriomeningitis. The protective effect of Ab is not restricted to neutralizing Abs (Thomsen et al., 1979; Thomsen and Marker, 1988; Wright et al., 1991).

Lassa, Mopeia, Junin, Machupo and Guanarito viruses have been associated with haemorrhagic disease in man (Table 1). Humans usually become infected by direct contact with contaminated rodent excretions (Sanchez et al., 1989; Stephensen et al., 1991; Salas et al., 1991). Person-to-person spread among family contacts has been also reported for Lassa virus (Keenlyside et al., 1983). These arenaviruses differ in their ability to elicit neutralizing Abs in humans

and other species. Junin and Machupo induce neutralizing Abs in rodents and humans, but neutralizing Abs develop rather late in infection (Casals, 1984; Peters, 1984). Serum from C. musculinus persistently infected with Junin has a high titre of neutralizing activity that is ineffective in clearing the virus (Weissenbacher et al., 1987). However, passive transfer of immune plasma has proven effective in reducing mortality and morbidity in patients acutely infected with Junin (Maitzegui et al., 1979; Peters, 1984). Also animal studies suggested that passive administration of serum containing neutralizing Abs can protect animals against primary infection with Junin (Maitzegui et al., 1979; Peters et al., 1987). Guinea pigs and marmosets immunized with Tacaribe develop low levels of neutralizing Ab to Junin that appear late after immunization. When animals were subsequently challenged with Junin, a secondary neutralizing anti-Junin response occurred, indicating that the animals were primed by cross-reacting Ag on Tacaribe (Weissenbacher et al., 1976; 1987; Coto et al., 1980).

The in vivo significance of neutralizing Ab to Lassa is unclear since infection of guinea pigs with Mopeia, Mobala or LCMV does not induce cross-reactive neutralizing Ab to Lassa, but these animals are protected against Lassa challenges (Peters et al., 1987). In humans neutralizing Ab to Lassa appears so late in convalescence that it can have little effect in virus clearance (Casals, 1984). Comprehensive data

on T cell responses to arenaviruses other than LCMV are yet to be provided.

6.) Immune responses to Pichinde virus

Although Pichinde virus does not cause disease in mice, acute infection results in a full array of responses such as interferon, NK, antiviral Abs and CTLs, and DTH is elicited after footpad inoculation (Wright et al., 1990a). The unusual feature about Pic in these model systems is that Pic fails to elicit neutralizing Abs. This is also true in the host rodent in nature and the only reports of neutralizing Ab in the literature are of low levels of neutralizing Abs arising very late after infection of rhesus monkeys and guinea pigs (Howard, 1987; Lewis et al., 1988). Following i.v. infection of adult mice, a strong T-cell-mediated immune response is elicited and Pic is rapidly cleared, but neutralizing Abs are not detected (Walker et al., 1984). Although it has not been clearly demonstrated, it is presumed that T cell responses are necessary for virus clearance. Ozols et al. (1988; 1990) have shown that the cytotoxic response is not directed to Pic NP, and recently it has been demonstrated that no CTL epitopes exist on GPC either (DeMille, personal communication). Other gene products such as L or Z proteins may contain the major target epitope(s) recognized by Pic-specific CTL.

Immune responses to Pic have been assessed in other rodents. The virus produces an infection in the MHA strain of Syrian hamsters which simulates human Lassa fever (Buchmeier and Rawls, 1977; Murphy et al., 1977). As in human arenavirus haemorrhagic fever syndromes, the immune response appears to serve only a protective role and is not implicated in causing pathology (Peters et al., 1987). Antibodies to viral antigens appear at 10-14 days in both susceptibe MHA and resistant LVG hamsters (Buchmeier and Rawls, 1977). However, the susceptibility of MHA hamsters to Pic may be related to their failure to mount a DTH response, in contrast to resistant strains of hamster (Gee et al., 1981; Chan et al., 1983; Peters et al., 1987). Protective T cell epitopes may exist on NP; a vaccinia construct containing Pic NP was able to modulate disease and delay death in MHA hamsters after challenge with Pic. These results are consistent with earlier results by Wright et al. (1987) where transfer of IL-2 into susceptible hamsters prior to infection slowed death and suggested that immunization with NP may activate helper T-cells that produce IL-2.

Infection of strain 13 guinea pigs with Pic has also been employed to serve as a model for human haemorrhagic fever. Antibodies appear on day 10 p.i. simultaneously with viraemia (Jahrling et al., 1981) but presence of antibodies does not prevent mortality, which is constantly 100% between 12 and 25 (mean 14.8) days after infection. Disease does not appear to

be due to immunopathological processes in this model (Jahrling et al., 1981; Peters et al., 1987; Lucia et al., 1989; 1990; Katz and Starr, 1990). The relative sensitivity of inbred strain 13 guinea pigs to Pic infection compared with outbred guinea pigs is presently unexplained, and may be due to differences in cellular immune responses.

7.1 Role of carbohydrate in immunogenicity and antigenicity

It has been suggested that glycosylation may play a role in directing and maintaining folding of proteins. For viral proteins this can be measured by determining whether N-linked glycosylation is essential for formation of conformational epitopes. Such a role for glycosylation has been demonstrated for the glycoproteins of several viruses, including LCMV (Wright et al., 1989; 1990b), Sendai virus (Vidal et al., 1989), HIV-1 (Fennie and Lasky, 1989), BHV-1 (vanGrunen-Little-Van Den Hurk and Babiuk, 1985), VSV (Gibson et al., 1978; 1980), Semliki Forest virus (Kaluza and Pauli, 1975), Bovine leukemia virus (Bruck et al., 1984), murine leukemia virus (Pierotti et al., 1981), Newcastle disease virus (Long et al., 1986), and influenza C (Sugawara et al., 1988). These viruses possess conformational neutralizing epitopes that are absent after growth of the viruses in tunicamycin. However, once an epitope is formed, removal of carbohydrates does not affect conformation, as shown for parainfluenza (Gorman et

al., 1991) and murine leukemia virus (Pierotti et al., 1981).

Most conformational epitopes on viral proteins that have been examined rely on the normal addition of carbohydrate during synthesis; this suggests that increasing or decreasing the number of glycosylation sites that are utilized could alter the conformation of the protein. This has been demonstrated for a conformational epitope on gD of HSV-1, where the loss of a single carbohydrate through mutagenesis of a consensus sequence led to altered conformation of the protein as determined by binding of monoclonal antibodies (Sodora et al., 1989).

Addition of extra carbohydrate molecules could also directly block linear epitopes. This has been demonstrated in several virus systems after growth of virus in the presence of neutralizing MAb, for example, influenza (Skehel et al., 1984) Sindbis (Davis et al., 1987; Strauss et al., 1991) and rotavirus (Caust et al., 1987; Shaw et al., 1988; Mackow et al., 1988).

This type of modulation of neutralization epitopes by carbohydrate has also occurred in arenaviruses. Two conformational neutralizing epitopes (A and D) are present on GP1 of LCMV Armstrong strain, and both are dependent on N-linked glycosylation. However, epitope D is absent from certain isolates of the Armstrong strain as well as from LCMV WE strain (Buchmeier and Parekh, 1987). Strains of the virus that lack this epitope have one extra N-linked carbohydrate at

aa 171 (Figure 2), (Wright et al., 1989) indicating that this epitope is either directly blocked or has an altered conformation due to the extra carbohydrate moiety.

8.) Vaccinia expression system

Vaccinia virus is a member of the poxvirus family which contains complex DNA viruses that infect both vertebrates and invertebrates. Infectious poxvirus particles contain a transcriptional system (Kates and McAuslan, 1967a, Munyon et al., 1967; Caplan and Holoczak, 1983) that can synthesize mRNA that is polyadenylated (Kates and Beeson, 1970), capped and methylated (Wei and Moss, 1975). Expression of foreign genes by recombinant vaccinia was first described in 1982 (Mackett et al., 1982). A similar approach has been used with other members of the poxvirus family, most notably fowlpox (Boyle and Coupar, 1988; Taylor et al., 1988a; 1988b). The genome of vaccinia virus is composed of a single linear dsDNA molecule of approximately 186 Kb (Moss, 1990). Hairpin structures connecting the two strands are present at each end of the DNA (Geshelin and Berns, 1974). A considerable portion of vaccinia genome has been sequenced (Moss, 1985; 1992). Most of the essential genes are present within the central region of the DNA which is highly conserved. Many of the genes that are dispensable for replication and those involved in host range are near the ends (Earl and Moss, 1987). The apparent

absence of introns, the relatively short promoter sequences, and the relatively small sizes of many open reading frames account for the packing of an estimated 150-200 genes into vaccinia virus.

Within minutes after infection of cells with vaccinia about 100 early mRNAs can be detected (Kates and McAuslan, 1967b; Kates and Beeson, 1970). Synthesis of DNA polymerase and other early gene products permits replication of vaccinia DNA to occur in the cytoplasm of the infected cells (Cairns, 1960; Harford et al., 1966). DNA replication signals the start of late gene expression, which can be detected about 4h p.i., and the decline in expression of early genes. There are about 100 late genes which encode the major structural polypeptides as well as some of the enzymes incorporated into virus particles. Infectious vaccinia particles can be detected in the cytoplasm 6h p.i. and continue to accumulate for about 48h and must be released by cell lysis (Moss, 1985; 1992).

The large size of the vaccinia virus genome makes homologous recombination a possible way of inserting foreign genes. This is carried out by transfecting engineered plasmids into cells that have been infected with vaccinia virus. In this manner, foreign DNA of up to 25 Kb has been incorporated into the vaccinia genome (Smith and Moss, 1983). Insertion of the foreign gene must be into a silent region of the genome or into nonessential genes, in order to retain

infectivity. The prototype vector, pGS20, was originally designed to facilitate the construction of recombinant viruses (Mackett et al., 1984). The prototype vector and its derivatives such as pSC11 (Chakrabarti et al., 1985) contain a segment of vaccinia virus DNA within which an expression cassette, consisting of a vaccinia promoter adjacent to one or more unique restriction endonuclease sites for gene insertion, has been placed. The vaccinia virus DNA flanking the cassette serves to guide recombination to a nonessential site in the genome. Since poxviruses have their own transcription system, the use of poxvirus promoters is obligatory.

The most extensively used promoter, P7.5, is a compound promoter with both early and late transcription start sites, thereby providing for continued expression throughout the growth cycle (Venkatensan et al., 1981; Cochran et al., 1985). The late gene promoter, P11, (Bertholet et al., 1985) provides delayed higher levels of expression after DNA replication. Higher levels of expression have been achieved using a synthetic late gene promoter (Davidson and Moss, 1991) and with the hybrid vaccinia/bacteriophage T7 expression system (Fuerst et al., 1987). Insertion of two heterologous genes into vaccinia virus during a single recombination event also has been successful using a vector (pDAVAC2) that contains a bidirectional P7.5 promoter (Morrison et al., 1990). The most popular insertion site has been the thymidine kinase (TK) gene (Hruby et al., 1983; Weir and Moss, 1983) since, as will be

discussed below, it provides for a method of selecting recombinant viruses.

Vaccinia virus has proven to be a very useful tool for expressing heterologous genes. It has been used to express more than a hundred different foreign genes including both viral and non-viral genes (reviewed by Piccini and Paoletti, 1988). Proteins made by recombinant vaccinia viruses appear to be processed and transported in a manner similar if not identical to that occurring in uninfected cells (reviewed by Mackett, 1987; Moss and Flexner, 1987; Moss, 1992).

Numerous recombinant vaccinia viruses have been also generated to study proteins or peptides of arenaviruses, including Pic (Ozols et al., 1990), Lassa virus (Clegg and Oram, 1985; Clegg and Lloyd, 1987; Auperin et al., 1988; Fisher-Hoch et al., 1989; Morrison et al., 1989; 1990) and LCMV (Whitton et al., 1988a; 1988b; 1989a; 1989b; Hany et al., 1989; Shultz et al 1989).

9.) Hypothesis

The major glycoprotein of Pic is heavily glycosylated and Pic fails to elicit neutralizing antibodies. The failure to elicit neutralizing antibodies may be due to the masking of epitopes or effects on the conformation of GP1 by the presence of extra carbohydrate(s), as shown for LCMV.

The hypothesis states that removal of carbohydrates before immunization will expose or create new epitopes, or produce epitopes with increased immunogenicity that might be targets for neutralizing antibody.

10.) Objectives

The general objective is to study the role of glycosylation in modulating the antigenicity and immunogenicity of Pichinde virus glycoproteins.

Specific Aims

1. Cloning of the glycoproteins of Pichinde virus.

2. Expression of Pichinde virus glycoproteins in a vaccinia virus expression system.

3. Removal of a potential glycosylation site from wild type GP1 by site directed mutagenesis of amino acid 183.

4. Expression of the mutant Pichinde virus glycoproteins in vaccinia virus expression system.

5. Assessment of immunogenicity of wild type and mutant Pichinde virus glycoproteins expressed by vaccinia virus.

CHAPTER 2 MATERIALS AND METHODS

I. Cells, viruses and virus infection

I.1) Cells

BHK-21 (baby hamster fibroblast) and Vero (African green monkey kidney fibroblasts) cell lines were kindly provided by Dr. M.J. Buchmeier, Research Institute of Scripps Clinic, La Jolla, California. CV-1 (African green monkey kidney) and 143B (human osteosarcoma thymidine kinase negative) cells were kindly provided by Dr. K.Dimock, University of Ottawa, Ottawa, Ontario. Cells were grown as monolayers in 25 cm^2 tissue culture flasks (Corning, Glass Works, Corning, NY). All media and reagents were purchased from GIBCO BRL, Burlington, Ont. Canada.

Cells were grown in DMEM or MEM media supplemented with 7% or 10% (CV-1) heat inactivated FBS, 1% L-glutamine with or without anti-PPLO agent (1X) and penicillin-streptomycin (100 units/ml penicillin G sodium, 100 ug/ml streptomycin sulfate). The monolayers were incubated at 37°C in 5% CO_2.

I.2) Viruses

Stocks of Pichinde virus strain AN3739 were obtained from Dr. M.J. Buchmeier, Research Institute of Scripps Clinic, La Jolla, California. Wild type vaccinia virus, WR strain, and the recombinant vaccinia virus containing the *lacZ* gene, vSC8,

were kindly provided by Dr. D. Harnish, McMaster University, Hamilton, Ontario, with permission of Dr. B. Moss, National Institute of Allergy and Infectious Diseases, Bethesda, MD.

I.3) Infection and Preparation of Virus Stocks

Infection of cells with Pichinde virus was performed at a MOI of 0.1, unless otherwise mentioned. The virus was allowed to adsorb for 1 h (37°C, 5% CO_2) with occasional rocking. The inoculum was aspirated and fresh medium was added. Cells were further incubated for 24-48 h, before the supernatant was harvested and stored at -80°C.

Infection of cells with vaccinia virus was performed in a similar manner at a MOI of 0.1-0.5 for 1 h in MEM without FBS. The inoculum was removed and fresh MEM supplemented with FBS was added for 24-72 h. Vaccinia virus infected cells, scraped from tissue culture flasks, were frozen, at -80°C, and thawed three times. Prior to infection, vaccinia virus was incubated with 10% trypsin at 37°C for 15 min to reduce clumping of the virus (Mackett et al., 1985).

I.4) Pichinde Virus Purification

Pichinde virus was purified as described by Buchmeier and Oldstone, (1979). Confluent monolayers of BHK cells were infected at a MOI of 0.1. After 1h adsorption at 37°C, the inoculum was replaced with DMEM medium. Extracelluler fluid was harvested at 48h postinfection. After a clarifying spin

at 7000 rpm (Beckman JA10 rotor) for 30 min, the supernatant was adjusted to 7% polyethylene glycol. After sitting at 4°C for 2h, the suspension was pelleted at 8000 rpm for 30 min. The pellets were resuspended in cold TNE buffer (0.01 M Tris-HCl, pH 7.5, 0.1 NaCl, 0.001 M EDTA), and layered onto a 50% (10 ml) and 25% (20 ml) discontinuous MD-76 (diatrizoate meglumine / diatrizoate sodium, Mallinckrodt Canada, Pointe Claire, Que.) gradient in TNE buffer. After centrifugation at 24,000 rpm (Beckman SW28 rotor) for 16h at 4°C, the visible virus band, at the interface of the 25% and 50% solutions, was collected, diluted 1:3 with TNE buffer and layered onto a continuous MD-76 gradient (25, 35, 45%). After centrifugation at 24,000 rpm for 16h at 4°C, the visible virus band was collected and diluted 1:1 with TNE buffer. After a final spin at 28,000 rpm (Beckman SW28 rotor) for 90 min, the supernatant was removed and the pellets were resuspended in 300 ul of TNE buffer. Purified virus was stored at -80°C.

I.5) Plaque assays

All assays were performed in duplicate and the average score was recorded. Pic virus was titrated on monolayer cultures of Vero cells in 6 well tissue culture plates (Nunc/Gibco, Montreal, Quebec). Ten-fold serial dilutions in MEM media in a volume of 0.3 ml/well were used to infect cells. One hour post infection, the inoculum was removed and cells were overlaid with 3 ml of 1 X 199 media with 0.8% agar and incubated at 37°C (5% CO_2). Three days later, 2 ml of a

second overlay, which contained 0.1 mg Neutral Red, were added. Cells were further incubated for 24 h. Clear plaques were counted.

Vaccinia virus was titrated on CV-1 cells as above except that agarose rather than agar was used in the overlay. In this case the cells were incubated for 48 h after the first overlay, then fixed with 1 ml of 10% formaldehyde and 0.8% NaCl in H_2O for 1 h at 37°C. The overlay was removed and cells were stained with 0.1% crystal violet.

I.6) Production of recombinant vaccinia virus

CV-1 cells were infected with wild type vaccinia virus, WR strain, at a MOI of 0.1 PFU/cell. One hour post infection, the cells were transfected with the recombinant plasmid, pSC-GPC, as follows: 20 ug of DNA was mixed with 0.25 M CaCl in 500 ul then added to an equal amount of 2 X BBS (50 mM N,N-bis [2-Hydroxyethyl]-2 aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM Na_2HPO_4). The mixture was incubated at room temperature for 20 min. The DNA mix was added, dropwise, to the monolayer. Eight hours post infection-transfection, medium was removed and fresh medium was added. Cells were incubated for another 36 h, then the virus was harvested.

I.7) Selection of recombinant vaccinia virus

Ten-fold serial dilutions of vaccinia virus were performed in serum free media and cells were infected as above, except that TK^- 143 cells were used in 100 mm plates (Corning). The monolayer was overlaid with 12 ml of 1 X 199 media, 0.8% agarose and 25 ug/ml BudR. After 2 days the cells were overlaid with 12 ml of 1 X 199 media, 0.8% agarose and 300 ug/ml Bluo-Gal. Blue plaques were picked after 24 h using a sterile pasture pipet. Each vaccinia plaque was resuspended in 0.5 ml of serum free MEM media, frozen and thawed 3 times, and plaque purified 3 times.

I.8) Immunization of animals

Antiserum to recombinant vaccinia virus was obtained from a rabbit which was initially injected with $5x10^6$ pfu intravenously. A second injection ($2.5x10^6$ pfu) was administered 4 weeks later. The animal was bled 10 days later and serum was prepared and stored at -20°C in 1 ml aliquots.

I.9) Neutralization assay

Neutralization assays were performed in six-well tissue culture plates seeded with Vero cells. Four-fold serial dilutions of sera were incubated with virus (approximately 150 PFU) for 1h at 37°C. A negative control was included which contained no serum. Each mixture was added to a Vero monolayer in a six-well plate which was approaching 100%

confluence. Following adsorption for 1h at 37°C, cells were overlaid with 3 ml of 1x199 media and 0.8% agar. Three days later a second overlay, which contained 0.1 mg Neutral Red was added. Plaques were counted 24h later. Plaque reduction and inhibition of neutralization were done in duplicate. A serum was considered to neutralize virus when plaque number was reduced in a linear fashion with increasing amounts of serum compared to the negative control.

II. Cloning techniques

II.1) Isolation of Pic virus genomic RNA

Purified Pic virus was resuspended in TNE buffer containing 50 ug/ml proteinase K (Boehringer Mannheim, Canada). An equal volume of vanadium-NSD (20 mM) was added and the suspension was transferred to a 1.5 ml microfuge tube. Following incubation at 37°C for 30 min, the mixture was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). Two volumes of ethanol were added to precipitate the RNA, which was stored at -80°C.

II.2) Electrophoresis of nucleic acids

Electrophoresis was performed in the Hoefer Scientific Instruments MINNIE or MAX Submarine Gel Units. Nucleic acids were separated in 0.8-1% agarose gel in 10 mM phosphate

buffer, pH 7.0 (6.1 mM Na_2HPO_4, 3.9 mM NaH_2PO_4) (RNA) or TBE buffer (89 mM Tris, 89 mM Boric acid, 2 mM EDTA) (DNA). Electrophoresis was at 50-100 Volts for 2-3 h or 35 Volts overnight with constant recirculation of buffer (RNA). RNA was denatured in the presence of 0.9 M (6%) glyoxal (BDH), 50% DMSO and 10 mM phosphate buffer at 60°C for 15 min. DNA samples were prepared for electrophoresis by mixing with a 1/6 volume of DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol). After electrophoresis, nucleic acids were stained either with 30 ug/ml acridine orange (RNA) or 0.5 ug/ml Ethidium Bromide (EtBr) (DNA). Bands of RNA or DNA were visualized by short - wave UV irradiation. Gels were photographed using polaroid type 667 film.

II.3) Reverse transcription

Oligonucleotides synthesized on an Applied Biosystem Model 380B DNA Synthesizer (Mississauga, Ont.) were used to prime cDNA synthesis using viral RNA as a template and avian myeloblastosis virus (AMV) reverse transcriptase (Life Science Inc., Petersburg, Florida, USA). The cDNA reaction consisted of the RNA in 100 mM Tris-HCl, pH 8.3, 130 mM KCl, 10 mM $MgCl_2$ and 2.5 mM DTT, 100 ng of primer, 1 mM of each of the four dNTPs and 22 units of reverse transcriptase in a total volume of 40 ul. The mixture was incubated at 42°C for 1 h then cDNA was extracted once with phenol/chloroform/isoamyl alcohol and

once with chloroform/isoamyl alcohol. The cDNA was precipitated in the presence of 2.5 volumes of ethanol and 0.5 volumes of 8 M NH_4 Ac.

II.4) Polymerase chain reaction

The cDNA was amplified by the polymerase chain reaction (PCR) using a PERKIN ELMER CETUS DNA thermal cycler (Norwalk, Connecticut). The reaction consisted of 10 ng cDNA in 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 1.5 mM $MgCl_2$ and 0.01% (w/v) gelatin, 100 ng of each of the two primers, 0.5 mM of each of the four dNTPs and 2.5 units of Taq DNA polymerise (BRL, Gaithersburg, MD, USA) in a total volume of 100 ul. Samples were overlaid with 100 ul of mineral oil. Denaturation of the template was at 94°C for 2:30 min. Afterwards the cycle profile was 1 min at 94°C (denaturation), 2 min at 48°C (annealing) and 3 min at 72°C (extension). An automatic extension time of 00:10 min was added to the 72°C extension step. Thirty cycles were performed. At the end of the 30th cycle, the 72°C extension step was prolonged for an additional 7 min. Samples were cooled to 4°C and DNA was extracted once with chloroform/isoamyl alcohol, once with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol. Samples were analyzed by agarose gel electrophoresis.

II.5) Site directed mutagenesis

Polymerase chain reaction was employed in site directed mutagenesis according to Kuipers et al., (1991) and Landt et al., (1990). A mutagenic oligonucleotide (Pic-183) that contained a single mismatch was designed. This would result in the desired mutation when used as a primer in PCR. PCR was carried out as described before except that approximately 10 ng of plasmid DNA harbouring wild type GPC gene was used as template for PCR and 100 ng of each of the mutagenic primer (Pic-183) and the universal primer (T7) were used in 22 cycles. Each cycle consisted of a denaturation step at 94°C for 1 min., an annealing step at 52°C for 1:30 min. and an extension step at 72°C for 1 min. Samples were analyzed by agarose gel electrophoresis and DNA fragments were eluted using Gene Clean II (Bio/Can Scientific Inc, Toronto, Ont.). Approximately 10 ng of the eluted PCR product was used as a primer in a second phase of PCR along with a second universal primer (Sp6) and 100 ng of plasmid DNA harbouring the wild type GPC gene. PCR was carried out for 27 cycles, as described before except that the annealing step was at 54°C for 1:30 min and the extension step was at 72°C for 2 min.

II.6) Electroelution

Gels were stained with EtBr and DNA was visualized under long wave UV irradiation. Bands of interest were cut out with a scalpel. The agarose slices were placed in the cup of the

IBI Model UFA Electro-Eluter (IBI) and the apparatus was filled with 500 ml of 0.5 X TBE buffer. The salt bridge was filled with 125 ul of 7.5 NH_4 Ac. and 0.01% Bromophenol blue through the diagonal opening. Electroelution was performed for 1 h at 100 Volts. The electroeluted sample (300 ul) was removed using a small piece of intermedic tubing attached to the end of a yellow micropipet tip. The sample was extracted twice with an equal volume of H_2O saturated n- butanol. The DNA was then precipitated with 2.5 volumes of ethanol and stored at -20°C.

II.7) Restriction endonuclease digestion

DNA was digested with restriction enzymes (Pharmacia, Promega or New England Biolabs) in a total volume of 20 ul following the manufacturer's recommendations for reactions. At the end of the incubation period, the restriction enzyme was heat inactivated at 65-85°C and DNA was precipitated with 2.5 volumes of ethanol.

II.8) Fill in of 3' overhangs

After restriction digestion, DNA was incubated in 0.05 M Tris, pH7.6, 0.01 M $MgCl_2$, 0.02 mM of each dNTP and 2.3 units of Klenow fragment of DNA polymerase (Pharmacia LKB Biotechnology, Pharmacia Canada Inc., Baie d'Urfe, Quebec) at 24°C for 30 min in a total volume of 16 ul.

II.9) Dephosphorylation of plasmid DNA

After restriction digestion, plasmid DNA was incubated in 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 10 mM Tris-HCl, pH 8.3, and 1u/pmole calf intestinal alkaline phosphatase (BRL) at 37°C for 15 min in a total volume of 100 ul. The dephosphorylated vector was extracted once with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol.

II.10) Phosphorylation of PCR products

cDNA amplified by the polymerase chain reaction (PCR) was phosphorylated prior to ligation. cDNA was incubated in one-phor-all buffer (Pharmacia), 0.01 mM ATP and 2.5 units of polynucleotide kinase (PNK) (Pharmacia) for 1 h at 37°C in a total volume of 20 ul. PNK was then heat inactivated at 75°C for 10 min.

II.11) Ligation

250 ng of a restriction fragment or a PCR product and 150 ng of the linearized plasmid were incubated in 0.05 M Tris, pH 7.5, 0.01 M $MgCl_2$, 5% PEG, 1mM ATP, 1 mM DDT, 0.5 ug/ul of BSA and 3.25 u of T_4 DNA ligase (New England Biolabs, Beverly, MA, USA) at 16°C for 4-16 h in a final volume of 10 ul.

III. Transformation of E. coli and plasmid preparation

III.1) Preparation of competent E. coli

A single colony of Escherichia coli, DH5αF', (BRL, Burlington Ontario) was used to inoculate 10 ml of 2YT media (1.6% tryptone, 1% yeast extract, 0.55 NaCl) and cells were allowed to grow at 37°C for 16 h without shaking. The over night culture was used to inoculate 100 ml of P medium (20 mM KPO_4, pH 7.0, 15 mM $(NH_4)_2$ SO_4, 1.8 uM $FeSO_4$, 10 mM $MgSO_4$, 1% Casamino acid, 0.2% Glucose) at 37°C with shaking until OD_{600} reached 0.4. The culture was cooled to 4°C and centrifuged at 4000 rpm for 5 min at 4°C in a Beckman JA20 rotor. The pelleted cells were washed with 100 ml of ice cold 10 mm NaCl and centrifuged as above. E. coli were resuspended in 50 ml of 50 mM $CaCl_2$ and placed on ice for 15 min. The cells were centrifuged one more time as above and resuspended in 10 ml of 50 mM $CaCl_2$ in 16% glycerol. Competent cells were immediately frozen in an ethanol/dry ice bath and placed at - 80°C in 200 ul aliquots.

III.2) Transformation of E. coli

Plasmid DNA, 10-20 ng, were mixed with 200 ul of competent cells. The mixture was kept on ice for 30 min, heat shocked at 42°C for 3 min, 800 ul of LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) were added to cells which were shaken at 37°C for 1 h. Transformed cells were spread on LB

agar plates containing 50ug/ml ampicillin. Plates were incubated for 16 h at 37°C.

III.3) Small scale preparation of plasmid DNA

A single colony of transformed E.coli was inoculated into 5 ml of LB medium containing 50 ug/ml ampicillin. Cells were allowed to grow at 37°C for 16 h with shaking. Cells from 1.5 ml of overnight culture were pelleted at 12,000 rpm for 2 min. The supernatant was aspirated and cells were resuspended in 500 ul of STET solution (8% sucrose, 0.5% triton X-100, 50 mM EDTA, pH 8, 10 mM Tris-HCl, pH 8) containing 350 ug lysozyme. Cells were disrupted at 95°C for 40 sec then cell debris was pelleted at 12,000 rpm for 15 min at 24°C. The supernatant was transferred to fresh microfuge tubes and 1 ug of RNase was added. Samples were incubated at 37°C for 30 min then DNA was extracted once with phenol/chloroform/isoamyl alcohol and once with chloroform /isoamyl alcohol. DNA was precipitated with 35 ul of 3 M NaAc and 420 ul of isopropanol at - 20°C for 30 min.

III.4) Large scale preparation of plasmid DNA

A single colony of transformed E. coli was used to inoculate 5 ml of LB medium containing 50 ug/ml ampicillin and cells were allowed to grow at 37°C for 16 h with shaking. 200 ul of the over night culture were used to inoculate 25 ml of LB medium containing ampicillin at 50 ug/ml. Cells were

allowed to grow as above then the culture was used to inoculate 1 litre of LB medium containing ampicillin. Three hours later 170 ug/ml of chloramphenicol were added and the incubation was continued over night. Cells were pelleted at 6000 rpm for 15 min at 4°C in a Beckman JA10 rotor and resuspended in 6 ml of solution I (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8) containing 20 mg lysozyme. Cells were incubated at 24°C for 10 min then 12 ml of solution II (200 mM NaOH, 1% SDS) were added and cells were further incubated at 24°C for 10 min. To this mixture 9 ml of NH_4Ac were added and the cells were placed on ice for 20 min. Cell debris was spun down at 20,000 rpm for 25 min at 4°C in a Beckman SW28 rotor. DNA was precipitated using 10 ml of isopropanol. The pellet was resuspended in 4.2 ml of TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8), CsCl and EtBr were added to give final concentrations of 1.1 g/ml and 0.8 g/ml respectively. The plasmid fraction was centrifuged to equilibrium at 55,000 rpm for 16 h at 20°C in a VTi 65 rotor (Beckman Ultracentrifuge L8-55M). The plasmid band was collected and EtBr was removed by extraction 6 times with an equal volume of H_2O saturated n-butanol. DNA was precipitated by adding 2 volumes of ethanol. After incubation at - 20°C for 30 min, DNA was pelleted and resuspended in TE (10 mM Tris-HCl, pH 7.2, and 1 mM EDTA) buffer. Quantitation of DNA was done using a DMS 200 UV-visible spectrophotometer (Varian Canada Inc., Georgetown, Ont.) and the concentration of DNA was adjusted to 1 mg/ml.

III.5) Sequence analysis

Dideoxynucleotide sequence analysis (Sanger et al., 1977) was performed using the Sequenase kit (United States Biochemical Corporation) essentially as described by the manufacturer. Plasmid DNA was denatured with 200 mM NaOH, 0.2 mm EDTA at room temperature for 5 min. The DNA mixture was neutralized with 300 mm Na Ac, PH 5.0 and DNA was precipitated with 2 volumes of ethanol. Three ug of plasmid DNA was annealed with 50 ng of primer (synthesized with an Applied Biosystem 380B DNA Synthesizer, University of Ottawa Biotechnology Research Institute) in 10 mM Tris-HCl, pH 8.0 and 10 mM $MgCl_2$ in a volume of 9 ul. The annealing mix was incubated for 2 min at 65°C and slowly cooled down to 35°C. The DNA /primer hybrid was aliquoted into 4 tubes for the different A, C, G, and T reactions. The A reaction contained 12 uM ddATP and 25 uM dCTP, dGTP and dTTP; the C reaction contained 40 uM ddCTP, 3.5 uM dCTP, 35 uM dGTP and dTTP; the G reaction contained 200 uM ddGTP, 3.2 uM dGTP and 35 uM dCTP and dTTP; and the T reaction contained 600 uM ddTTP, 3.2 uM dTTP, 35 uM dCTP and dGTP. Each reaction contained 6.5 uCi (3.2 uM, final concentration) [α-^{32}S]dATP (Amersham) and 0.5 units of Sequenase™ enzyme. Reaction mixtures with a final volume of 3.5 ul were incubated at room temperature for 5 min and chased at 37°C for 7 min with approximately 300 uM dNTPs in a final volume of 6 ul. The reactions were stopped with 4 ul of 90% formamide buffer containing 10 mm EDTA, 0.3% xylene

cyanol, bromophenol blue and orange G. The reactions were heated at 95°C for 3 min immediately before loading onto 6% polyacrylamide (5.7% acrylamide - 0.3% bis-acrylamide) - 7 M urea in TBE buffer. Electrophoresis was performed in the IBI (New Haven, CT.) model STS-45 Thermoplate Sequencing Apparatus at a constant power of 50 Watts.

IV) Protein analysis

IV.1) Radiolabelling of infected cells

Infected monolayers were washed with PBS (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$) then starved for 1 h in 1.5 ml of either methionine - free MEM or sugar - free DMEM. Cells were labeled for 15 - 60 min with 100 uCi of L- [^{35}S] methionine or glucosamine hydrochloride, D-[1,6-^3H(N)] (DU PONT Canada Inc., Markham, Ont.) respectively.

IV.2) Preparation of cell lysates

Cells were washed twice with 2 ml of PBS then 1 ml of lysis buffer (20 mM Tris, 137 mM NaCl, 1mM $CaCl_2$, 0.5 mM $MgCl_2$, 1% (v/v) NP-40, 10% (v/v) glycerol, 1% (v/v) aprotinin, 1mM phenylmethylsulfonyl fluoride, 1 mM benzamidine hydrochloride) was added. Cells were lysed on ice for 10 min and transferred to microcentrifuge tubes. Cell debris was cleared by

centrifugation at 14,000 rpm for 20 min at 4°C and the supernatant was frozen at - 80°C.

IV.3) Immunoprecipitation

Cell lysates were shaken for 2 h at 4°C in the presence of antibody at a final dilution of 1:100 and 100 ul of washed protein A sepharose CL-4B beads at 30 mg/ml (Sigma). Precipitates were collected by centrifugation and washed 4 times in wash buffer (100 mM Tris, 500 mM LiCl) before resuspending in 20 ul of sample buffer (0.125 M Tris, 2% SDS, 20% glycerol, 2% 2-mercaptoethanol). Antisera used in immunoprecipitation were as follows; mouse anti-Pic, prepared by Dr. K.E.wright, hamster anti-Pic, from Dr. W.E.Rawls, guinea pig anti-Pic, and anti-LCMV monoclonal 33.6, obtained from Dr. M.Buchmeier.

IV.4) SDS - Polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analyzed by SDS-PAGE (Laemmli, 1970) on slab gels. Separating gels were composed of 10% acrylamide, 0.13% bis-acrylamide, 0.095% SDS, and 0.35 M Tris, pH 8.8. Stacking gels were composed of 5% acrylamide, 0.065% bis-acrylamide, 1% SDS, and 0.125 M Tris, pH 6.8. Electrophoresis buffer consisted of 0.025 Tris, pH 8.3, 0.192 M glycine and 0.1% SDS. Samples were heated to 90°C for 3 min before loading onto the gel. Electrophoresis was performed at constant current, 7 mA, for 16 h using the Bio-rad Protean II

Slab Electrophoresis Cell. After electrophoresis, gels were fixed in 7.5% acetic acid and 20% methanol for 60 min and incubated in Amplify (Amersham) for 30 min. Gels were dried using the Bio-rad Model 583 Gel Dryer for 75 min prior to exposure to X-ray films at -80°C.

IV.5) Western blotting

SDS-PAGE was performed on 10% slab gel as described before, section IV.4. Purified virus was loaded at 5.7 ug/well in 1% SDS sample buffer and electrophoresis was carried out in the cold as described in Swack et al., 1987. Proteins were electrophoretically transferred at 4°C onto nylon membrane (Millipore) at 0.5 A for 3h in blotting buffer (25 mM Tris, 192 mM glycine, 20% (V/V) methanol and 0.1% SDS, pH 8.3, (Burnette, 1981). Free protein binding sites were blocked with 2% milk powder in PBS-0.05% Tween-20 for 3h at room temperature. Blots were incubated with varying amounts of antibody in PBS-Tween for 1h. Following three 15 min washes in PBS-Tween, membranes were incubated with 1:2000 dilution of protein A alkaline phosphatase (Sigma) for 1h at room temperature. Membranes were washed three times and antigens were visualized by incubating membranes in carbonate buffer (100 mM $NaHCO_3$, 1 mM $MgCl_2$, pH 9.8) containing 0.3 mg/ml of NBT (p-nitroblue tetrazolium chloride) (Sigma) and 0.15 mg/ml of BCIP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt) (Sigma) for 10-15 min. The colour development

was stopped by placing the membrane in distilled water.

IV.6) Intracellular Immunofluorescence

BHK-21 cells were seeded on coverslips (Canlab, Mississauga, Ont.) on a six well tissue culture plate. Twenty four hours later, cells were infected at a MOI of 1 for Pic virus and 0.2 for vaccinia virus for 24h. Coverslips were washed 3 times in PBS prior to fixation in 100% acetone at -20°C for 10 min. Coverslips were allowed to air dry washed with PBS. Primary antibody was added to each triplet of coverslips at final dilutions of 1:30, 1:90, and 1:270 in PBS. Following incubation at for 1h, at 37°C and 5% C_2O, slides were washed 3 times with PBS and fluorescein-conjugated sheep anti-mouse (Jackson Immunoresearch Laboratories Inc., West Grove, PA) was added at a final dilution of 1:100 in PBS containing 2% BSA. After further incubation for 1h cells were washed 3 times, air dried and mounted with 90% glycerol in PBS, pH 9.0. Slides were examined using a fluorescent microscope.

IV.7) ELISA

Purified virus at a concentration of 0.5ug/well was coated onto 96-well flat-bottomed plates (Linbro) in PBS at room temperature. Following overnight incubation, plates were blocked for 1h with 2% skim milk powder in PBS containing 0.05% Tween-20. Antibodies were titrated in fourfold

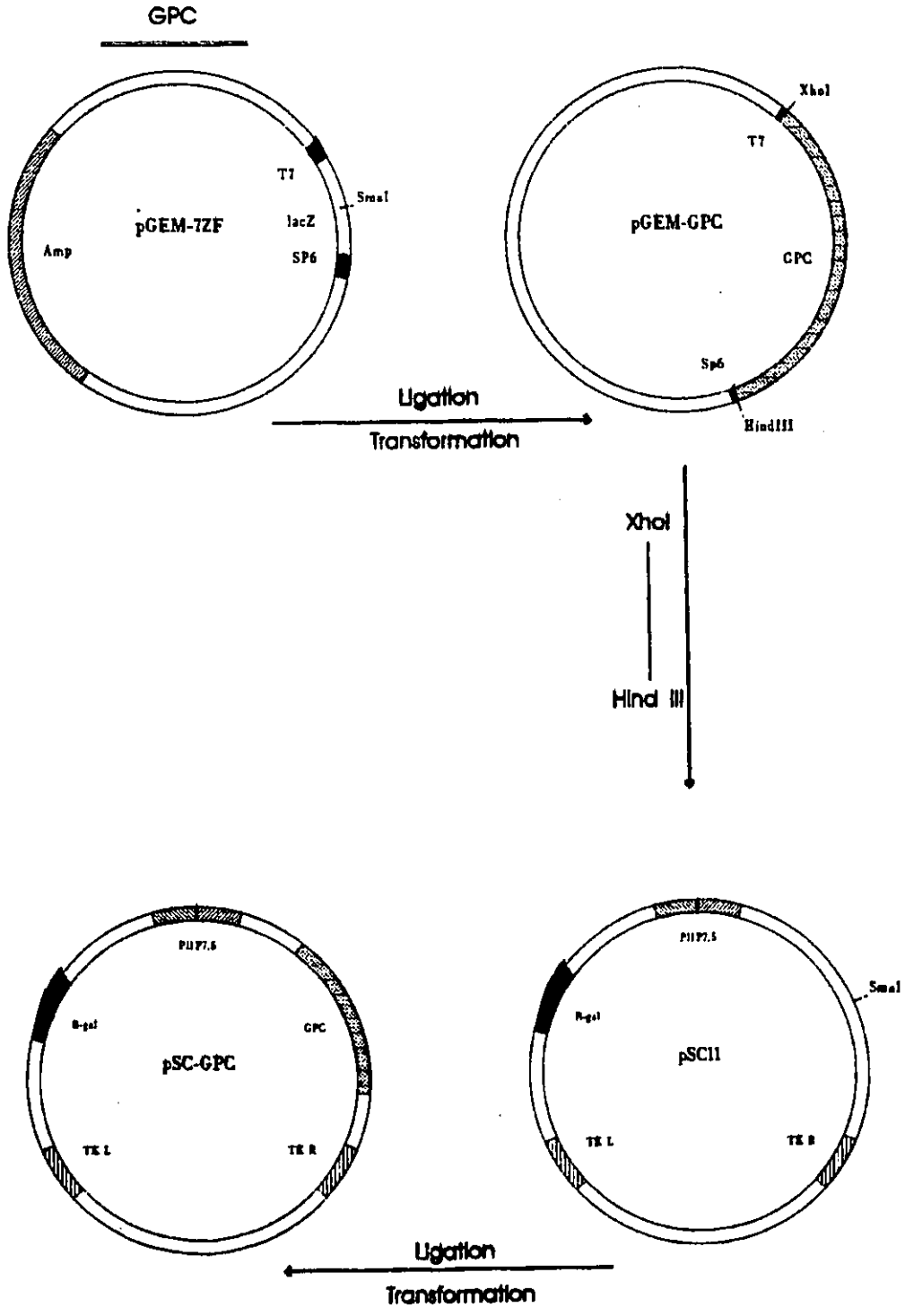
dilutions in a volume of 100 ul. After a 60 min. incubation, the plates were washed and bound antibody was detected with protein A-peroxidase (1:1500) (Sigma) and O-phenylenediamine substrate (Bio-Rad) as described by Parekh and Buchmeier, (1986). Wells were considered positive for antibody when O.D. was greater than O.D. for pre-immune serum by 2 standard deviations.

CHAPTER 3 RESULTS

1.) Cloning and Sequencing of Pichinde Virus Glycoprotein Coding Sequences

The strategy used to clone the coding sequences of Pichinde virus glycoprotein precursor, GPC, is summarized in Figure 3. To clone the coding sequence of Pichinde virus glycoproteins, a synthetic oligonucleotide, Pic-1, (Appendix 1) complementary to the 24 nucleotide sequence at the 3' terminus of Pichinde virus GPC gene (Auperin et al., 1984a) was used to prime cDNA synthesis. cDNA was amplified by PCR using Pic-1 and a second 24 mer primer, Pic-2, complementary to the 5' end of the coding sequence of Pichinde virus S RNA (Auperin et al., 1984a). The PCR product was electrophoresed and the expected product of 1512 bp was electroeluted. Pic-1 and Pic-2 were designed with ApaI and XhoI restriction sites for sticky-end cloning into the unique ApaI/XhoI sites of pGEM-7ZF (Appendix 1). Attempts to sticky-end clone the PCR product were not successful, so it was blunt ended using the Klenow fragment of DNA polymerase I. The GPC fragment was inserted into the dephosphorylated SmaI site of pGEM-7ZF which was used to transform E. Coli strain DH5αF'. Recombinant clones (pGEM-GPC) were screened by α-complementation (Maniatis et al., 1989). DNA from minipreps of positive colonies was analyzed by restriction digestion

Figure 3: Construction of pSC-GPC clone containing the wild type Pichinde virus GPC gene. The coding sequence of GPC gene (1512 bp) was inserted into the SmaI site of pGEM-7ZF to produce pGEM-GPC. The XhoI/HindIII fragment (1534 bp) was released and inserted into pSC11 to construct pSC-GPC.


GPC
pGEM-7ZF
T7
SmaI
lacZ
SP6
Amp
pGEM-GPC
XhoI
T7
GPC
Sp6
HindIII
Ligation
Transformation
XhoI
Hind III
pSC-GPC
GPC
TK L
TK R
pSC11
SmaI
TK L
TK R
Ligation
Transformation

with enzymes (Figure 4): PstI/ScaI (lanes 2, 6 and 10); XhoI(lanes 3, 7 and 11); and BamHI (lanes 4, 8 and 12). The expected fragments, 2648 bp and 1845 bp after PstI/ScaI digestion, linear 4500 bp after XhoI digestion and 3598 bp and 902 bp after digestion with BamHI, were obtained. Dideoxynucleotide sequence analysis was carried out for 4 pGEM-GPC clones using oligonucleotides (Pic-1, Pic-3,4,5,6, and Pic-7) (Appendix 1) complementary to the published sequence of the GPC gene (bases 1549-1563, 1249-1269, 941-960, 675-694, 426-445 and 183-202 respectively) (Auperin et al., 1984a). Only the viral sense DNA strand was sequenced. The start and stop codons were in frame. Three clones (EW-1, EW-3 and EW-4) were similar in sequence, while the fourth clone, EW-2, was different. The amino acid sequence of EW-1 and EW-2 were compared to the published sequence (Bishop and Auperin, 1987) and to the GPC gene of a temperature sensitive mutant of Pic, ts13, which was cloned and sequenced independently in our laboratory (Figure 5). When compared to the published sequence, EW-1 was found to have 6 nucleotide changes which led to 5 amino acid changes (aa 63, 77, 161, 426 and 477). EW-2 had the same changes except that aa 477 was preserved. The same changes observed in EW-1, plus some extra changes, were also found in ts13.

Figure 4: Agarose gel electrophoresis of pGEM-GPC. Plasmid DNA of 3 different clones was digested with PstI/ScaI, XhoI or BamHI, electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. Lane 1: λDNA digested with HindIII, lanes 2, 6, 10: pGEM-GPC cut with PstI/SacI, lanes 3, 7, 11: pGEM-GPC cut with XhoI, lanes 4, 8, 12: pGEM-GPC cut with BamHI, lanes 5, 9, 13: uncut pGEM-7ZF.

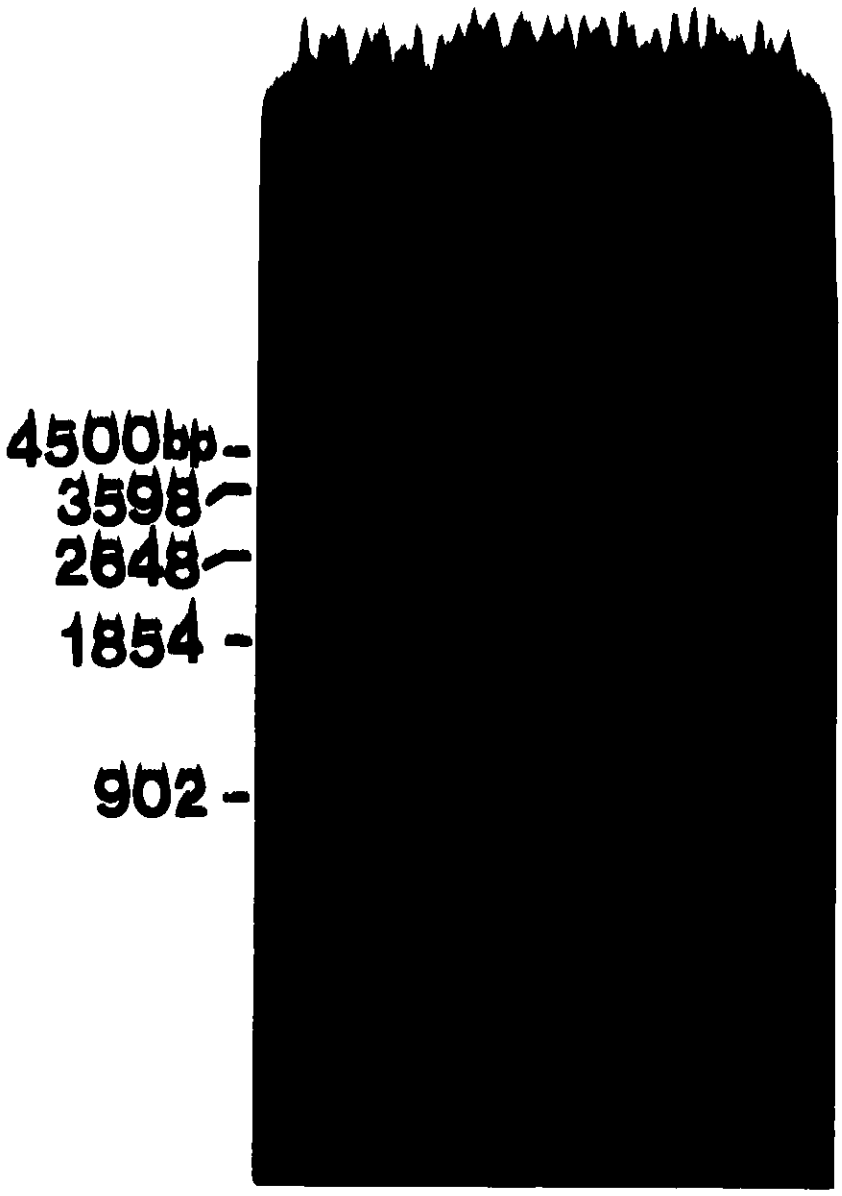

4500bp
3598
2648
1854
902

Figure 5: Amino acid sequence of the coding region of the glycoprotein precursor molecule of Pichinde virus. Two separate clones of Pichinde virus GPC, EW-1 and EW-2, derived from the same cloning experiment were sequenced and compared to the published sequence of Pic GPC (Bishop and Auperin, 1987). The GPC of a temperature sensitive mutant of Pichinde virus, ts13, was cloned and sequenced independently in our laboratory. Conserved amino acids are noted by dashes and areas not yet resolved are blank.

```
PIC      1 MGQIVTLIQS IPEVLQEVFN VALIIVSVLC IVKGFVNLMR
EW-1       ---------- ---------- ---------- ----------
EW-2       ---------- ---------- ---------- ----------
ts13       ---------- ---------- ---------- ----------

        41 CGLFQLVTFL ILSGRSCDSM MIDRRHNLTH VEFNLTRMFD
           ---------- ---------- --N------- ------Q---
           ---------- ---------- --N------- ------Q---
           ---------- ---------- --N------- ------Q---

        81 NLPQSCSKNN THHYYKGPSN TTWGIELTLT NTSIANETSG
           ---------- ---------- ---------- ----------
           ---------- ---------- ---------- ----------
           ---------- ---------- ---------- ----------

       121 NFSNIGSLGY GNISNCDRTR EAGHTLKWLL NELHFNVLHV
           ---------- ---------- ---------- ----------
           ---------- ---------- ---------- ----------
           -----R---- ---------G ---------- ----------

       161 TRHIGARCKT VEGAGVLIQY NLTVGDRGGE VGRHLIASLA
           A--------- ---------- ---------- ----------
           A--------- ---------- ---------- ----------
           A--------- ---------- ---------- ----------

       201 QIIGDPKIAW VGKCFNNCSG DTCRLTNCEG GTHYNFLIIQ
           ---------- ---------- ---------- ----------
           ---------- ---------- ---------- ----------
           ---------- ---------- ---------- ----------

       241 NTTWENHCTY TPMATIRMAL QRTAYSSVSR KLLGFFTWDL
           ---------- ---------- ---------- ----------
           ---------- ---------- ---------- ----------
           ---------- ---------- ---------- -       --

       281 SDSSGQHVPG GYCLEQWAII WAGIKCFDNT VMAKCNKDHN
           ---------- ---------- ---------- ----------
           ---------- ---------- ---------- ----------
           ---------- ---------V ---------- ----------

       321 EEFCDTMRLF DFNQNAIKTL QLNVENSLNL FKKTINGLIS
           ---------- ---------- ---------- ----------
           ---------- ---------- ---------- ----------
           D--------- ---------- ---------- ----------

       361 DSLVIRNSLK QLAKIPYCNY TKFWYINDTI TGRHSLPQCW
           ---------- ---------- ---------- ----------
           ---------- ---------- ---------- ----------
           ---------- S--------- -------- ----------

       401 LVHNGSYLNE THFKNDWLWE SQNLYNEMLM KEYEERQGKT
           ---------- ---------- -----S---- ----------
           ---------- ---------- -----S---- ----------
           ---------- ---------- -----S---- -----I----

       441 PLALTDICFW SLVFYTITVF LHIVGIPTHR HIIGDGCPKP
           ---------- ---------- ---------- ------Y---
           ---------- ---------- ---------- ----------
           ---------- ---------- ---------- ------Y---

       481 HRITRNSLCS CGYYKYQRNL TNG
           ---------- ---------- ---
           ---------- ---------- ---
           --------
```

2). Production of recombinant vaccinia virus expressing Pichinde virus glycoproteins.

In order to study the immunogenicity and antigenicity of Pichinde virus glycoproteins, we used the vaccinia virus expression system based on the shuttle vector pSC11 (Chakrabarti et al., 1985). The vector contains 2 vaccinia virus promoters flanked by sequences from the vaccinia virus thymidine kinase gene. The P7.5 promoter, a compound promoter, is derived from a viral gene expressing a 7.5 KD polypeptide with both early and late transcription start sites, thereby providing for continued expression throughout the growth cycle (Cochran et al., 1985). The late P11 promoter is derived from a late gene encoding an 11 KD polypeptide (Bertholet et al., 1985). pSC11 also contains unique restriction endonuclease sites placed downstream of the promoters. To facilitate selection of recombinant vaccinia, the E. Coli B-galactosidase gene was placed under the control of the P11 promoter (Chakrabarti et al., 1985).

The GPC gene was released from the pGEM-GPC (clone EW-1) multicloning site by XhoI/HindIII digestion. This resulted in GPC flanked either by 6 (Pic-2 sequence) or 19 extra (Pic-2 and pGEM-7ZF sequences) nucleotides at the 5′ end and 16 nucleotides of pGEM-7ZF at the 3′ end. The 5′ overhanging ends were filled in using the Klenow fragment of DNA polymerase I and subjected to electrophoresis. The appropriate fragment was electroeluted and inserted into the

dephosphorylated SmaI site of pSC11. After transformation, different recombinant clones of pSC-GPC, the pSC11 vector harbouring the GPC gene, were analyzed by digestion with restriction enzymes (Figure 6) : BamHI (lanes 2, 4 and 6) and XbaI (lanes 3, 5, and 7). The expected fragments, 5150 bp, 3000 bp, 650 bp and 500 bp after BamHI digestion and 4600 bp, 4000 bp and 900 bp after digestion with XbaI, were obtained. Lanes 6 and 7 show a clone in the wrong orientation. The sequence of the pSC-GPC was also confirmed by sequence analysis.

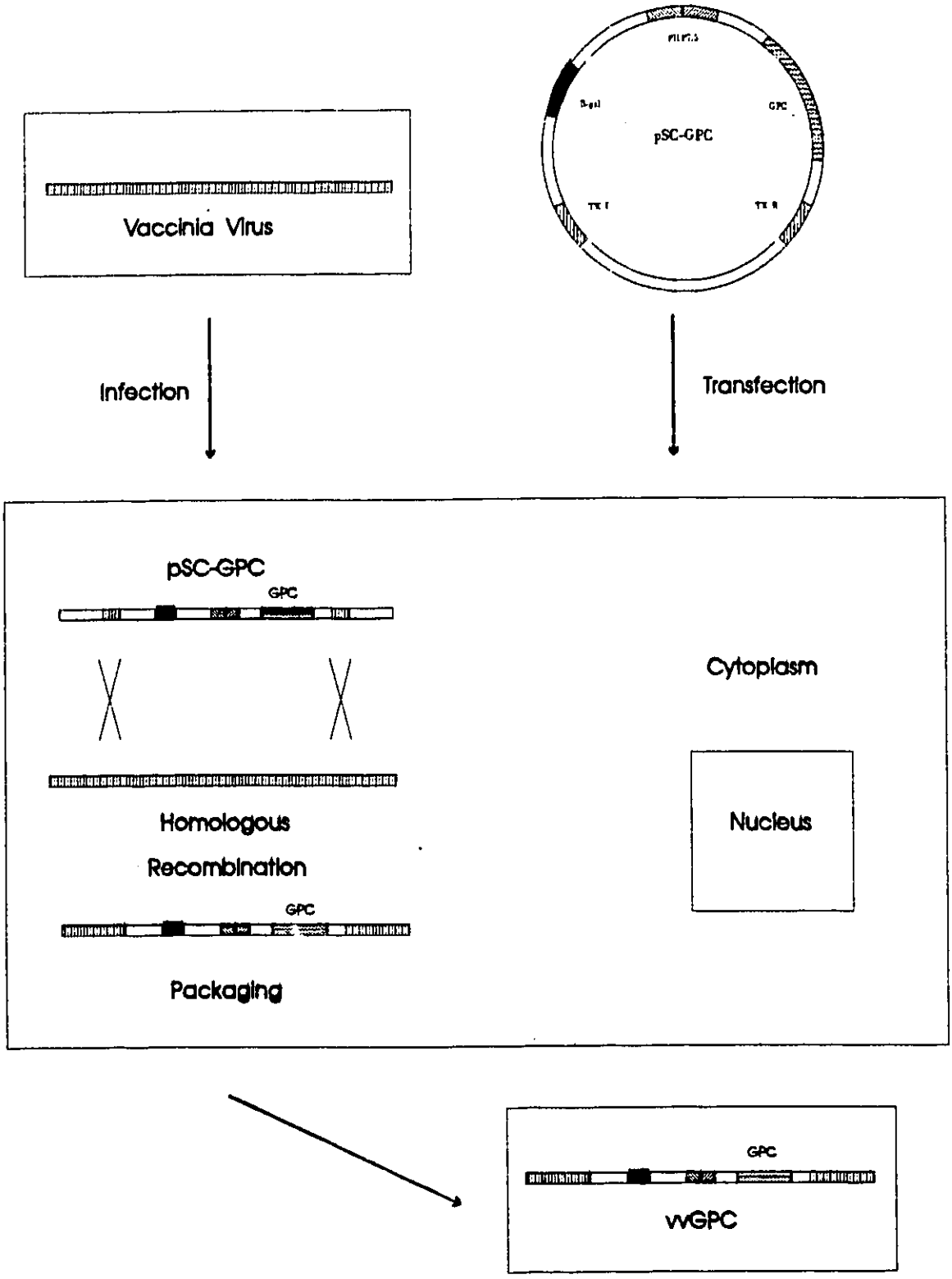
A schematic representation of the strategy used to produce recombinant vaccinia virus (vvGPC) is shown in Figure 7. The pSC-GPC construct was prepared in large scale and resuspended in water. A monolayer of CV-1 cells was infected with wild type vaccinia virus, WR strain, or with vSC8 as a control at a MOI of 0.1/cell. An hour after infection, the inoculum was aspirated, 10 ml of fresh MEM was added, and transfection of the monolayer with pSC-GPC was performed. Cells were incubated for 48 hours to allow homologous recombination between the thymidine kinase gene sequences on the plasmid and the vaccinia genome to occur. Homologous recombination allows insertion of the foreign GPC and B-galactosidase genes into the vaccinia virus genome.

The infected-transfected cells were harvested, frozen and thawed three times. Following this step, it is crucial to

Figure 6: Agarose gel electrophoresis of pSC-GPC clones. Plasmid DNA was digested with BamHI or XbaI, electrophoresed on 1% agarose gel and stained with ethidium bromide. Lane 1: a mixture of a HindIII digest of λDNA and a HaeIII digest of φX174 DNA. Lanes 2, 4, 6: pSC-GPC clones cut with BamHI. Lanes 3, 5, 7: pSC-GPC cut with XbaI. Lanes 8 and 9: pSCII cut with BamHI and XbaI respectively. Lane 10: closed circular pSC11. Lanes 11 and 12: closed circular pSC-GPC.


5.15kb
4.6
4
3
900bp
650
500

Figure 7: Schematic representation of recombinant vaccinia virus production. CV-1 cells were infected with wild type vaccinia virus, WR strain, at a MOI of 0.1 PFU/cell then transfected with the pSC-GPC contruct. Homologous recombination between vaccinia virus DNA and the thymidine kinase sequences on the plasmid DNA produces the recombinant vaccinia virus (vvGPC).


Vaccinia Virus
pSC-GPC
Infection
Transfection
pSC-GPC
GPC
Cytoplasm
Nucleus
Homologous
Recombination
GPC
Packaging
GPC
vvGPC

select recombinant vaccinia virus harbouring GPC and B-galactosidase genes (vvGPC) from the mixture of harvested virus which includes wild type vaccinia virus, spontaneous thymidine kinase mutants and recombinant vaccinia virus (vvGPC). Negative thymidine kinase selection (Mackett et al., 1982) and B-galactosidase expression allowed selection of recombinant vvGPC. Human TK- 143B cells were infected with the harvested virus, overlaid with 0.8% agarose containing 1 X 199 media and 5-bromdeoxyuridine (BUdR). Plaques were allowed to develop for 48 hours then cells were overlaid again with 0.8% agarose containing 1 X 199 media and Bluo-gal and incubated for 6-24 hours. The presence of BUdR, a nucleotide analogue, in the first overlay allows selection of thymidine kinase negative (TK-) viruses. Phosphorylation of BUdR by thymidine kinase promotes its incorporation into viral DNA, a lethal event to wild type vaccinia (TK^+). Recombinant vaccinia virus expressing GPC (vvGPC) as well as spontaneous thymidine kinase mutants, no longer contain a functional thymidine kinase gene. The presence of Bluo-gal in the second overlay allows recombinant vaccinia plaques to turn blue within 6 hours after being processed by B-galactosidase. Blue plaques were picked and recombinant virus was plaque-purified three times and amplified for production of recombinant vaccinia virus stocks to be used for the expression assays.

PCR was employed to assay for the presence of the Pic GPC gene in vvGPC. Viral DNA was used as template for

amplification of the GPC gene using two oligonucleotides (Pic-1 and Pic-2) complementary to the 3′ and 5′ sequences of the GPC gene. vSC8 (a recombinant vaccinia virus expressing only the B-galactosidase gene) was used as a negative control. vvGPC showed a PCR product (1512 bp) representing the GPC gene (Figure 8, lane 3) while vSC8 showed no products (lane 2).

3.) Expression of Pichinde virus glycoproteins by vaccinia virus.

To test for expression of Pic GPC, cell lysates infected with either Pic or vvGPC were incubated with several anti-Pic antibodies for immunoprecipitation (mouse anti-Pic (Figure 10), guinea pig anti-Pic (Figure 9) and hamster anti-Pic). A molecule migrating at 79 KD was observed consistently with GPC precipitated from Pic infected lysates. Kinetic analysis of GPC expression in vvGPC infected cells was assayed by immunoprecipitation at 3, 6, 12, 24 and 48 hours p.i. [^{35}S]-Met labeled proteins immunoprecipitated with guinea pig anti-Pic serum from lysates ($5x10^6$ cell equivalents) of mock-infected, vSC8-infected, vvGPC-infected and Pichinde virus-infected CV-1 cells were assayed by SDS-PAGE as shown in Figure 9. The glycoprotein precursor molecule, GPC, 79 KD, was immunoprecepitated from vvGPC infected cells as early as 3 hours p.i. and reached maximum expression at 12 hours p.i. Neither mock infected nor vSC8 infected cells expressed this

Figure 8: Agarose gel electrophoresis of GPC gene. Viral DNA from 10^5 PFU of recombinant vaccinia virus harbouring the GPC gene was used as template for PCR. The DNA fragments were electrophoresed on 0.8% agarose gel and stained with ethidium bromide. Lane 1: a mixture of a HindIII digest of λDNA and a HaeIII digest of φX174 used as markers, Lane 2: PCR product of vSC8 (negative control vaccinia), Lane 3: PCR product of vvGPC, Lane 4: PCR product of vvGPC-183.

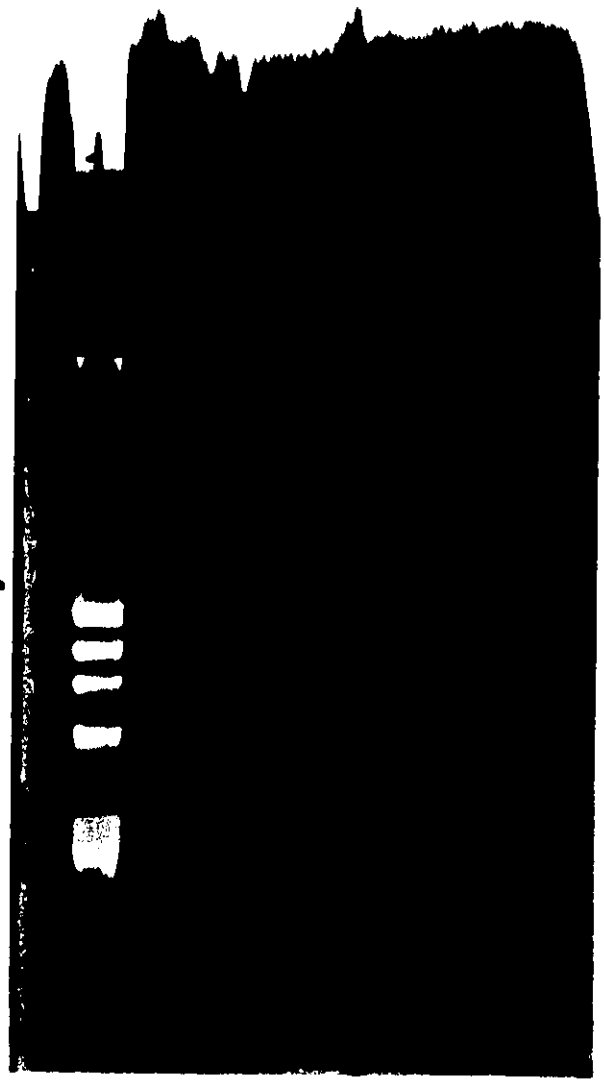
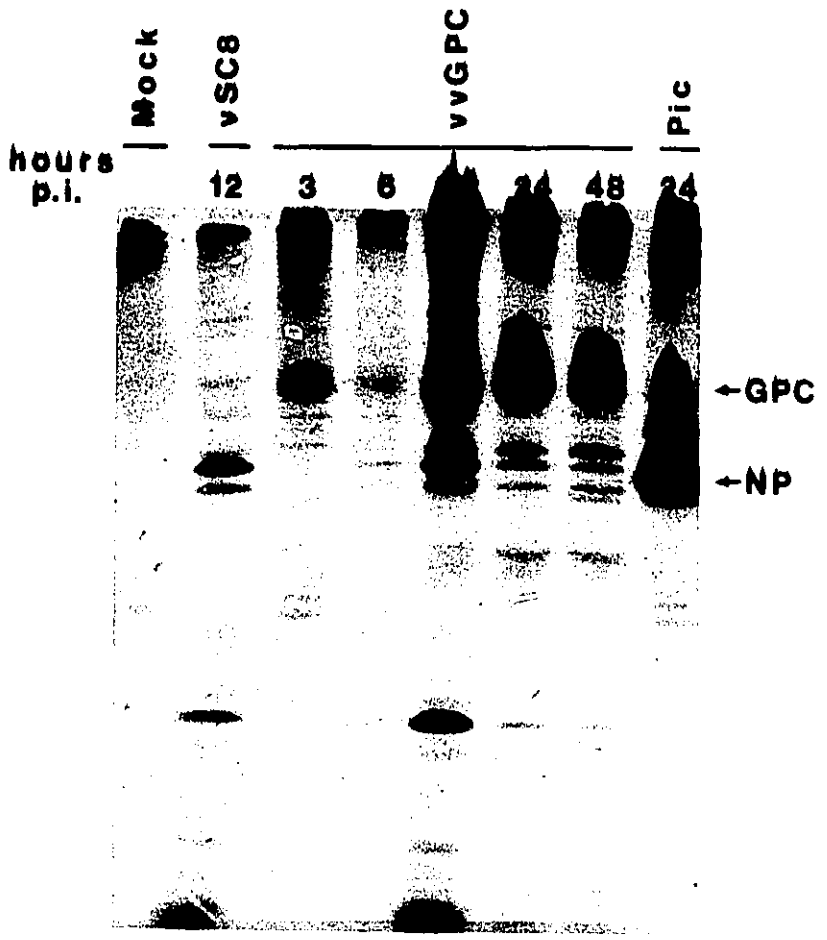

1512 bp

Figure 9: Immunoprecipitation of Pic glycoproteins expressed by recombinant vaccinia virus. CV-1 cells were infected with vvGPC at a MOI of 0.5 for indicated times. Control cells were infected with Pic at a MOI of 1.0 for 24 hours, vSC8 at a MOI of 0.5 for 12 hours or Mock (M). Infected cells were starved for 1 hour then labeled for 3 hours with [^{35}S]-Met., then lysed. Labeled proteins were immunoprecipitated with guinea-pig anti-Pic serum and electrophoresed on a 10% polyacrylamide gel containing SDS.


Mock
vSC8
vvGPC
Pic
hours p.i.
12
3
6
24
48
24
GPC
NP

protein. GPC is the lower of the two bands present in this figure. The upper band is non-specific background, as it appears in the lane with control precipitates, and disappears when immunoprecipitations are carried out with monoclonal Ab (Figure 10). These results indicate that Pic GPC expressed by vvGPC is fully glycosylated, as it co-migrates with Pic GPC and was detected immediately after pulsing.

In order to assess processing of Pic-GPC expressed by recombinant vvGPC, cells infected with vvGPC or Pic were pulsed for 30 minutes with [^{35}S]-Met., then chased for intervals of 15, 30, 60, 90, 120 and 180 minutes before immunoprecepitation with a monoclonal antibody (MAb 33.6)specific for an epitope on GP2 of LCMV that cross reacts with GP2 of all other arenaviruses (Weber and Buchmeier, 1988). The results of such a pulse-chase experiment are illustrated in Figure 10. Presence of GP2, indicative of GPC cleavage, was evident in lysates of cells infected with Pic after 120 minutes of chase. Cleavage was not evident in lysates of vvGPC infected cells at any pulse time. A second experiment labelling infected cells with [^{3}H] glucosamine was carried out (Figure 11). Cells infected with vSC8, Pic or vvGPC were pulsed for 1 hour with [^{3}H] glucosamine then chased for intervals of 90, 120 and 180 minutes before immunoprecipitation with monoclonal antibody 33.6. Cleavage of Pic GPC was evident during the one hour pulse while

Figure 10: Pulse-chase methionine labelling of Pic GPC expressed by vvGPC. BHK-21 cells were infected with vvGPC at a MOI of 0.5 for 3 hours, Pulsed (P) with [^{35}S]-Met for 30 minutes, then chased for indicated times. Control cells were infected with Pic at a MOI of 1.0 for 24 hours. Mock (M) infected cells were pulsed only. Cells were lysed and Pic GPC was immunoprecipitated with a monoclonal antibody (33.6) that recognizes a conserved epitope on GP-2 of arenaviruses. Immunoprecipitable proteins were electrophoresed on a 10% polyacrylamide gel containing SDS.

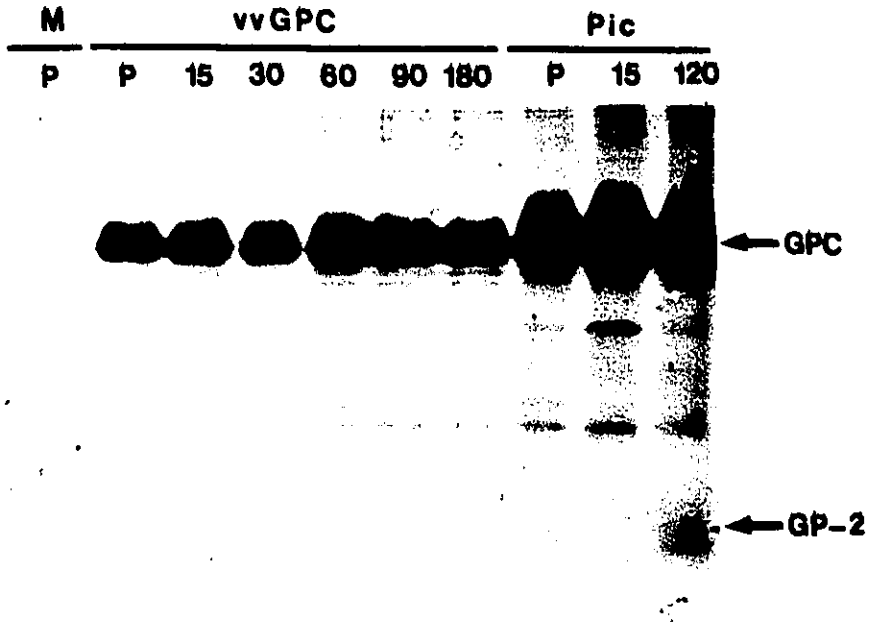

M
vvGPC
Pic
P
P
15
30
60
90
180
P
15
120
GPC
GP-2

Figure 11: Pulse-chase glucosamine labelling of Pic GPC expressed by vvGPC. BHK-21 cells were infected with vvGPC at a MOI of 0.5 for 3 hours, Pulsed (P) with [^{3}H] glucosamine for 1h, then chased for indicated times. Control cells were infected with Pic at a MOI of 1.0 for 24 hours. vSC8 infected cells were pulsed only. Cells were lysed and Pic GPC was immunoprecipitated with a monoclonal antibody (33.6) that recognizes a conserved epitope on GP-2 of arenaviruses. Immunoprecipitable proteins were electrophoresed on a 10% polyacrylamide gel containing SDS. vvGPC-infected cells (last lane) were incubated at 33°C.


vSC8
Plc
vvGPC
33°c
P
120
P
90
180
180
GPC
GP2

cleavage was not observed in lysates of vvGPC infected cells even after a 180 min chase. This may be due to the reduced amount of GPC observed, or less efficient cleavage. Using double the amount of vvGPC-infected cell lysate (10^7 cell equivalents) in immunoprecipitation with prolonged exposure of gels to x-ray films did not reveal any cleavage (data not shown).

The GPC of the temperature sensitive mutant of Pic (ts13) shows cleavage at a lower temperature (33°C) compared with the wild type (37°C). Another pulse-chase experiment was carried out at 33°C to determine if the GPC expressed by vvGPC might also be processed more efficiently at low temperature . As shown in Figure 11, when infected cells were incubated at 33°C, cleavage of GPC of vvGPC was not evident even after a 180 min chase.

Expression of Pic GPC after infection of cells with vvGPC was also determined by indirect immunofluorescence. CV-1 cells infected with vvGPC showed strong intracellular immunofluorescence using mouse anti-Pic serum (data not shown).

4.) Production and characterization of anti-vvGPC serum.

In order to assess the immunogenicity of Pic GPC expressed in vaccinia virus, a rabbit was immunized with 5 X 10^6 PFU of vvGPC intravenously. A second injection (2.5 X 10^6

PFU) was administered 4 weeks later. The animal was bled 10 days later and serum was collected.

Fourfold dilutions of rabbit anti-vvGPC were tested for binding to purified PIC by ELISA. Mouse anti-Pic serum and rabbit pre-immune serum were used as controls. Rabbit anti-vvGPC had a titre of 1/24,800 for binding Pic.

The next step was to assess recognition of Pic glycoproteins in the native and denatured forms by rabbit anti-vvGPC. This was performed by immunoprecipitation and Western blot. The results from immunoprecipitation are shown in Figure 12. [^{3}H] glucosamine-labeled proteins from lysates of mock-infected cells (M) or Pic-infected cells (Pic) were immunoprecipitated with control hamster anti-Pic serum or rabbit anti-vvGPC then electrophoresed on a SDS-PAGE. Rabbit anti-vvGPC was able to detect both Pic GPC and GP1 and only weakly GP2. In other experiments (not shown) vvGPC did not precipitate proteins from mock-infected cells, and pre-immune serum did not precipitate proteins from Pic-infected cell lysates.

The results from Western blotting are illustrated in Figure 13. Purified Pic was separated on a 10% polycrylamide gel containing SDS. Proteins were electrophoretically transferred onto nitro-cellulose paper and probed with mouse anti-Pic, rabbit pre-immune serum or rabbit anti-vvGPC. Rabbit pre-immune serum did not bind to any Pic proteins.

Figure 12: Immunoprecipitation of Pic glycoproteins by anti-vvGPC serum. BHK-21 cells were infected with Pic at a MOI of 1.0 for 24 hours before a 3 hour labeling with [^{3}H]-glucosamine. Viral proteins were electrophoresed on a 10% SDS-polyacrylamide after being immunoprecepitated with hamster anti-Pic serum or rabbit anti-vvGPC serum. M represents mock-infected cells.

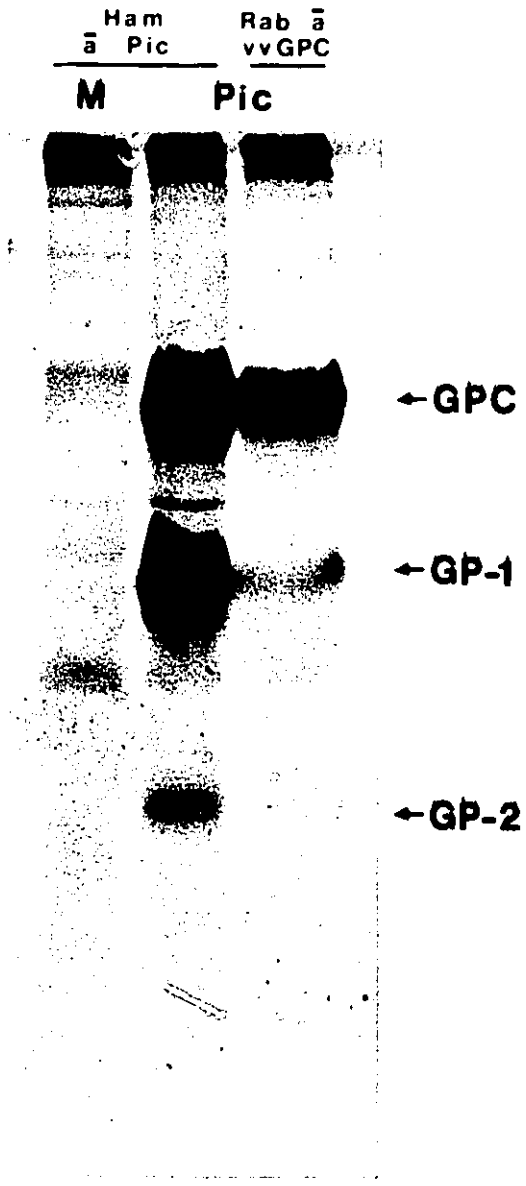
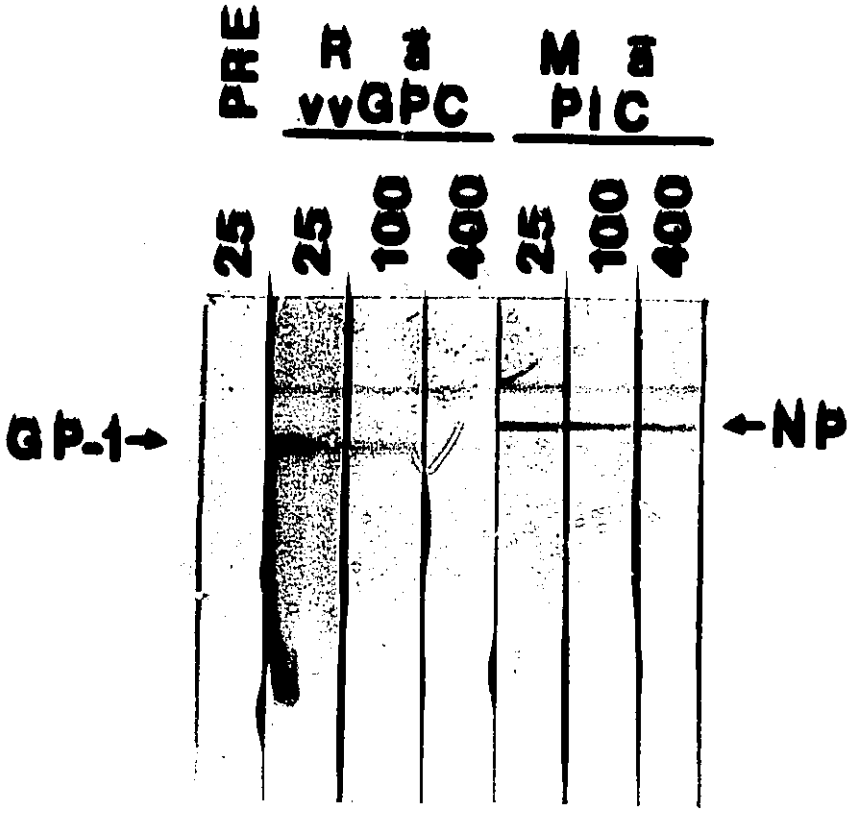

Ham
ā Pic
Rab ā
vvGPC
M
Pic
←GPC
←GP-1
←GP-2

Figure 13: Western blot analysis of Pic glycoproteins. Purified Pic virus was loaded onto a 10% polycrylamide gel containing SDS (10 ug/well) and electrophoresed. Proteins were transferred to nitro-cellulose by electroblotting at 4°C. Blots were probed with mouse anti-Pic (M á Pic), rabbit pre-immune serum (Pre) or rabbit anti-vvGPC (R á vvGPC) at the dilutions given.


PRE
R a vvGPC
M a PIC
25
25
100
400
25
100
400
GP-1
NP

Rabbit anti-vvGPV detected Pic GP1 out to a dilution of 1/400. Although not evident in this Figure, GP2 could be observed at high concentrations of Ab in other experiments. Mouse anti-Pic detected mainly the major structural protein, NP, and only weakly recognized Pic glycoproteins at high concentrations. This is the usual observation with polyclonal antibodies to whole Pic.

Pichinde virus fails to elicit neutralizing antibodies in several animal species (Howard, 1987; Walker et al., 1984). To determine whether anti-vvGPC serum had any neutralizing activity, fourfold serial dilutions of each of rabbit anti-vvGPC, rabbit pre-immune serum and hamster anti-Pic were used in neutralization assay against approximately 150 PFU of Pic. The control wells received virus incubated with media in the absence of any sera. As shown in Table 2, no neutralization activity was detected with any of the sera.

5.) Site-directed mutagenesis of amino acid 183.

After cloning and expression of wild type Pic GPC in vaccinia virus, the next step was to eliminate a potential N-linked glycosylation site (aa 181-183) on GP1. This potential glycosylation site is conserved among Pic, Lassa and certain strains of LCMV and was chosen because absence of this glycosylation site in certain isolates of the Armstrong strain

Table 2: Neutralization assay. Fourfold serial dilutions of each of rabbit pre-immune serum (Prebleed), hamster anti-Pic (Ham $\bar{a}$ Pic) and rabbit anti-vvGPC (Rab $\bar{a}$ vvGPC) were incubated with approximately 150 PFU of Pic for 1 hour at 37°c. Each mixture was then added to a Vero monolayer in duplicate. Following adsorption for 1 hour, cells were overlayed with media and 0.8% agar. Three days later a second overlay containing Neutral Red was added. Plaques were counted 24 hours later. The control monolayer received virus incubated with media in the absence of any sera.

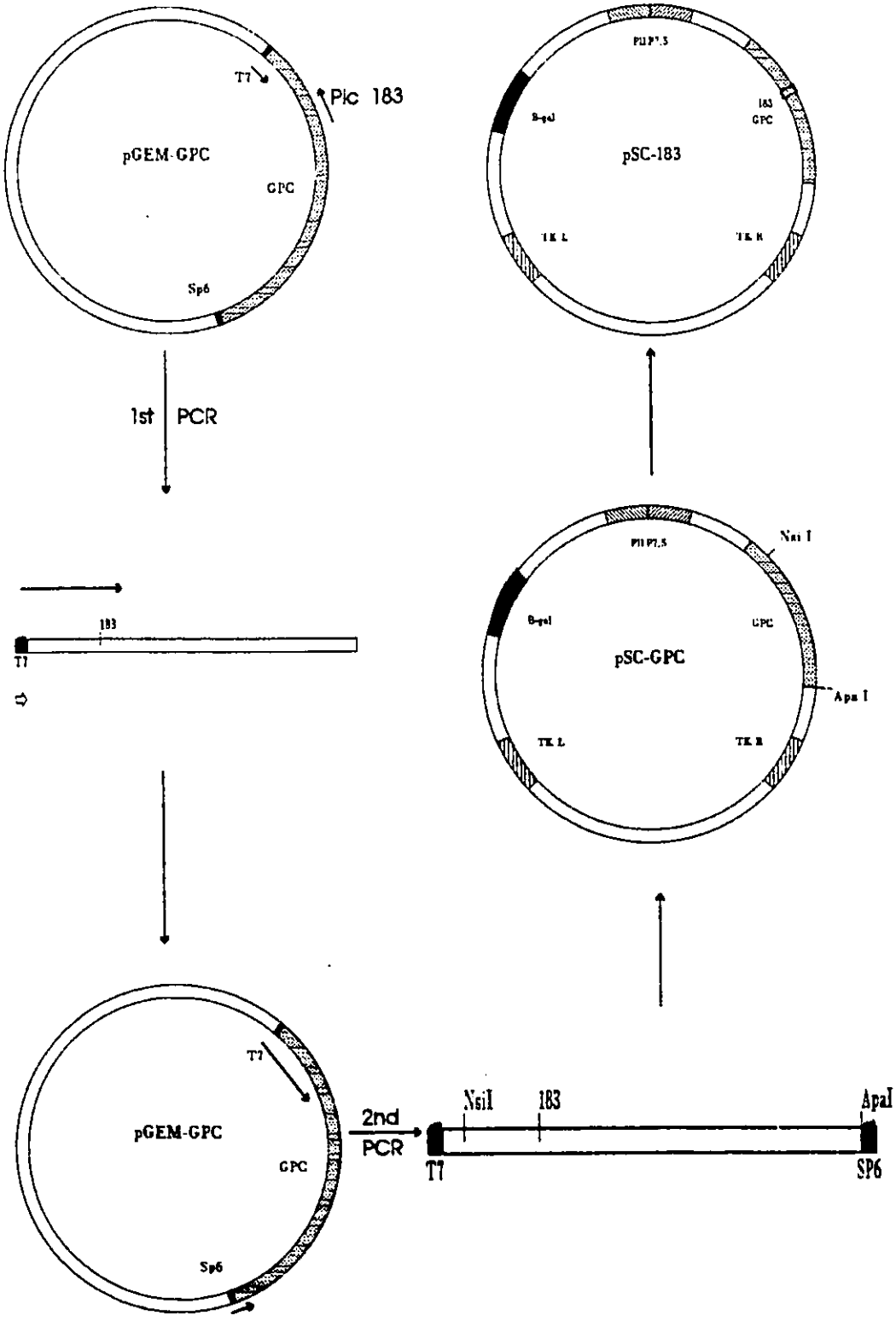
Sample		Plaques*	% Reduction
Media		146	0
Prebleed	1/25	132	9%
	1/100	138	5%
Rab ā vvGPC	1/25	138	5%
	1/100	140	4%
	1/400	137	6%
	1/1600	123	15%
Ham ā Pic	1/25	140	4%
	1/100	126	14%
	1/400	134	8%
	1/1600	125	14%
Mock		0	0

* Represents average from 2 wells.

of LCMV led to the presence of a neutralizing epitope (Wright et al., 1989; Buchmeier and Parekh, 1987).

The mutation was carried out to alter the N-linked glycosylation site (aa 181-183) from Asn-Leu-Thr to Asn-Leu-Ala by changing the first base of the Thr codon (ACA) from A to G. Site-directed mutagenesis using the PCR technology (Kuipers et al., 1991; Landt et al., 1990) was employed to carry out the mutation. The pGEM-GPC construct harbouring wild type GPC gene was used as a template for PCR using a mutagenic primer (Pic-183) (Appendix 1) and a universal primer (T7) in a first round of PCR. The strategy for mutation and subcloning of mutant GPC into pSC11 is illustrated in Figure 14. The mutagenic primer was designed in such a way that the first 5′ nucleotide of the primer follows a T- residue in the same strand of template sequence, thus overcoming the problem of the addition of an untemplated residue at the 3′ end of the first amplified fragment by Taq-polymerase (Kuipers et al., 1991). The product of the first round of PCR (600 bp) which contains the desired mutation was used in a second round of PCR along with another universal primer (Sp6) to amplify full length mutant GPC by using pGEM-GPC as template. The second PCR product, 1552 bp, was digested with NsiI/ApaI to release a 1397 bp fragment containing the mutant base. The pSC-GPC construct was double digested with NsiI/ApaI to release 2 fragments, 1397 bp and 8103 bp, which were electrophoresed.

Figure 14: Strategy for site-directed mutagenesis and subcloning of mutant GPC sequences. A mutagenic oligonucleotide primer (Pic-183) and a universal primer (T7) were used in a first round of PCR to amplify and mutate 600 bp of the GPC gene of the pGEM-GPC construct. The mutant fragment was used with another universal primer (Sp6) in a second round of PCR to amplify full length mutant GPC gene. The NsiI/ApaI fragment of the pSC-GPC construct was replaced by the mutant NsiI/ApaI to generate the mutant pSC-183.


T7
Plc 183
pGEM-GPC
GPC
Sp6
1st PCR
183
T7
pSC-183
pSC-GPC
Nsi I
Apa I
pGEM-GPC
GPC
Sp6
2nd PCR
NsiI
183
ApaI
T7
SP6

14 A

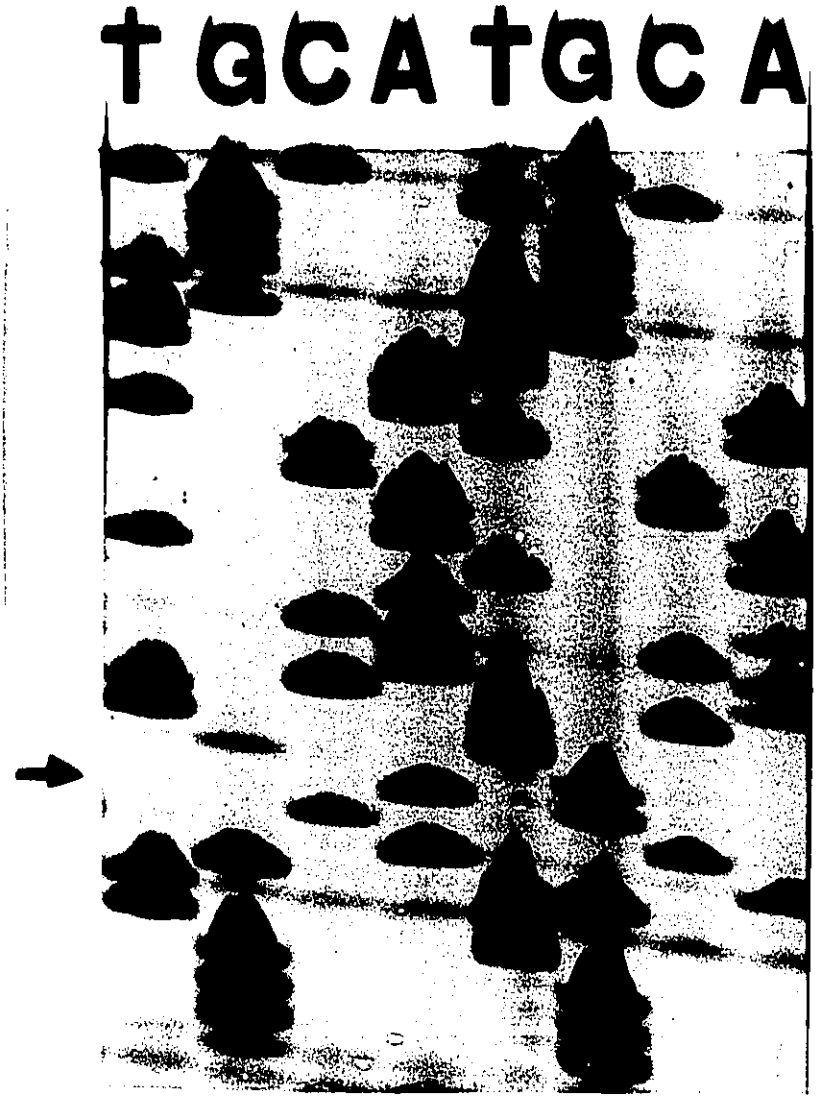
The large fragment, 8103 bp, was eluted by The GeneClean II® Kit (Bio 101 Inc.) and ligated to the NsiI/ApaI fragment purified from the PCR product to generate mutant pSC-183.

6.) Construction of recombinant vaccinia virus expressing mutated GPC.

pSC-183 harbouring the mutant Pic GPC gene was analysed by digestion with restriction enzymes (data not shown). The expected fragments 5150 bp, 3000 bp and 650 bp after BamHI digestion and 4600 bp, 4000 bp and 900 bp after digestion with XbaI were observed. The mutation was confirmed by sequence analysis as shown in Figure 15. pSC-183 DNA was prepared on a large scale and resuspended in water. Infection - transfection of a monolayer of CV-1 cells was performed as described above to produce the mutant virus, vvGPC-183. The infected - transfected cells were harvested, frozen and thawed three times. Following this step, recombinant virus was selected in the presence of BudR and bluo Gal. Blue plaques were picked, purified three times and amplified for production of virus stocks to be used for the expression assays.

Several anti-Pic sera were used to immunoprecipitate labeled proteins of vvGPC-183 infected cells. No mutant GPC was evident even with prolonged exposure (data not shown). Presence of GPC was also tested by immunization of mice with vvGPC-183. No evidence of binding to Pic was detected when

Figure 15: Sequence analysis of mutant Pic glycoprotein gene. Sequencing reactions using a specific primer (Pic-5) to the GPC gene were done in the presence of dideoxynucleotides. The dideoxynucleotide used in each of the 4 reactions is indicated above the corresponding lane. The reactions were loaded onto a 6% polyacrylamide gel containing 7M urea and separated by electrophoresis. The first set of 4 reactions represents the wild type sequence and the second set represents the mutant sequence. The arrow indicates the position of the mutation from A to G.


tgcatgca

serum was tested by ELISA. Because GPC-183 was not expressed in vvGPC-183, PCR was employed to characterize the mutant virus, vvGPC-183, (Figure 8, lane 4) as well as the vvGPC harbouring wild type Pic GPC (lane 3). The expected 1512 b prepresenting the GPC gene was detected in vvGPC but was absent in the mutant vvGPC-183.

CHAPTER 4 DISCUSSION

The general objective of this project was to shed light on the role of glycosylation in modulating the antigenicity and immunogenicity of Pichinde virus glycoproteins. The immediate objectives of the study described herein were:

1. Cloning of the glycoprotein coding sequences of Pichinde virus.
2. Expression of Pichinde virus glycoproteins using a vaccinia virus expression system.
3. Removal of a potential glycosylation site from wild type GP1 by site directed mutagenesis of amino acid 183.
4. Expression of mutant Pichinde virus glycoproteins using a vaccinia virus expression system.
5. Assessment of immunogenicity of wild type and mutant Pic glycoproteins expressed in vaccinia.

Cloning and sequencing of Pichinde virus glycoprotein coding sequences

The coding sequence of Pichinde virus GPC gene (Auperin et al., 1984a) was cloned into the plasmid vector, pGEM-7ZF, and subcloned into the shuttle vector pSC-11. The authenticity of the GPC gene was verified by restriction enzyme digestion and sequence analysis. The amino acid sequence was compared to the published sequence of Pic GPC

(Bishop and Auperin, 1987) and the GPC gene of a temperature sensitive mutant of Pic, which was cloned and sequenced independently in our laboratory (Figure 5). EW-1 was found to have six nucleotide changes which led to five amino acid changes (aa 63, 77, 161, 426 and 477). EW-2 showed the same changes except for that corresponding to aa 477, which was identical to the published sequence. ts13 was found to be the same as EW-1 at these sites, and in addition had other differences predicted to result in additional amino acid changes. Three changes occur within GP1, and two within GP2, and none of these occurs within 100 aa of the cleavage site (Figure 5). The residues that are altered are not conserved among arenaviruses except for a cysteine at aa 477 (Figure 2). The changes do not result in alteration of amino acid charges except at aa 63 (from negatively charged to uncharged) and aa 77 (from positive to negative). All N-linked glycosylation sites found in GPC protein of the published sequence are conserved in EW-1 and EW-2 and the amino acid changes did not lead to creation of new potential N-linked glycosylation sites. Although cysteine is important in formation of disulphide bonds and folding of the glycoprotein, aa 477 lies in the hydrophobic domain of GP2 which is thought to represent the membrane anchor (Burns and Buchmeier, 1991). Hence loss of this cysteine may not lead to major changes in conformation. The strain of Pic originally sequenced (Bishop and Auperin, 1987) is the same strain that was cloned and

sequenced for these studies. The observed nucleotide and amino acid changes suggest variation in the same strain of virus occurring as virus is passaged in separate laboratories. This is also consistent with findings by Clegg et al., (1990) where comparison of the S RNA of different Lassa virus isolates has revealed nucleotide differences predicted to lead to amino acid changes. Alternatively, errors might have been introduced during the PCR reaction. The Taq DNA polymerase lacks editing functions and appears to incorporate incorrect nucleotides at an overall error frequency of 0.25% in a 30-cycle amplification (Saiki et al., 1988). However the presence of the same nucleotide changes in ts13 supports the assumption that the changes in EW-1 and EW-2 are not due to PCR errors.

Production of recombinant vaccinia virus expressing Pic glycoproteins

The vaccinia virus expression system has proven to be very useful in expressing heterologous genes, including both viral and non-viral genes (reviewed by Piccini and Paoletti, 1988). Numerous recombinant vaccinia viruses have been generated to study proteins or peptides of arenaviruses, including Pichinde virus (Ozols et al., 1990), Lassa virus (Clegg and Oram, 1985; Clegg and Lloyd, 1987; Auperin et al.,

1988; Morrison et al., 1990) and LCMV (Whitton et al., 1988a; 1988b; 1989a; 1989b; Shultz et al., 1989).

The EW-1 construct was used to generate the recombinant vaccinia virus (vvGPC) expressing the glycoproteins of Pic and PCR was employed to characterize the foreign gene (Figure 8, lane 3). GPC expressed by vvGPC was found to be authentic as this molecule could be immunoprecipitated by several anti-Pic Abs. GPC expressed in vaccinia was the fully glycosylated as it comigrated with Pic GPC at 79 KD. Time course of expression of GPC expressed by vvGPC was carried out to assess the kinetics of GPC synthesis in this system. The glycoprotein precursor molecule (GPC) was immunoprecipitated from vvGPC-infected cells 3 hours post infection and reached a maximum 12 hour post infection when MOI was 0.5 (Figure 9). Pulse chase experiments were conducted to assess processing of Pic GPC expressed in vvGPC (Figure 10). A monoclonal antibody (MAb 33.6) specific for an epitope on GP2 of LCMV that cross reacts with GP2 of all other arenaviruses (Weber and Buchmeier, 1988) was used. GP2, indicative of GPC cleavage, was evident with lysates of cells infected with Pic after 120 minutes chase had elapsed. Cleavage was never observed with vvGPC even when immunoprecipitation was carried out with twice as much infected cell lysate and gels were exposed for up to 2 months. The glycoprotein precursor molecule (GPC) of the temperature sensitive mutant of PIC (ts13) shows cleavage at 33°C compared with the wild type (37°C). Another pulse-chase

experiment was carried out at 33°C to determine if the GPC expressed by vvGPC might also be more efficiently processed at low temperature, but again was negative for cleavage (Figure 11). Cells infected with vvGPC showed strong intracellular immunofluorescence (data not shown).

Other proteins made by recombinant vaccinia viruses appear to be processed and transported in a manner similar if not identical to that occuring in the natural situation (reviewed by Mackett 1987; Moss and Flexner, 1987; Moss, 1992). The GPC genes of other arenaviruses (LCMV and Lassa) have been expressed in vaccinia virus. The Lassa virus GPC gene, including the non coding sequence at the 5′ end was expressed, and its product was cleaved into GP1 and GP2 (Morrison et al., 1989; 1990). Auperin et al., (1988) and Fisher-Hoch et al., (1989) have also shown cleavage of Lassa GPC expressed in vaccinia although the amount of Lassa GPC and the cleavage products was reduced as compared to Lassa infected cells. On the other hand, cleavage of glycoprotein precursor molecule of LCMV GPC expressed in vaccinia was not shown by immunoprecipitation and repeated attempts to display surface expression of LCMV GPC determinants by immunofluorescence gave weakly positive results (Whitton et al., 1988b). This was also the case for Pic GPC expressed in vaccinia despite its being fully glycosylated, a prerequisite for cleavage of LCMV GPC as demonstrated by Wright et al., (1989 and 1990b). The absence of GP2 in lysates of vvGPC

infected cells at any pulse time may be due to the reduced amount of GPC observed, although this was taken into account in some experiments. However, reduced expression of GPC may be due to the presence of 356 bases between the start codon of the gene and the promoter sequences. Because of the nature of the pSC11 plasmid (Chakrabarti et al., 1985) the RNAs containing the Pic GPC sequences should be a combination of four features: the DNA insert, 1512 bases, which does not include the noncoding sequence, an additional 350 bases encoded by pSC11 sequences, 6 bases derived from pGEM, and a poly (A) tail of intermediate length (often 200-400 bases). This would make the initiation codon of GPC 356 bases downstream from the P7.5 promoter sequences. Alternatively efficiency of expression might be affected by the absence of upstream noncoding sequence of the GPC gene or the presence of extra sequences in pSC-GPC derived from pGEM-7ZF as described in results. Lack of cleavage might also be the result of altered conformation of Pic GPC. As noted above, none of the observed aa changes occurred close to the cleavage site. However, loss of the conserved cysteine (aa 477) in GPC expressed in vvGPC might affect cleavage by leading to changes in the initial folding of GPC in ER, so that improperly folded GPC fails to reach the site of cleavage (Golgi) or fails to be recognized by the cleavage enzyme. Experiments were not conducted to examine location of Pic GPC in cells infected with vvGPC. Surface immunofluorescence was attempted to

examine surface expression, but a good anti-Pic serum for use as a positive control in these experiments was not available.

Production and characterization of anti-vvGPC serum

In order to assess the immunogenecity of Pic GPC expressed in vaccinia, rabbit anti-vvGPC serum was raised and the serum was proven to bind to Pic by ELISA. The next step was to assess recognition of Pic glycoproteins in the native and denatured forms by rabbit anti-vvGPC. Rabbit anti-vvGPC was shown to detect authentic Pic GPC and GP1 and only weakly GP2 by both immunoprecipitation and Western blot analysis (Figures 12 and 13). The fact that rabbit anti-vvGPC reacts more efficiently to GP1 in Western blots than mouse anti-Pic suggests that GP1 as expressed by vaccinia is more immunogenic and/or has altered conformation so that more linear epitopes are exposed. This finding supports the previous suggestion that lack of cleavage is due to changes in conformation. However, the increased reactivity to GP-1 in this antiserum could also be due to the host animal, and this was not tested as a rabbit anti-Pic serum was not available at the time of these experiments.

Pichinde virus fails to elicit neutralizing antibodies (Howard, 1987; Walker et al., 1984). Neutralization assays were performed to determine whether anti-vvGPC serum had any neutralizing activity. As shown in Table 2 no neutralization

activity was detected with any of the sera tested, indicating that this serum is like polyclonal anti-Pic serum in this respect.

Construction of recombinant vaccinia expressing mutated Pic GPC

Site-directed mutagenesis was employed to remove a potential N-linked glycosylation site (aa 181-183, which is conserved among Pic, Lassa and some strains of LCMV) on GP1 of wild type Pic (Figure 14). The mutant GPC gene was subcloned into pSC11 to be expressed in vaccinia virus. Mutant clones were further analysed by restriction enzyme digestion and the mutation was confirmed by sequence analysis.

Mutant vaccinia , vvGPC-183, harbouring the mutant Pic GPC gene was constructed but attempts to characterize the foreign gene by PCR (Figure 8, lane 4) or its products by immunoprecipitation using different anti-Pic sera (data not shown) were not successful. Assessment of the immunogenicity of mutant Pic-GPC expressed by vaccinia was also carried out by raising anti-vvGPC-183 in mice. However, binding of anti-vvGPC-183 to Pic was not evident by ELISA.

The failure to detect the mutant gene by PCR or to express it in vaccinia virus may be due to: 1. absence of the foreign gene itself i.e. the rare event of homologous recombination between the thymidine kinase gene sequences on

pSC-183 and the vaccinia genome was not successful, 2. homologous recombination occurred but most of the harvested virus was either wild type or spontaneous thymidine kinase mutants, 3. homologous recombination occurred but blue plaques (presumably representing vvGPC-183) were contaminated with wild type and spontaneous mutants when picked. This might have led to expression of undetectable amounts of the mutant GPC-183.

Conclusions

The immediate objectives of this study have been accomplished. This includes cloning of the glycoproteins of Pichinde virus, expression of Pichinde virus glycoproteins in vaccinia virus expression system and removal of a potential glycosylation site from wild type GP1 by site directed mutagenesis of amino acid 183. EW-1 has six nucleotide changes, compared to the published sequence of Pic GPC, which led to five amino acid changes. The GPC gene of Pic ts13, which was cloned and sequenced independently in our laboratory, has the same changes as EW-1 in addition to some other amino acid changes. The observed nucleotide and amino acid changes indicate variation in the same strain of Pic occurring as virus passaged in separate laboratories. vvGPC expresses authentic Pic GPC as it could be immunoprecipitated by MAb and several anti-Pic sera. GPC expressed in vaccinia

is fully glycosylated as it comigrates with Pic GPC at 79 KD. vvGPC shows maximum expression of Pic GPC 12 h p.i. when MOI is 0.5. Cleavage of Pic GPC expressed in vaccinia was never observed even when immunoprecipitation was carried out with twice as much infected cell lysate and gels were exposed for up to 2 months. The antiserum raised against vvGPC recognizes Pic glycoproteins in the native and denatured forms. The more efficient reactivity of rabbit anti-vvGPC towards GP1 than anti-Pic sera, suggests that GP1 as expressed in vaccinia is more immunogenic and/or has altered conformation so that more linear epitopes are exposed. For the final two objectives: the mutant pSC-183 was constructed, but attempts to obtain recombinant virus were unsuccessful, which should be feasible, and consequently assessment of its immunogenicity was not carried out.

Assessment of the immunogenicity of both vvGPC and vvGPC-183 are relevant to infections with arenaviruses and in particular to Lassa virus that elicits low levels of neutralizing antibodies and causes significant disease in Africa. Understanding the role of glycosylation in the immunogenicity of Pic glycoproteins will extend to other viral systems where persistence is established and where surface glycoproteins are known to be heavily glycosylated. Ultimately, knowledge of what makes proteins immunogenic can help in development of vaccines and therapy of acute and persistent infections.

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Appendix I: Oligonucleotide Sequences

PIC-1	5′ TAA GGG CC⇓C TTA CCC ATT TGT AAG 3′
PIC-2	5′ TTG C↓TC GAG ATG GGA CAA ATT GTG 3′
PIC-3	5′ CGA GCC ATT GTG AAC CAG CC 3′
PIC-4	5′ TCC AGC CCA GAT AAT GGC CC 3′
PIC-5	5′ AGC ACT TCC CAA CCC ATG CG 3′
PIC-6	5′ TGC CAT ATC CAA GGC TCC CG 3′
PIC-7	5′ TGA GGA AGG TGA CGA GCT GG 3′
PIC-183	5′ CTG TCC CCA ACT GCC AAG TTG 3′
T7	5′ TAA TAC GAC TCA CTA TAG GGA GA 3′
SP6	5′ CAT ACG ATT TAG GTG ACA CTA TAG 3′

⇓ Represents ApaI restriction site.
↓ Represents XhoI restriction site.