IMMUNOLOGICAL STUDIES OF FRAGMENTS OF HERPES SIMPLEX TYPE 2

GLYCOPROTEIN B EXPRESSED IN ESCHERICHIA COLI

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by

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ABSTRACT

The herpes simplex virus (HSV) envelope glycoprotein gB is known to be highly immunogenic. Related glycoproteins, with conserved amino acid sequence, occur in members representative of all the herpesvirus subfamilies. In this work the immunogenicity of HSV-2 gB has been studied using molecular cloning techniques and in vivo and in vitro immuno-assays.

A 4.7kbp fragment of HSV-2 representing 0.345-0.376 MU was shown to hybridise to an HSV-2 messenger RNA of 3.2kb. This translated in vitro to a polypeptide of 92kDa which comigrated on SDS PAGE with immunoprecipitated gB-2. A restriction map of the 4.7kbp DNA was subsequently shown to correspond to a published gB-2 gene sequence. Fragments of this DNA were expressed fused to β-galactosidase in E.coli by random cloning into the vector pXY460. A number of HSV-2 serologically positive clones were characterised by DNA sequencing. The gB-2 specific sequences expressed by three clones were unambiguously determined. Their respective fusion proteins were immunopurified by anti-β-galactosidase affinity chromatography. These antigens were assayed in vitro by lymphoproliferative responses and in vivo by delayed-type hypersensitivity and protection studies. Ag59, representing gB-2 codons 339-394, was the most antigenic and immunogenic of the recombinant antigens and provided 36% protection in a 10x LD$_{50}$ lethal challenge.
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Other than where stated, the materials and results described in this thesis represent my own unaided work.
CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE</td>
<td>1</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>2</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>3</td>
</tr>
<tr>
<td>CONTENTS</td>
<td>4</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>12</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>18</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>19</td>
</tr>
<tr>
<td>1.) GENERAL INTRODUCTION</td>
<td>21</td>
</tr>
<tr>
<td>1.1.) HERPESVIRIDAE</td>
<td>21</td>
</tr>
<tr>
<td>1.2.) HERPES SIMPLEX VIRUS</td>
<td>24</td>
</tr>
<tr>
<td>1.2.1.) History</td>
<td>24</td>
</tr>
<tr>
<td>1.2.2.) General structure</td>
<td>25</td>
</tr>
<tr>
<td>1.2.3.) Virus replication</td>
<td>28</td>
</tr>
<tr>
<td>1.2.4.) Virology</td>
<td>29</td>
</tr>
<tr>
<td>1.2.5.) Latency</td>
<td>30</td>
</tr>
<tr>
<td>1.2.6.) Clinical Manifestations</td>
<td>34</td>
</tr>
<tr>
<td>1.2.7.) Epidemiology</td>
<td>37</td>
</tr>
<tr>
<td>1.2.8.) Transformation</td>
<td>38</td>
</tr>
<tr>
<td>1.3.) MOLECULAR BIOLOGY OF HERPES SIMPLEX VIRUS</td>
<td>40</td>
</tr>
<tr>
<td>1.3.1.) Genome</td>
<td>40</td>
</tr>
<tr>
<td>1.3.2.) HSV Transcripts</td>
<td>41</td>
</tr>
<tr>
<td>1.3.3.) Gene Locations</td>
<td>43</td>
</tr>
<tr>
<td>1.4.) HSV GLYCOPROTEINS</td>
<td>46</td>
</tr>
<tr>
<td>1.4.1.) Genes Encoding the Glycoproteins</td>
<td>46</td>
</tr>
<tr>
<td>1.4.2.) Structure of the Glycoproteins</td>
<td>49</td>
</tr>
<tr>
<td>1.4.3.) Glycosylation</td>
<td>51</td>
</tr>
<tr>
<td>1.4.4.) Synthesis of Glycoproteins</td>
<td>51</td>
</tr>
<tr>
<td>1.4.5.) Events and Sites Involved in Synthesis</td>
<td>52</td>
</tr>
</tbody>
</table>
1.4.6.) Glycoprotein Functions ........................................ 54
1.4.6.1.) Adsorption ............................................... 54
1.4.6.2.) Penetration ............................................... 55
1.4.6.3.) Cell Fusion ............................................... 56
1.4.6.4.) Envelopment and Egress of Virion ......................... 57
1.4.6.5.) Immune evasion ............................................. 58

1.5.) GLYCOPROTEIN B .................................................. 59
1.5.1.) A family of related proteins ................................. 59
1.5.2.) Identification of gB-1 and gB-2 and the genes encoding them ........................................ 60
1.5.3.) B-cell epitope structure of gB-1 ............................ 63

1.6.) IMMUNOLOGY OF HSV INFECTION .............................. 64
1.6.1.) Natural Resistance ........................................... 64
1.6.2.) Humoral Response ............................................. 67
1.6.3.) Cytotoxic T Lymphocytes .................................... 69
1.6.4.) Helper T Lymphocytes ........................................ 71
1.6.5.) Delayed-type Hypersensitivity T lymphocytes .......... 72
1.6.6.) Suppressor T Lymphocytes ................................... 73
1.6.7.) Immunization studies with individual HSV antigens ................................. 74

1.7.) AIMS OF THE THESIS ............................................. 76

2.) GENERAL MATERIALS AND METHODS ............................. 77
2.1.) Cells ........................................................... 77
2.2.) Virus .......................................................... 77
2.3.) Purification of virus .......................................... 78
2.4.) Virus plaque assay ............................................ 79
2.5.) Growth of hybridomas ......................................... 79
2.6.) Preparation of ascitic fluid ................................ 80
2.7.) Preparation of purified monoclonal antibody .............. 80
2.8.) Polyacrylamide gel analysis of proteins .................... 81
2.9.) Electrophoretic transfer of proteins to nitrocellulose. "Western blotting" .................. 82
2.10.) Immuno-staining of Western blot .................................................. 82
2.11.) Large scale preparation of DNA ..................................................... 83
2.12.) Mini preparations of DNA ........................................................... 84
2.13.) Restriction analysis of DNA ......................................................... 85
2.14.) Agarose gel electrophoresis of DNA ........................................... 86
2.14.1.) Analytical gels ................................................................. 86
2.14.2.) Preparative gels ................................................................. 87
2.15.) Electroelution of DNA fragments ................................................ 87
2.16.) Extraction and precipitation of DNA ........................................... 88
2.17.) Subcloning of DNA fragments ................................................... 88
2.18.) Transformation of E. coli with recombinant plasmids ...................... 89

3.) IDENTIFICATION OF THE qB-2 GENE .................................................. 90

3.1.) INTRODUCTION ................................................................................. 90

3.2.) MATERIALS AND METHODS ......................................................... 92
3.2.1.) Isolation of HSV-2 infected cell RNA ....................................... 92
3.2.2.) Preparation of messenger RNA .................................................. 93
3.2.3.) Agarose gel electrophoresis of RNA ......................................... 94
3.2.4.) Northern transfer ..................................................................... 95
3.2.5.) Hybridization of 32P probes to Northern blots ......................... 95
3.2.6.) Hybridization selection of RNA ................................................ 97
3.2.6.1.) Preparation of DNA solid phase ........................................... 97
3.2.6.2.) Hybridization ................................................................. 98
3.2.7.) In vitro Translation .................................................................. 99
3.2.8.) Immunoprecipitation of in vitro translation products .................. 100
3.2.9.) Radio-labelling of DNA probes .............................................. 101
3.3.1) RESULTS............................................. 102
3.3.1.) Identification of the gB polypeptides of
HSV-1 and HSV-2........................................ 102
3.3.2.) DNA subcloning to isolate the gB-2 gene...... 105
3.3.2.1.) Confirming restriction sites in the HSV-2
genomic clone pGR93................................. 105
3.3.2.2.) Subcloning the region of the genome
approximately 0.345-0.390 MU...................... 108
3.3.2.3.) Preparation of probes........................ 108
3.3.3.) Northern blot analysis........................... 111
3.3.4.) Subcloning of the 4.7kbp BamHI-KpnI fragment
representing 0.345-0.376 MU....................... 114
3.3.5.) In vitro translation............................. 114
3.3.6.) Restriction mapping of pUH10.................. 117

3.4.) DISCUSSION........................................... 124
3.4.1.) Monoclonal antibody recognition of the gB-1 and
gB-2 polypeptides................................. 124
3.4.2.) Northern blot analysis of HSV-2 infected cell
mRNA.................................................... 125
3.4.3.) In vitro translation of hybridization
selected 3.2kb mRNA................................. 125
3.4.4.) Restriction analysis of pUH10................... 127
3.4.5.) Summary.......................................... 130

4.) EXPRESSION OF FRAGMENTS OF THE gB-2 POLYPEPTIDE
IN ESCHERICHIA COLI..................................... 131

4.1.) INTRODUCTION...................................... 131

4.2.) MATERIALS AND METHODS.......................... 134
4.2.1.) The vector pXY460 and bacterial host......... 134
4.2.2.) Preparation of DNA for cloning............... 136
4.2.3.) Selection of clones expressing GZ activity.... 137
4.2.4.) Preparation of protein from clones.......... 137
4.2.5.) Immuno-affinity chromatography .............. 138
4.2.6.) Enzyme substrate affinity chromatography...... 138
4.2.7.) Lac Z microtitre assay.......................... 140
4.2.8.) DNA sequencing................................... 140
4.2.9.) DNA sequence analysis............................ 143

4.3.) RESULTS.............................................. 144
4.3.1.) Cloning of fragments from the 2.6kb XhoI
        fragment of pUH10.................................. 144
4.3.2.) DNA sequencing................................... 149
4.3.3.) Protein Purification.............................. 156
        4.3.3.1.) Enzyme substrate affinity chromatography.... 156
4.3.3.2.) Monoclonal antibody affinity
        chromatography.................................... 159

4.4.) DISCUSSION.......................................... 164
4.4.1.) Cloning for expression of gB-2 fusion
        proteins............................................ 164
4.4.2.) Sequence analysis of clones..................... 166
4.4.3.) Recombinant protein purification.............. 168

5.) STUDIES OF THE IMMUNE RESPONSE TO gB-2 AND THE
    RECOMBINANT ANTIGENS................................. 169

5.1.) INTRODUCTION...................................... 169
5.1.1.) T lymphocyte responses to HSV.................. 169
5.1.2.) The mouse model................................ 169
5.1.3.) In humans...................................... 171
5.1.4.) Aims........................................... 171
5.2.) MATERIALS AND METHODS............................ 172
5.2.1.) Mice.......................................... 172
5.2.2.) Collection of blood samples..................... 172
5.2.3.) Inactivation of virus............................ 173
5.2.4.) Extraction of infected cell proteins.......... 173
5.2.5.) Preparation of anti-gB immuno-affinity column .............................................. 174
5.2.6.) Purification of gB-2 .............................................. 175
5.2.7.) Lymphocyte proliferation assay ......................... 175
  5.2.7.1.) Priming .............................................. 175
  5.2.7.2.) Preparation of draining lymph node cells ....... 176
  5.2.7.3.) Plating out of DLN cells ............................... 176
  5.2.7.4.) Measurement of $^3$H-thymidine uptake ........... 177
  5.2.7.5.) Calculation ........................................... 177
5.2.8.) Delayed-type hypersensitivity assay .................... 178
  5.2.8.1.) Priming (inducing) .................................... 178
  5.2.8.2.) Eliciting ............................................... 178
  5.2.8.3.) Measurement of footpad swelling ..................... 179
  5.2.8.4.) Calculation ........................................... 179
  5.2.8.5.) The use of cyclophosphamide ......................... 179
5.2.9.) Protection studies ........................................ 180
  5.2.9.1.) Priming ............................................... 180
  5.2.9.2.) Challenge ............................................. 180
  5.2.9.3.) Calculation ........................................... 181
5.2.10.) Preparation of $^{125}$I-rabbit anti-mouse
  immunoglobulin ................................................ 181
5.2.11.) Radio-immunoassay (RIA) ............................... 182

5.3.) RESULTS .......................................................... 183
5.3.1.) Lymphocyte proliferation assay ......................... 183
  5.3.1.1.) Timecourse ............................................. 183
  5.3.1.2.) Response to recombinant antigens ................... 185
  5.3.1.3.) Effect of dose of antigen on in vitro T
  lymphocyte proliferation response ....................... 187
  5.3.1.4.) Immunogenicity of the Recombinant Antigens .... 189
5.3.2.) Delayed-type hypersensitivity (DTH) response ....... 192
  5.3.2.1.) Timecourse of response and effect of
cyclophosphamide treatment ................................ 192
  5.3.2.2.) Priming with recombinant antigens ................ 194
5.3.2.3.) Effect of cyclophosphamide on the induction of DTH response to HSV-2 by the recombinant antigens................................. 195
5.3.2.4.) Ability of the recombinant antigens to induce or elicit a virus specific DTH response in cyclophosphamide pretreated mice............. 197
5.3.2.5.) Effect of various combinations of copriming on the DTH response to HSV-2............................. 199
5.3.3.) Protection of mice against lethal challenge with HSV-2 strain BRY.................................................. 202
5.3.3.1.) Determination of LD50........................................... 202
5.3.3.2.) Protection of mice against lethal challenge with HSV-2 by immunization with HSV-2, gB-2 or the recombinant antigens............... 204
5.3.3.3.) Determination of anti-HSV antibody in the serum of mice in the challenge experiment.... 209

5.4.) DISCUSSION................................................. 211
5.4.1.) Lymphocyte proliferation assay.............................. 211
5.4.1.1.) Timecourse.............................................. 211
5.4.1.2.) Response to recombinant antigens in vitro (antigenicity).............................................. 211
5.4.1.3.) Response to recombinant antigens in vivo (immunogenicity).............................................. 212
5.4.2.) Delayed-type hypersensitivity............................... 214
5.4.2.1.) Timecourse.............................................. 214
5.4.2.2.) Response to gB-2 and the recombinant antigens............................... 215
5.4.2.3.) Effect of cyclophosphamide on the response to the recombinant antigens............................... 216
5.4.3.) Lethal challenge............................................ 217
5.4.3.1.) Protection by immunization with HSV-2 and derivative antigens............................... 218
5.4.3.2.) Antibody response........................................ 219
5.4.4.) Summary..................................................... 221
6.) GENERAL DISCUSSION.............................................. 222

6.1.) Identification of the gB-2 gene.......................... 222
6.2.) Expression of fragments of the gB-2 polypeptide... 225
6.3.) New methods applicable to recombinant gene
       expression.................................................. 226
6.4.) T cell immunogenicity/antigenicity of the
       recombinant gB-2 antigens.............................. 228

REFERENCES...................................................... 232

APPENDIX. DNA molecular weight markers............... 256
       Chemicals suppliers.................................... 256
LIST OF FIGURES.

Figure 1.1. Structure of the HSV virion...................... 26

Figure 1.2. General HSV DNA organization................... 27

Figure 1.3. Zosteriform spread of HSV infection............ 32

Figure 1.4. Clinical manifestation of HSV-2 infection..... 35

Figure 1.5. HSV-1 genome organization..................... 44

Figure 1.6. Map location of HSV glycoprotein genes........ 48

Figure 1.7. Structure of an HSV glycoprotein.............. 50

Figure 3.1. Western blot of tunicamycin treated and untreated HSV-1 strain KOS infected BHK cell lysates probed with the gB specific monoclonal antibody TI57.... 103

Figure 3.2. Western blot of tunicamycin treated and untreated HSV-2 strain BRY infected BHK cell lysates probed with the gB specific monoclonal antibody TI57.... 104

Figure 3.3. Restriction digests of the plasmid pGR93.... 105

Figure 3.4. Restriction map of the HSV-2 genomic clone pGR93 indicating especially BamHI, BglII and KpnI...... 106

Figure 3.5. Restriction digests of pH10 (7kbp BamHI-BglII fragment in pBR322) MU 0.345-0.376............... 109
Figure 3.6. Restriction map of pBH10 showing the locations of the PvuII, KpnI and BstEII sites including the locations of the probes used to examine the Northern blot of HSV-2 mRNA........................................... 110

Figure 3.7. Northern blots of HSV-2 BRY infected BHK cell mRNA.......................................................... 113

Figure 3.8. In vitro translation and immunoprecipitation of HSV-2 infected BHK cell poly A+ and hybrid-selected RNA.......................................................... 115

Figure 3.9. In vitro translation and immunoprecipitation of HSV-2 infected BHK cell poly A+ and hybrid-selected RNA heat denatured prior to translation...................................................... 116

Figure 3.10. The use of partial digestion of single end radiolabelled pUH10 DNA to construct a restriction map of the pUH10 insert.................................................. 117

Figure 3.11. Various single and double digests of pUH10 with the restriction enzymes Smal (M), SacI (C), XhoI (X), PstI (P), SphI (S), SalI (L), EcoRI (E), and HindIII (H)................................................................. 119/120

Figure 3.12. Further single and double digests of pUH10 with the restriction enzymes described in Fig.3.11...... 121/122

Figure 3.13. A restriction map of the insert of pUH10 derived from interpretation of the data shown in Figures 3.10, 3.11 and 3.12.................................................. 123
Figure 3.14. A comparison of the restriction map of pUH10 and a restriction map showing recognition sites for the same enzymes derived by computer analysis of the nucleotide sequence of gB-1 published by Bzik et al (1984) .......................................................... 128

Figure 3.15. A comparison of the restriction map of pUH10 with a map published by Person et al (1985) and a restriction map derived from the nucleotide sequence of gB-2 published by Bzik et al (1986) ......................... 129

Figure 4.1. The plasmid pXY460.................................................. 135

Figure 4.2. Nucleotide sequences surrounding the cloning site of pXY460 showing the location of the sequencing primers......................................................... 141

Figure 4.3. Restriction digestion of the 2.6kbp XhoI insert of pUH10 with ThaI (T), RsaI (R) and AluI (A) .... 144

Figure 4.4. Multiple partial restriction of the 2.6kbp XhoI fragment of pUH10 with ThaI, RsaI and AluI (MP) .... 145

Figure 4.5. SDS PAGE of IPTG induced culture lysates of clones pXH02-85 derived from the 2.6kbp XhoI fragment of pUH10 , together with pXY461, a) Coomassie stained, and b) Western blotted and stained with rabbit polyclonal anti-HSV-2 antisera and 125I-protein A......................... 146

Figure 4.6. Analysis of DNA insert size in a number of pXH clones......................................................... 148
Figure 4.7. An example of nucleotide sequence analysis of pXH clones showing the confirmation of reading frame of the insert with respect to the ATG initiation codon of the vector................................. 150

Figure 4.8. Summary of sequence data generated for clones pXH53-85........................................ 151/155

Figure 4.9. Elution profile of enzyme substrate-analogue affinity chromatography of cleared induced culture lysate from pXY461 (containing unfused GZ)...... 157

Figure 4.10. SDS PAGE analysis of fractions collected during elution of the enzyme substrate affinity column (Fig.4.9.)............................................. 158

Figure 4.11. Elution profile of BG79 immuno-affinity chromatography of cleared induced culture lysate from pXY461...................................................... 160

Figure 4.12. SDS PAGE analysis of fractions collected during elution of the immuno-affinity column (Fig.4.11.)..................................................... 161

Figure 4.13. SDS PAGE analysis of immunopurified samples of Ag461, Ag59, Ag65 and Ag71............... 162

Figure 4.14. A computer generated representation of the gB-2 sequences showing the stop codons in all six reading frames............................................. 165

Figure 5.1. Timecourse of the response of HSV-2 primed lymph node cells to HSV-2 or gB-2..................... 184
Figure 5.2. Response of HSV-2 primed DLN cells to varying doses of purified antigens......................... 186

Figure 5.3. Response of HSV-2 strain 333 primed lymph node cells to gB-2 or enzyme affinity purified recombinant antigens............................................. 188

Figure 5.4. Response of lymph node cells primed with different antigens to in vitro stimulation with HSV-2 strain BRY and gB-2....................................................... 190

Figure 5.5. Response of DLN cells primed with HSV-2 strain 333, recombinant antigen or ovalbumin (Ova) to in vitro stimulation with media alone (-Ag), ovalbumin, HSV-2 strain 333 viral antigen or gB-2....................... 191

Figure 5.6. Timecourse of the DTH response in the right hind footpad of mice primed with HSV-2 antigens from time of eliciting....................................................... 192

Figure 5.7. Timecourse of the footpad DTH response to HSV-2 antigens in mice primed with HSV-2 antigens, and the effect of cyclophosphamide on that response.......... 193

Figure 5.8. Footpad DTH response to HSV-2 antigens in cyclophosphamide pretreated mice primed with various antigens......................................................... 194

Figure 5.9. 24 hrs footpad DTH response to HSV-2 antigens in mice primed with various antigens: the effect of cyclophosphamide pretreatment......................... 196

Figure 5.10. Effect of priming or eliciting with various antigens on the 24 hrs footpad swelling DTH in cyclophosphamide pretreated mice......................... 198
Figure 5.11. Effect of various combinations of priming and eliciting antigens on the 24 hrs footpad DTH response in mice not treated with cyclophosphamide ................. 201

Figure 5.12. Estimation of the LD$_{50}$ for HSV-2 BRY in 10 weeks old BALB/c female mice ............................................. 203

Figure 5.13. Active protection of mice by immunization with various antigens ...................................................... 205

Figure 5.14. Optimization of RIA of HSV-2 antigen on solid phase (adsorbed live virus) to detect anti-HSV-2 antibodies .......................................................... 209

Figure 6.1. Distribution within gB-2 of characteristics common to known T cell epitopes ........................................ 230
LIST OF TABLES.

Table 1.1. Summary of characterized HSV-1 genes......... 45

Table 3.1. Comparison of the observed banding patterns from restriction digestion of pGR93 with the banding pattern predicted from a restriction map derived by Reyes (1982)................................................ 107

Table 4.1. Comparison of yields of GZ (Ag461) and recombinant antigens purified by enzyme affinity chromatography or immuno-affinity chromatography........... 155

Table 4.2. Details of characterisation of pXH clones.... 163

Table 5.1. Calculation of the LD$_{50}$ for HSV-2 BRY in 10 week old BALB/c female mice............................. 203

Table 5.2. Survival levels of mice primed with various antigens and challenged with 10x LD$_{50}$ of HSV-2 BRY...................................................... 206

Table 5.3. Life table for mice in the group primed with Ag461...................................................... 207

Table 5.4. Life table for mice in the group primed with Ag59 and statistical comparison with Table 5.3..... 208

Table 5.5. Anti-HSV-2 antibody titre in the serum of mice from the protection experiment (prior to challenge)......................................................... 210

Table 6.1. Potential sites for internal initiation of translation (AUG codons) in the gB-2 gene.......... 224
LIST OF ABBREVIATIONS.
Amp - ampicillin (Sigma)
BHK - baby hamster kidney (cells)
bp - (deoxyribonucleic acid) base pairs
BSA - bovine serum albumin (Pentax fraction V - Sigma)
CMV - cytomegalovirus
cpe - cytopathic effect
Cy - cyclophosphamide (Sigma)
cpm - counts per minute (scintilation)
ddH₂O - distilled deionised water
DLN - draining lymph node
DMSO - dimethylsulphoxide (Sigma)
dNTP - 2'-deoxynucleotide 5'-triphosphate (A - adenosine, G - guanosine, C - cytidine, T - thymidine)
ddNTP - 2',3'-dideoxynucleotide 5'-triphosphate
EBV - Epstein-Barr virus
EDTA - ethylenediaminetetraacetic acid
EGTA - ethylene-bis(oxy-ethylenenitrilo)tetraacetic acid
gB - glycoprotein B
GZ - lacZ gene product, β-galactosidase
HI - heat inactivated
HSV - herpes simplex virus
i.d. - intra dermal
IFN - interferon
i.p. - intra peritoneal
IPTG - isopropyl-β-D-thiogalactopyranoside (Sigma)
i.v. - intra venous
kb - (ribonucleic acid) kilo bases
kbp - (deoxyribonucleic acid) kilo base pairs
kDa - (protein) kilo Daltons
mRNA - messenger RNA
MU - (herpes virus genome) map units
NMS - normal mouse serum
NP40 - Nonidet P40 non-ionic detergent
PFU - plaque forming units
p.i. - post infection
PMSF - phenylmethysulphonyl fluoride (Sigma)
RaMIg - rabbit anti-mouse immunoglobulin
RRL - rabbit reticulocyte lysate (BRL)
s.c. - subcutaneous
SDS PAGE - sodium dodecylsulphate polyacrylamide gel electrophoresis
TEMED - N,N,N',N'-tetramethylethylenediamine (Sigma)
TLCK - Nα-p-tosyl-L-lysine chloromethyl ketone (Sigma)
TPEG - p-aminophenyl-B,D-thiogalactopyranoside (Sigma)
Tris. - tris(hydroxymethyl)-aminomethane (Sigma)
Tu - tunicamycin (Sigma)
UVI - ultraviolet irradiation inactivated
X-gal - 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma)
1.) GENERAL INTRODUCTION.

1.1.) HERPESVIRIDAE.

Herpes, from the Greek επνίλ "to creep", is a lay term which describes the clinical manifestation of an inflammatory skin disease of man, first reported by the Greeks over 25 centuries ago. The microbial agents that cause this characteristic infection are the herpes viruses, which are responsible for a great deal of morbidity and mortality in both humans and domesticated livestocks. They exploit the intimacy of social interaction between their hosts, such as direct facial, oral or genital contact for dissemination. Their success is also due in part to their ability to become latent, enhancing their survival in the individual and in the population as a whole. Members of the herpesvirus family, herpesviridae, have been found to infect almost every species investigated. They have been isolated from humans, primates, domesticated mammals (livestock and pets), birds, reptiles, fish and molluscs (reviewed in Roizman,1985a and Nahmias et al.,1981).

The six main human herpesviruses include representatives of each of the three herpesvirus subfamilies which are defined on the basis of host range, reproductive cycle, cytopathology and latency (discussed by Roizman,1985a). The herpes simplex viruses (HSV), type 1 and type 2, and varicella-zoster virus (VZV) belong to the alphaherpesvirinae, human cytomegalovirus (HCMV) belongs to the betaherpesvirinae and Epstein-Barr virus (EBV) belongs to the gammaherpesvirinae. Most recently identified is human herpesvirus 6 (HHV-6), its classification has yet to be defined.
VZV, EBV, HCMV and HHV-6 are described briefly below.

Primary infection with VZV usually occurs in childhood and results in varicella (chicken pox). This involves fever then generalized skin eruptions, progressing from erythematous areola to pustules and finally scabs. Like HSV, VZV shows a neural tropism and frequently becomes latent in the dorsal root ganglia. Herpes zoster is the result, in adulthood of a recurrence of the latent infection. This involves skin lesions running across the torso along the line of nerves serving the dermatome of associated ganglia (Weller, 1982).

EBV was first identified, in a cell line of lymphoblastoid origin from a Burkitt's lymphoma biopsy, as virus particles with a herpes-like morphology. EBV is B cell lymphotrophic and causes infectious mononucleosis primarily in adolescents and young adults. In West Africa it is associated with Burkitt's lymphoma, and in China with nasopharyngeal carcinoma. EBV will infect man and certain primates (Klein, 1974).

HCMV infection characteristically causes formation of enlarged cells (cytomegaly) containing intranuclear and cytoplasmic inclusions. HCMV is also lymphotrophic and specific in its host range to man. Infection may be asymptomatic or involve mild to severe fever (resembling infectious mononucleosis) and can in certain groups be fatal. HCMV infection during pregnancy may be considered equal to rubella virus as a threat to the developing fetus. Primary or reactivated HCMV infection is a serious, often life threatening, complication for surgical transplant recipients undergoing immunosuppressive therapy (Wright, 1974).
A novel lymphotrophic human herpesvirus has been identified in patients with lymphoproliferative disorders (Salahuddin, et al., 1986). Originally thought to be B cell specific, this virus appeared otherwise indistinguishable from a virus identified in Ugandan patients also suffering lymphoproliferative disorders. This virus was cultivatable in cells of T and B lymphoid, glial and fibroblastoid origin (Downing et al., 1987; Tedder et al., 1987). Its cytopathic effect resembles HCMV, infected cells become grossly enlarged and show non-virus intranuclear and cytoplasmic inclusions. However other than a short region of nucleic acid homology to HCMV (Efstathiou, et al., 1988), this virus shows no homology to the other human herpesviruses. This virus has been designated HHV-6.
1.2.) HERPES SIMPLEX VIRUS.

1.2.1.) History.

The vesicular nature of herpetic lesions was well characterized in the early 19th century but it was not until 1893 that Vidal recognized that infection was transmitted by contact between individuals (reviewed by Wildy, 1973). Detailed scientific investigation of the disease and its causative agent began in the 20th century. Unna, in 1896, studying the histopathology of herpes infection observed multinucleated giant cells (reviewed by Whitley, 1985). In 1919 Lowenstein clearly demonstrated the infectious nature of HSV, by transmission of the infection to rabbit cornea from lesions of human herpes keratitis and vesicles of herpes labialis (Whitley, 1985). The neuropathology of these infections was later established by work on the transmission of the virus along nerves of infected rabbits (Goodpasture and Teague, 1923).

The occurrence of neutralizing antibody to HSV was noted (Andrews and Carmichael, 1930) in the serum of adults known to have been infected. Interestingly these patients were found to suffer recurrent lesions, despite this apparent indication of immunity. HSV was associated with many forms of disease over the following years. The virus was found to be shed by infants displaying herpetic stomatitis, by Dodd and his colleagues in 1938, and to be involved with eczema herpeticum, by Seidenberg in 1941, herpes simplex encephalitis, by Smith and his colleagues in 1941, and primary keratoconjunctivitis, by Gallardo in 1943 (reviewed by Whitley, 1985).

The first association between neuropathology, recurrent disease and the virus were made by observations on the
development of oral and facial lesions following surgery on the fifth cranial nerve in 90% of individuals treated (Carlton and Kilbourne, 1952). However, latency of virus in the root of this nerve, the trigeminal ganglion, was not demonstrated for many years. Then virus was recovered from the trigeminal ganglia of rabbits displaying recurrent eye infection (Stevens et al., 1972), and subsequently also from humans (Lewis et al., 1980), in both cases by explantation of ganglion tissue and its cocultivation with susceptible cells.

Clinical observations made in the early decades of this century suggesting that there may be two forms of this virus were confirmed in terms of antigenic and biological differences by Nahmias and Dowdle (1968). These authors showed that of the two types, HSV-1 and HSV-2, type 1 was more frequently associated with oral and facial disease, whereas type 2 was associated with infections of the genitalia.

1.2.2.) General structure.

In general members of the herpesviridae have a similar structure. Under the electron microscope the virion (Figure 1.1) are between 120-200nm in diameter. Enclosing the virion is a lipid bilayer membrane with surface projections (the virally encoded glycoproteins), which is impermeable to negative stain. This surrounds a tegument of globular material which is amorphous, variable in amount and distributed asymmetrically around the icosahedral nucleocapsid. This contains a core of DNA wound round a fibrillar spool, the fibres of which attach to the inside of the capsid shell. The capsid shell is 100-110nm in diameter, has 5 capsomeres on each edge and contains 150 hexameric and 12 pentameric capsomeres.
Figure 1.1. The structure of HSV-1 visualised by negative staining and transmission electron microscopy. Clearly visible are the icosahedral nucleocapsid and the lipid bilayer envelope, which appears thickened by projecting glycoproteins (Courtesy of Dr. J. Almeida).
The genome of herpes simplex virus is composed of linear double stranded DNA of molecular weight between 97-99 x 10^6 daltons, or approximately 150kbp (Kieff et al., 1971; McGeoch et al., 1988). Genome organization was classified as group E (Roizman, 1985a) comprising a short and long segment, each being unique and flanked by reiterated sequences (Wadsworth et al., 1975). Four isomeric forms of the genome occur naturally (Figure 1.2) of which the first (i) is known as the prototype. Conventionally all physical and genetic information is displayed on the prototype, with map location from right to left in map units (M.U.) from 0.000-1.000.

**Figure 1.2.** The HSV genome. The linear double stranded DNA genome ≈ 150kbp long is composed of one unique long and one unique short region, both flanked by their own specific inverted repeat sequences (b/b' and c/c') which in turn are flanked by a common inverted repeat sequence (a/a'). The organization of the four isomeric form of the genome which occur naturally are shown.
The genome is predicted to contain 70 distinct genes encoding structural and functional proteins ranging in molecular weight from 12 kDa to 200 kDa. A number of the structural proteins found embedded in the envelope are glycosylated, in the rough endoplasmic reticulum and the Golgi apparatus of the host cell, this being essential to their function. They are responsible for the phenomenon of cell agglutination and fusion caused by these viruses. The glycoproteins are a major antigenic component of the virus, appearing on the surface of productively infected cells as well as on the viral envelope.

1.2.3.) Virus multiplication.

The first stage of viral multiplication involves the adsorption and entry of the virion. The virus attaches by interaction of the viral envelope glycoproteins with receptors on the host cell plasma membrane, ultimately leading to fusion of the viral envelope with the plasma membrane. This releases the nucleocapsid into the cytoplasm. The DNA-protein complex is translocated to the nucleus where the viral DNA is transcribed. The viral mRNA is translated in the cytoplasm whilst the viral DNA is replicated in the nucleus and wound onto a fibrillar spool to form immature nucleocapsid. These become encapsidated and the mature nucleocapsids gain tegument and an envelope as they bud through the inner lamella of the nuclear membrane into the cisternae of the endoplasmic reticulum. The virus is then transported through the endoplasmic reticulum to the Golgi apparatus. Mature virion then pass in what are thought to be lyzosomal vesicles to the cell surface where they are released as infectious virion. The membrane constituting the envelope of the virion, originating from the inner lamella of the nuclear membrane, has embedded in it the virally encoded glycoproteins.
1.2.4.) Virology.

HSV-1 and HSV-2 are classified as Alphaherpesvirinae, characterized by a short reproductive cycle, which in in vitro cell cultures leads to a rapid spread of infection (a matter of hours), changes in cellular morphology or cytopathic effect (cpe) and cytolysis. In vivo they show neural tropism with frequent establishment of latency in the ganglia of the peripheral nervous system.

Although in nature HSV has only been found to infect man, this apparently narrow host range is not reflected in the laboratory where primates, rabbits, guinea pigs, rats and mice are all found to be susceptible to infection. Mice are the most common experimental host since they make excellent models in which to study the immune response to the virus. This has allowed a great advancement in knowledge and understanding in this field. The range of in vitro tissues culture systems which are permissive for HSV infection is also wide. Most commonly used are the human epitheloid line HEp2, African green monkey kidney cells (Vero), and baby hamster kidney cells (BHK). These cells allow propagation of established laboratory strains, new clinical isolates and manipulated recombinant viruses. Viral strains can be maintained through many serial passages and still retain infectivity and lethality in experimental animals. Again this has greatly enhanced the possibilities for advancement of understanding of the biology and immunology of these viruses.

As described there are two types of HSV known as type 1 and 2 which share a similar genome organization, etiology and serology. However there are subtle differences in their serology and in a number of their biological properties, for example tissue tropism, epidemiology and virulence. They are
clearly distinguishable on the basis of both serology and
detail of genetic constitution.

1.2.5. Latency.

An interesting aspect of HSV infection is the recurrence
of virus and symptoms without exogenous reinfection, which
suggests the existence within the individual of a reservoir
of latent virus. As discussed earlier (section 1.2.1) the
evidence from human disease clearly indicated the sensory
ganglia as the seat of latency (Hill, 1985).

Latent infection appears to reside primarily in the cell
body of the sensory neuron in the PNS but also in neuronal
cell bodies in the CNS. Reactivation of latent virus in the
trigeminal ganglia of mice was shown to result in the
presence of HSV specific antigens in neurons but not
satellite glial cells (McLennan and Darby, 1980). The low
levels of inoculum passed naturally probably require growth
at the site of primary infection prior to infecting the
nerve endings innervating the area (though experimentally a
large inoculum can do this directly). The virus then travels
by microtubule driven retrograde intra-axonal transport
(towards the CNS) to the neuronal cell body, at a rate of 2-
10 mm/hour (Hill, 1985). This has been clearly demonstrated in
animal models by zosteriform spread. From the site of
inoculation the virus infects the sensory ganglion, then
travels (via intra-axonal transport) to the CNS, infecting
neighbouring neurones, then back via the sensory ganglion to
areas of the dermatome adjacent to the site of infection
(Blyth et al, 1984; Simmons and Nash, 1984); as illustrated in
Figure 1.3.

Recent work by Clements and Subak-Sharpe (1988) seems
to indicate the presence of latent virus within the original
site of inoculation with infectious virus. Virus could be recovered from the site of inoculation (footpad) from latently infected mice treated with acyclovir or by surgical section of the nerve supplying the leg, to prevent appearance of virus in the footpad due to reactivation of latent virus in associated ganglia. Virus was not recovered directly but required at least 6 days incubation before virus was detectable.

Both viral DNA and RNA have been found in latently infected ganglia (Baringer, 1981). Viral DNA from latently infected mouse brain stems appeared not to be extensively methylated (Dressler et al, 1987) but appeared to be associated with nucleosomes as characteristic banding patterns were produced by micrococcal nuclease digestion (Deshmane and Fraser, 1989). The significance of these observations is still unclear and there is still no evidence to demonstrate whether the DNA exists in an integrated or episomal form. A particular RNA species has been implicated in the establishment and maintenance of latency. This latency associated transcript (LAT) is the only detectable HSV specific RNA product in latently infected neurons and is restricted to the nucleus (Stevens et al, 1987). The LAT gene has been identified and appears to be spliced and to lack a polyadenylation signal – LAT is known to be very poorly selected by oligo-dT (Wechsler et al, 1989). Two forms are found, one is 1.8-2.0 kb, the other is 1.3 kb; the former is detectable during productive infection, the latter is only associated with the establishment of latency (Wagner et al, 1988). They are derived from the complementary strand adjacent to the IE110 (ICPO) gene and both forms show an overlap with the 3' end of its coding sequence (≈46 codons) (Spivack and Fraser, 1987). LAT has been detected in the PNS (trigeminal ganglia) and in various connected regions of CNS (in both neurons and glial cells) in latently infected mice.
(Deatly et al, 1988). In addition to the mouse model LAT has been observed in latently infected ganglia in rabbits (Rock et al, 1987) and humans (Krause et al, 1988).

**Figure 1.3.** Zosteriform spread of HSV infection from an inoculum at the epidermis to the sensory ganglion via intra-axonal transport then to the central nervous system and by cell to cell transmission (probably involving astrocytes and oligodendrocytes) to other neurones finally leading to recurrent infection at the epidermis.
The question of whether a protein encoded by LAT or a mechanism of anti-sense regulation of IE gene expression controls the establishment and maintenance of latency may now be redundant. Mutant viruses lacking the LAT gene are able to establish latency in vivo and can be recovered from explanted ganglia (Sedarati et al, 1989; Steiner et al, 1989). The finer details of the latent infections established by these viruses have yet to be revealed, such as numbers of neurones involved, extent of transcription and genome copy number per cell. The role of LAT in latency is not clear.

Recrudescence, the reappearance of clinical lesions after resolution of primary infection results from the recurrence of infectious virus in the peripheral tissue following the reactivation of productive viral synthesis in a latently infected ganglion. Since the virus can remain latent for the lifetime of the host and may reactivate at any time, the latent and recurrent infections are very important. Alterations of host immune status may allow a reactivation of productive infection. This may be triggered by unrelated infection (e.g. influenza virus, rhinovirus, Human Immunodeficiency Virus HIV), stress (for example exposure to ultraviolet [U.V.] light) or immunosuppressive drug therapy. The latter is particularly important in patients recovering from transplant surgery, in whom recurrent herpesvirus infections may become life threatening (Borysiecwicz and Sissons, 1989).

Latency is a strategy the virus has employed to evade the host immune response, and to increase the cumulative duration of expression of infectious virion. This increases the opportunity for transmission of the virus between individuals. Latency is a very important factor in the persistence and spread of the virus in the population.
1.2.6.) Clinical Manifestations.

Clinical manifestations of primary HSV infection show great variability ranging from being totally asymptomatic to combinations of fever, pharyngitis, ulcerative and vesicular lesions, gingivostomatitis, localized lymphadenopathy and general malaise. In the case of both serotypes of HSV the most common clinical manifestation is a vesicular lesion on an erythematous base (Figure 1.4) ranging in size and severity between individuals, with a duration of 10-16 days. Primary infection leads to seroconversion; patients produce HSV specific antibodies which have virus neutralizing titres ranging from >1:1280 to <1:8. A number of individuals will later show recurrent infection with associated lesions, irrespective of the presence or absence of neutralizing antibodies. However, because of the primed immune response, recurrent lesions are often less severe and of shorter duration. Tissue damage at the site of a lesion is due to a combination of viral tissue necrosis and the response of lymphocytes and macrophages which are recruited to the site and activated.

Although there is no absolute tissue tropism, there are important trends in the sites the two virus types have been reported to infect. Between 93-97% of non-genital infections have been identified as HSV-1 compared to HSV-2, with the exception of infections of the hands and arms of which 53% are due to HSV-1 and 47% to HSV-2. Urinogenital infections are in the range 91-97% due to HSV-2 compared to HSV-1. Of the severe to fatal diseases and complications 75-98% are caused by HSV-1, except in newborns where 72% are caused by HSV-2, transmitted through an infected birth canal. An exception to the reported occurrence of both virus types at
various sites is the observation that viruses isolated from
the trigeminal ganglia are always HSV-1, whereas viruses
isolated from the sacral ganglia are always HSV-2 (Nahmias

Figure 1.4. Clinical manifestation of HSV-2 genital herpes,
showing vesicular lesions on an erythematous base (by
courtesy of Wellcome Research Laboratories, Beckenham).
HSV-1 is most commonly associated with lesions of the face and oral cavity. Primary infection may be overt but is commonly asymptomatic or experienced only as a mild pharyngitis. Recurrent infection is also often asymptomatic, but still transmissible. More serious complications often involve a generalization of infection producing eczema herpeticum, spread of infection to the central nervous system causing herpes encephalitis, or spread to the visceral organs, all of which may prove fatal if untreated. These conditions can often occur in patients experiencing immunosuppression, such as that used subsequent to transplant surgery, or infection with HIV. Neonates who are not yet immune competent are also often susceptible. Another serious complication of HSV-1 infection is keratoconjunctivitis in which the virus is spread to the conjunctiva, whether by cross contamination from an expressing facial lesion or by neural transmission. This may lead to deeper infection causing chorioretinitis, cataracts and ultimately blindness.

HSV-2 most commonly affects the glans and shaft of the penis and the vulva, vagina and cervix, producing vesicular lesions (Figure 1.4). It is thought likely that primary infections may in some instances be asymptomatic, which is commonly the case for recurrent infections. Primary infections are often accompanied by fever, dysuria, inguinal adenopathy and discharges. Recurrent lesions may affect the primary sites but frequently spread to the perineum, anus, buttocks and thighs. Complications are uncommon and usually benign, unless neonates are infected at partition. Some of the more severe complications are aseptic meningitis, or occasionally ocular infections following oral-facial HSV-2.
1.2.7.) Epidemiology.

Man is the only natural host for HSV and infection occurs world wide, even in isolated and primitive groups (Black, 1975). Its persistence in isolated populations is a reflection of its persistence in individuals (due to latency) and thus its ability to spread between successive generations, unlike epidemic viral diseases such as mumps and measles. The disease is rarely fatal and very often not physically debilitating, so infected individuals have never been quarantined as often is the case with fatal infectious diseases such as smallpox and cholera. Estimates suggest that more than one third of the human population suffers overt recurrent infection. This high prevalence remains in spite of increased awareness of the importance of both social and personal hygiene, which has drastically reduced the incidence of many other disease.

Analysis of the epidemiology of the two HSV types is made difficult by the frequency of asymptomatic infection and antigenic cross reactivity between the types. A number of serological techniques, such as neutralization, complement fixation, passive hemagglutination, indirect immunofluorescence and enzyme linked immunosorbent assay (ELISA) have been applied to the determination of epidemiology. More recent developments of type specific monoclonal antibodies and the identification of specific glycoproteins have allowed clarification of the specificity of serological response (Eberle and Courtney, 1981; Periera and Baringer, 1981).

The influence of age, nationality, geographic location and socioeconomic status on the prevalence of HSV infection has been reviewed and shows many interesting trends (Whitely, 1985; Rawls and Campione-Piccardo, 1981). Brazilian
aboriginal indians harboured infection in over 95% of children by the age of fifteen. Similarly in the poor black communities of America two thirds of children aged up to five were seropositive, a proportion which increased to 70-80% by early adolescence. By comparison middle class individuals of industrialized nations showed only 20% seropositive within the first 5 years of life, proceeding to 40 and 60% respectively in the years 10-20 and 20-30. This has been reviewed in more detail by Rawls (1985).

1.2.8.) Transformation.

Observations have frequently been made of cellular transformation by herpes simplex viruses. When treated with ultraviolet irradiation to inactivate structural and function genes, thereby preventing lytic replication of the virus, "the infectious" particles are able to induce both morphological and biochemical transformation of cells in culture (Rapp,1981). HSV transformed cells of various types contain detectable viral DNA and RNA, as well as cytoplasmic and membrane antigens, but no nuclear antigens. When inoculated into syngeneic animals these cells can form malignant tumours which will readily metastasize and spread. Animals carrying such tumours will produce antiviral antibodies. This is a quite different pattern to that generally seen with cells transformed with Adenoviruses and Papovaviruses (oncogenic viruses) in which viral antigens do not appear in the cytoplasm but do appear in the nucleus and on the membrane in association with transplantation rejection antigens (Rapp,1981).

Fragments of the HSV genome, when transfected into cells, can cause transformation. These fragments, refered to as morphological transformation regions (MTRs) are not
colinear between HSV-1 (MTR I - 0.29-0.45 MU) and HSV-2 (MTR II - 0.58-0.62 ; MTR III - 0.54-0.58) (Galloway and McDougal, 1983). Many sub-fragment of these regions can transform although expression of viral antigens is not necessarily observed; some of the fragments are too small to encode viral proteins (Macnab, 1987). It has also been noted that viral DNA can be lost during serial passage of these cells though the transformed phenotype is retained. The long and short repeat regions of HSV have homology with cellular DNA which may facilitate intergative recombination, allowing mutagenesis of the cellular DNA by changes in gene expression or by rearrangements of structure (Macnab, 1987).

Neither HSV-1 nor HSV-2 contain recognisable oncogenes. Protein kinase activity has been associated with oncogenic transformation and many oncogenes are active protein kinases: v-src, v-abl, v-fos and v-yes (Hunter, 1984). It is therefore possible that the 53 kDa product of the US3 gene of HSV-1, proposed to be a protein kinase, may play a role in transformation. However, this gene is not included in the known MTR regions (Macnab, 1987).

Interest in the transforming potential of HSV stems from epidemiological evidence associating HSV-2 infection with human cervical cancer (Whitely, 1985 ; Rawls, 1985). Experimentally HSV-2 has been shown to induce cervical neoplasia and cervical carcinoma in mice. In humans HSV DNA can be detected in cervical invasive neoplastic and cervical carcinoma tissues; however, Human Papilloma Virus (HPV) DNA is more frequently identified in these types of tissue (Macnab, 1987).

Another role for HSV-2 in cervical neoplasia/carcinoma may be in synergising with HPV (zur Hausen, 1982). It is recognised now that human cytomegalovirus (HCMV), another
human herpesvirus with transforming potential (Tevithia, 1985), can be sexually transmitted and may also synergise with HPV. HCMV DNA has also been identified in cervical neoplastic/carcinoma tissues (Macnab, 1987)

There is clearly a potential for the involvement of HSV-2 in malignant transformation of human tissues, however if it truly does play a role the exact mechanisms remain to be defined.

1.3.) MOLECULAR BIOLOGY OF HERPES SIMPLEX VIRUS.

1.3.1.) Genome.

The structure and organization of the HSV genome has been described (Section 1.2.1). The herpes simplex viruses both have G+C rich nucleotide composition: HSV-1 68.3% G+C (McGeoch et al., 1988) and HSV-2 69% G+C (Kieff et al., 1971). They share about 47% sequence homology and when co-infected can undergo intertypic recombination which may yield viable progeny.

Clonally purified progeny which are the products of intertypic recombinational events between HSV-1 and HSV-2 can be generated during coinfection with the two virus types. Analysis of these has allowed a correlation to be made between the restriction enzyme maps of the two viruses and a number of virus encoded infected cell polypeptides (ICPs) (Marsden et al., 1978; Morse et al., 1978; Ruyechan et al., 1979; Haliburton, 1980). This type of analysis was the beginning of work that has led to the assignment of map positions for several of the genes encoding these polypeptides. Marker rescue of defects in gene products by recombination or cotransfection of specific restriction
fragments has also added to the localization of specific HSV
genes on the genome map (Stow et al, 1978; Parris et al, 1980).

More recently the nucleotide sequence of HSV-1 has been
determined. Initially sequence was determined for the unique
short region (McGeoch et al, 1985), then the short repeat
(McGeoch et al, 1986) and the long repeat (Perry et al, 1986), and finally for the long unique region (McGeoch
et al, 1988). These works represent significant advances in
the understanding of HSV genome organisation and the
location of virus encoded gene products and functions (see
Figure 1.5).

1.3.2.) HSV Transcripts.

Investigation of viral specific mRNA has shown great
similarity between the transcripts of HSV-1 and HSV-2. HSV
mRNA, like host cellular mRNA, is synthesized in the nucleus
and is capped at the 5' end, polyadenylated at the 3' end
and internally methylated. Some HSV mRNAs are spliced during
their biosynthesis, particularly the large nuclear
associated RNAs, though many are not. A similar picture is
found with EBV and CMV (Wagner, 1985). Only two HSV-1 genes
contain introns and require splicing, the IE110 (ICP )
(Perry et al, 1986) and a gene of unknown function
designated UL15 (Costa et al, 1985). The primary transcripts
of the remaining genes are therefore fully functional.

HSV mRNAs fall into three general classes, which reflect
the temporal regulation of HSV gene expression. The α mRNAs
are the first to be expressed in an unmodified host. They
are expressed in the absence of de novo protein synthesis.
They are few and map at or near the long or short repeat
regions. One of the polypeptides translated from α mRNAs,
IE175 (ICP4), can replace the adenovirus early gene Ela function (Feldman et al, 1982), and activates transcription of the HSV early and late genes. IE110 (ICP0) activates transcription of late genes and can act independently or in cooperation with IE175. For this reason the β mRNAs require protein synthesis for their expression but are detected in the absence of DNA replication. There is an overlap of the expression of these intermediate β mRNAs and the true late ψ mRNAs which absolutely require viral DNA replication for expression. Inability to distinguish clearly between these mRNA classes is partly due to difficulties in successfully inhibiting viral DNA synthesis by treatment with drugs such as hydroxyurea, mitomycin C and arabinosylcytosine and partly due to the fact that many mRNAs are detectable before viral DNA synthesis but much more abundant thereafter. These mRNAs are referred to as "leaky late" or βψ mRNAs. There are many β, βψ and ψ mRNAs and they map throughout the non-repeated regions of the long and short genome sequences (Spear and Roizman, 1980).

Soon after infection host polysomes are found to be dispersed and stable RNA synthesis is inhibited (Sydiskis and Roizman, 1967). Up to 90% of the polysomes forming after this event are associated with viral mRNA. The "host shut off" effects of infection were found to be a property of both the virion particle itself and elements transcribed from the genome (Fenwich and Clark, 1982). A mutant virus defective in "host shut off", vhs, was found not to degrade host mRNA and to have longer viral mRNA half lives (Kwong et al, 1988). This mutant was shown to lack the UL41 gene and it has been suggested that the protein encoded by UL41 may interact with host factors determining mRNA half life, causing an overall reduction in half life (Kwong and Frenkel, 1989).
Other features of HSV mRNAs are that despite the GC rich nature of the genome the AATAAAA transcription termination signal or a near variant is found in the 3' end of nearly all transcripts so far analysed (Wagner, 1985). These sequences commonly appear fairly close to the translation termination codon often 50-80 bases downstream. This means that specific mRNAs are frequently only a few hundred bases larger than their DNA coding sequences, and the additional RNA is mainly 3' poly A.

1.3.3. Gene Locations.

The availability of recombinant DNA methodology has been invaluable in the localization, isolation and characterization of specific HSV transcript. Specific fragments of DNA from a known genomic location can be cloned and used to probe Northern blots of HSV mRNA and to hybrid select those mRNAs identified. Cell free translation system can then be used to translate the polypeptide precursors of the gene products, which can be identified with suitable antisera or monoclonal antibodies. In this way a polypeptide can be mapped to a specific genome location. Some transcripts of HSV have been mapped, to the nucleotide level, by use of SI nuclease and exonuclease VII to digest hybrids of genomic DNA and mRNA (reviewed by Wagner, 1985). Around 22 HSV-1 specific polypeptides have been located by characterisation of their transcripts. These include early genes such as ICP4 regulatory protein, the capsid protein VP5 and the glycoproteins gB, gC, gD, and gE. Also several enzymic functions have been located, such as alkaline exonuclease, thymidine kinase, ribonucleotide reductase, deoxypyrimidine triphosphatase and a DNA binding protein (reviewed by Wagner, 1985).
Among the genes identified a number have been mapped to the same coordinates in HSV-1 and HSV-2. The thymidine kinase, ribonucleotide reductase, capsid protein VP5 and a number of glycoproteins (including gB, gC, gD and gG) have been comapped. This data and the localization of transcript classes suggest very similar arrangements of the HSV-1 and HSV-2 genomes.

Figure 1.5. The organisation of genes within the HSV-1 genome (from McGeoch et al, 1988). The upper scale represents map units, the lower scale represents kilobasepairs. The size and orientations of proposed functional open reading frames are indicated by arrows. The locations of origins of DNA replication are shown as X. The unique regions are shown as solid lines and the repeated elements as open boxes. Vertical lines represent proposed locations of polyadenylation sites. 41 of these genes have been characterised (to varying extents) and are noted over leaf, in Table 1.1 (N.B. size is for unprocessed polypeptide). Relevant references are detailed by McGeoch et al (1988).
Table 1.1. Summary of characterized HSV-1 genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of amino acids</th>
<th>Size kDa</th>
<th>Function or property</th>
</tr>
</thead>
<tbody>
<tr>
<td>IE110</td>
<td>(19)</td>
<td></td>
<td>IE transcriptional regulator</td>
</tr>
<tr>
<td></td>
<td>(222)</td>
<td>78.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(534)</td>
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<td></td>
</tr>
<tr>
<td>UL5</td>
<td>882</td>
<td>98.7</td>
<td>DNA replication (ATP utilising?)</td>
</tr>
<tr>
<td>UL6</td>
<td>676</td>
<td>74.1</td>
<td>Virion protein</td>
</tr>
<tr>
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<td>750</td>
<td>79.9</td>
<td>DNA replication</td>
</tr>
<tr>
<td>UL9</td>
<td>851</td>
<td>94.2</td>
<td>DNA replication</td>
</tr>
<tr>
<td>UL12</td>
<td>626</td>
<td>67.5</td>
<td>Deoxynucleoside</td>
</tr>
<tr>
<td>UL19</td>
<td>1374</td>
<td>149.1</td>
<td>Major capsid protein</td>
</tr>
<tr>
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<td>838</td>
<td>90.3</td>
<td>Glycoprotein gH</td>
</tr>
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<td>376</td>
<td>40.9</td>
<td>Thymidine kinase</td>
</tr>
<tr>
<td>UL25</td>
<td>580</td>
<td>62.6</td>
<td>Virion protein</td>
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<td>635</td>
<td>62.4</td>
<td>Capsid protein</td>
</tr>
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<td>100.1</td>
<td>Glycoprotein gB</td>
</tr>
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<td>1196</td>
<td>128.3</td>
<td>Major DNA binding protein</td>
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<td>1235</td>
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<td>596</td>
<td>63.9</td>
<td>Locus of immune cytolysis resistance mutant</td>
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<td>Virion protein</td>
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<td>124.0</td>
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<td>88</td>
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The locations of gene coding sequences in HSV-1 is now known in absolute terms. There are 56 potential coding sequences in the unique long region, another 12 in the unique short region and two copies each of a gene in the long repeat and a gene in the short repeat (McGeoch et al., 1988). There are 70 possible polypeptide products. A number of genes have been assigned to known viral products and functions, however, many genes remain to be clearly characterized.

1.4.) HSV GLYCOPROTEINS.

The envelope glycoproteins encoded by HSV not only appear on the surface of the infectious virion but also appear on the cell membrane of the infected cell. They therefore constitute a major target for the immune response to the virus and to virus infected cells. They play important functional roles in the infectivity and spread of the virus.

The nomenclature of the glycoproteins was arrived at by agreement of workers in the field attending the International Herpesvirus Workshop in 1983 (described by Spear, 1985). All are prefixed with "g" then named alphabetically and suffixed with 1 or 2 to indicated which serotype of virus they are derived from. gA-1 was found to be a form of gB-1 carrying an immature oligosaccharide (Eberle and Courtney, 1980) and is now designated pgB-1 ("p" - precursor). gF of HSV-2 was found to map to the same region as gC-1 (Zezulak and Spear, 1983), and is now designated gC-2.

1.4.1.) Genes Encoding the Glycoproteins.

Seven distinct glycoproteins (gB, gC, gD, gE, gG, gH and gI) have been identified and characterized with
monoclonal antibodies and by nucleotide sequencing. Sequence analysis of the entire genome suggests that though a number of as yet uncharacterized genes may be membrane associated only these seven genes had the characteristics of membrane anchored surface glycoproteins (McGeoch et al, 1988).

In early studies five genetic loci encoding glycoproteins have been mapped on HSV-2 and four on HSV-1. The locations were originally inferred by analysis of variations of electrophoretic mobility of glycoprotein products from intertypic recombination between HSV-1 and HSV-2 (Marsden et al, 1978; Ruyechan et al, 1979). The positions of the loci have been confirmed and details of the products have been ascertained by a number of methods. gB-1 was mapped by marker rescue of temperature sensitive mutations affecting its production (Ruyechan et al, 1979; DeLuca et al, 1982; Holland et al, 1983) and mutation affecting its antigenic structure (Holland et al, 1983; Kousoulas et al, 1984). gC-1, gD-1 and gE-1 map positions were confirmed by selection of their respective mRNAs and identification of their in vitro translation products (Lee et al, 1982; Frink et al, 1983). This type of analysis was also carried out for mRNA encoding gB-1 (Rafield and Knipe, 1984). Finally the nucleotide sequence of the genes have been determined for gB-1 (Bzik et al, 1984; Pellet et al, 1985a), gB-2 (Bzik et al, 1986; Stuve et al, 1987), gC-1 (Frink et al, 1983) and gD-1 (Watson et al, 1982).

The gene for gB-2 has been mapped and sequenced and is colinear with gB-1. The genes for gD-2 and gE-2 have not yet been mapped in such detail but have an implied colinearity, with gD-1 and gE-1 respectively, from intertypic recombination (Ruyechan et al, 1979), an approach which successfully comapped gB-1 and gB-2. gC-2 was initially misnamed gF as its gene product did not show antigenic...
homology with gC-1 (Balachandran et al, 1981, 1982). Antigenic relatedness of gC-2 to gC-1 was eventually demonstrated (Zezulak and Spear, 1983) and the two genes have been mapped to the same region of the respective genomes (Zezulak and Spear, 1984a; Frink et al., 1983). It appears that the two genes have diverged during their evolution to a much greater extent than the genes of the other glycoproteins.

Figure 1.6. Map locations of the HSV glycoprotein genes.

A novel 124 kDa glycoprotein from HSV-2 was designated gG-2 (Roizman et al., 1984). Although no antigenically related counterpart could be identified in HSV-1 it was believed to correspond to HSV-1 US4 gene which has been designated gG-1 (McGeoch et al., 1987; McGeoch et al., 1988). The glycoprotein gH was first identified by characterization of the specificity of an anti-HSV monoclonal antibody LP11, and has now been mapped and sequenced (Gompels and Minson, 1986). Most recently gI has been identified as part of a complex involved in Fc binding (Johnson and Feenstra, 1987) and has been mapped to the US7 gene (McGeoch et al., 1985; McGeoch et al., 1988).
1.4.2.) Structure of the Glycoproteins.

The polypeptides of a number of glycoprotein genes have been characterized by in vitro translation of the related mRNAs and by inference from the published nucleotide sequence (described below). In each case the open reading frames contained no introns and correlated with the size of the translation product. They each had a hydrophobic signal sequence at the amino-terminus (Figure 1.7). This would ensure the association of the polysomes with the rough endoplasmic reticulum (RER), so that the protein would pass into the lumen of the RER concomitant with synthesis, as expected for a membrane protein. In the case of gD-1 and gD-2 these sequences are known to be cleaved off, by comparison of predicted amino acid sequence with that determined from analysis of the actual protein (Eisenberg et al, 1984).

Another feature of the predicted amino acid sequences of these glycoproteins is a region near the carboxy-terminus with a high degree of hydrophobicity, characteristic of the cell membrane spanning anchor sequences of membrane proteins. In each case there is a hydrophilic sequence before the carboxy-terminus assumed to be the cytoplasmic domain of the protein. These assumptions are based on experimental evidence, an example of which is the G protein of VSV in which these features were demonstrated (Rose et al, 1980). The assumption of this orientation in the membrane of the glycoproteins is supported by the mapping of mutations affecting the antibody recognition of gB-1 to the amino-terminal domain (DeLuca et al, 1982). Also all of the potential N-linked glycosylation sites (Asn-X-Thr or Asn-X-Ser) occur in the amino-terminal domain (Figure 1.7), and it is expected that carbohydrate side chains are exposed on the outside of the cell.
Figure 1.7. Predicted structural organization of HSV glycoproteins showing an extracellular domain (ECD), carrying the potential attachment site for carbohydrate side chains, a hydrophobic transmembrane (TM) region and a cytoplasmic anchor domain (CA). Adapted from Pellet et al (1985b).
1.4.3.) Glycosylation.

An essential feature of these glycoproteins is the cumulative sugar residues covalently bound to the polypeptide. Addition of N-linked oligosaccharide to the glycoproteins has been shown by use of the specific inhibitor tunicamycin (Spear, 1985). Blocks of preformed carbohydrate become linked to the amino group of certain asparagine residues. These groups are then processed by specific cleavage e.g. α-mannosidase cleaves (mannose)_9 (N-acetylglucosamine)_2 to (mannose)_5 (N-acetylglucosamine)_2 (Kariainen and Pesonen, 1982). High mannose immature forms of the glycoproteins are sensitive to cleavage by endo-β-N-acetylglucosaminidase H (Endo H) (Spear, 1985). Trimming of mannose residues and addition of complex sugar residues results in glycoprotein which is insensitive to Endo H. In the case of gB-1 some carbohydrate can be cleaved from the mature form, suggesting that it remains partially unprocessed. The use of alkaline borohydride, which only releases O-linked carbohydrate, indicates that gB-1, gC-1, gC-2, gD-1, gD-2 and gE-1 all contain O-linked carbohydrate (Johnson and Spear, 1983; Zezulak and Spear, 1983; Serafini-Cessi et al., 1988). Metabolic inhibitors have been used to investigate glycoproteins in related herpesvirus, VZV gpII (Montavolo and Grose, 1987) and HCMV gB (Britt and Vugler, 1988) which undergo essentially the same processing. Sulphate and fatty acid are both found coupled to gE-1. There is, however no evidence for the addition of such prosthetic groups to the other HSV glycoproteins.

1.4.4.) Synthesis of Glycoproteins.

It might be expected that synthesis of the glycoproteins would be related to the appearance of their respective
mRNAs. In a study of HSV-2 the glycoproteins gB-2, gD-2 and gE-2 were detected by monoclonal antibody precipitation as soon as 1-3 hours post infection (p.i.), at which time gC-2 and gG-2 were not detectable. After 5-7 hours the synthesis of gD-2 and gE-2 declined, whilst gB-2 increased steadily up to 11 hours p.i., and gC-2 and gG-2 also increased to 11 hours p.i. (Balachandran et al, 1982). Similar observations have been made for the synthesis of some of the HSV-1 glycoproteins (Spear, 1985).

The mRNAs encoding gD-1 and gB-1 are both $\beta$ class transcripts, not requiring viral DNA replication, whereas gC-1 is a $\tau$ class transcript, requiring viral DNA replication. Thus gC-1 appears later in infection (Wagner, 1985). This evidence reflects observations on polypeptide synthesis. It has been noted that gD-1 synthesis declines after 6 hours p.i. despite continued synthesis of gD-1 mRNA which is still functional in in vitro translation (Johnson and Spear, 1984). This is an indication that there may be other mechanisms controlling translation that are as yet uncharacterized.

1.4.5.) Events and Sites Involved in Synthesis.

The glycoproteins are presumed to be produced on the polysomes associated with the RER. gD-1 and gD-2 produced in vitro will associate with dog pancreas microsomes and become glycosylated, but only if the microsomes are present at the initiation of translation. This suggests that the amino-terminal signal sequences are involved in initiating the membrane association. This membrane association is supported by the observation that newly synthesized glycoproteins cannot be extracted from cells without the use of detergents. These proteins are found to be glycosylated as soon as they are detectable (Spear, 1985).
Signal sequences are cleaved off, probably before the completion of translation. Changes in the electrophoretic mobility of individual gene products are observed as processing of the carbohydrate takes place. Usually migration during sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) becomes slower reflecting an increase in the molecular weight and a change in the relative charge due to the sugars. This change in mobility is not necessarily representative of the change in molecular weight, since glycoproteins are known to migrate anomalously on SDS PAGE.

Maturation of the carbohydrate is thought to be due to conversion of the high mannose type oligosaccharide, added in the RER, to complex type oligosaccharide (as described above). This process has been shown to take place in the Golgi apparatus (Spear, 1985). Use of Monensin, which inhibits the transport of material from the Golgi to the cell surface and interferes with normal Golgi functions, inhibited the addition of O-linked oligosaccharide and transport of the glycoproteins to the cell surface (Johnson and Spear, 1982). In cells treated in this way virions accumulated in intracellular vesicles probably derived from the Golgi apparatus. These virions were infectious even though they contained only immature forms of the glycoproteins' carbohydrates. It is thought that glycoproteins may be processed and matured in situ on the surface of virion which have acquired the immature forms whilst budding through the inner nuclear membrane. This is supported by the observation that cells lacking glycosyltransferases and cells blocked in maturation of the glycoproteins, by use of ammonium ions or Monensin, accumulate virion containing the immature forms (Spear, 1985).
1.4.6.) Glycoprotein Functions.

The primary function of the glycoproteins is to mediate attachment to the cell, adsorption and penetration of the virus. Thus they will control the cell types which can be targets, and determine the particular host specificity and range and any tissue tropism of the virus. By mediating membrane fusion, required for penetration of the virion, they may also enhance the cell to cell spread of the virus, producing the foci of infection commonly observed in vitro and in vivo, and the formation of syncytia. They are capable of acting as receptors of immunoglobulin Fc region and the C3b component of complement (Spear, 1985). They will almost certainly play a role in the envelopment of the nucleocapsid, and the egress of the completed virion.

1.4.6.1.) Adsorption.

The host cell surface receptor for adsorption of the virion has not been identified. This is in part due to the fact that the virion component responsible for adsorption also has not been identified. The glycoprotein gC has been ruled out since mutants of both HSV-1 (Manservigi et al., 1977) and HSV-2 (Zezulak and Spear, 1984b) completely lacking the respective gene products are still infectious. Another candidate could have been gB. There are a number of mutations within gB which affect virion infectivity, none of which prevent binding of mutant virion to the target cells. One piece of evidence is available regarding the cell surface receptor for HSV. It has been noted that for each serotype, binding of the homologous virus is competitive, but binding of the heterologous virus is not (Vahlne et al., 1979). This suggests that the cell surface receptor differs for the two serotypes. This may also be due to a
difference in the viral antireceptor used by the two virus types.

1.4.6.2.) Penetration.

As already mentioned there are a number of mutations affecting the gB-1 product which do not affect adsorption. These mutations do however affect the penetration and rate of entry of the virion and its ability to promote cell fusion (DeLuca et al., 1982). The positions of these mutations within the gB-1 open reading frame have been defined at the nucleotide sequence level (Bzik et al., 1984). The lack of infectivity of these virions is complemented by the use of polyethylene glycol, a well known promoter of cell membrane fusion. Together with the defect in the ability of these virions to cause cell fusion this implies that gB-1 has the function of inducing fusion of the virion envelope with the cell membrane, thereby placing the infectious nucleocapsid in the cytoplasm. A series of studies have shown that the absence from virions of gB-1 (Cai et al., 1988), gD-1 (Ligas and Johnson, 1988), gH-1 (Desai et al., 1988) or gE-1 (Chatterjee et al., 1989) does not prevent adsorption of virions to the cell surface but does prevent penetration. In addition, specific monoclonal antibodies directed against gB-1 (Highlander et al., 1988), gD-1 (Highlander et al., 1987), gH-1 (Fuller et al., 1989) and gE-1 (Chatterjee, et al., 1989) reduce the rate of or prevent penetration. This suggests that there are a number of important interactions following adsorption which lead to membrane fusion between the virion and the target cell, involving at least these four glycoproteins.

It is interesting that cells expressing gD-1 alone show a resistance specifically to HSV-1 infection, though binding of virus to these cells was not affected (Johnson and Spear,
It was proposed that the recombinant gD-1 may sequester a cell surface receptor which is required for interaction with gD-1 on the virus to induce penetration.

Penetration by membrane fusion may occur at the cell surface or may first require endocytosis and associated changes in pH and ion concentrations. As discussed by Spear (1985), the latter appears to be the case for viruses such as VSV and influenza, but for HSV the evidence points to cell surface fusion of membranes. Electron micrographic evidence suggests that attached virus does not become endocytosed (Offey and Spear, 1989).

1.4.6.3. Cell Fusion.

There are a number of genetic loci on the HSV genome in which mutations have caused the promotion of cell fusion, or syncytia formation, the so called "syn" loci (Ruyechan et al., 1979; Little and Schaffer, 1981). Only one of five loci lies within a known glycoprotein gene - the syn3 locus in gB-1. Sequence analysis has determined that the mutation is in the predicted cytoplasmic domain, and not the extracellular domain (Bzik et al., 1984). The nature of the gene products involved in the other syn mutations has not been clarified. The functional significance of the syn3 mutation in gB-1 is not yet clear and therefore little is known about the mode of action of gB-1 in inducing cell fusion.

A number of monoclonal antibodies have been shown to inhibit cell fusion (Noble et al., 1983). Eight showing specificity for gD-1 blocked cell fusion, implying a role for gD-1. However five antibodies with specificity for gB-1 failed to inhibit cell fusion significantly. This may be a consequence of lack of overlap between the epitopes and the
fusion functional domain(s). In this work anti-gE-1 and anti-gC-1 antibodies also failed to inhibit fusion, though again this does not rule out a role for gE-1 and gC-1. Other monoclonal antibodies against gE-1 have been shown to inhibit cell fusion (Chatterjee et al, 1989) thus implying a role for gE-1. Cells expressing gB-1 alone have been shown to undergo cell fusion when exposed to low pH as compared to cells containing the expression vector alone, which do not (Ali et al, 1987).

Further evidence for a role for glycoproteins in cell fusion events comes from the observation that use of inhibitors of glycosylation or maturation of glycoproteins also inhibits cell fusion (Spear, 1985).

1.4.6.4.) Envelopment and Egress of Virion.

The predicted amino acid sequence for the glycoproteins so far characterized by nucleotide sequencing all show a domain carboxy-terminal to the putative transmembrane region. This carboxy-terminal domain should be localized facing the cytoplasm or nuclear matrix of the infected cell or the tegument of the virion (Figure 1.6). It is possible that these protein sequences are involved in directing the envelopment of the nucleocapsid as it buds through the inner nuclear membrane. This arrangement would ensure that the virion has the full complement of glycoproteins necessary to provide it with its essential biological functions.

Since mutants completely lacking gC-1 are still infectious, the implication is that they do not affect envelopment. The mutations in gB-1 described above do not cause complete absence of gB-1 protein from the virion. However gB-1 may be present in the virion in an aberrant conformation. So whilst a role for gC-1 in envelopment can
be ruled out, a role for gB-1 cannot.

Once enveloped, the virion must travel from the perinuclear lumen to the outside of the cell. A natural route for proteins is from the RER via the Golgi apparatus to the cell surface by exocytosis. The glycoproteins may aid egress simply by introducing the virion into this system and engaging a system of "reverse phagocytosis", as discussed by Spear (1985). Evidence for this route of egress, previously mentioned, included the use of Monensin to inhibit Golgi function, resulting in accumulation of virions in intracellular vesicles; however, a specific role for glycoproteins in egress has not been demonstrated.

1.4.6.5.) Immune evasion.

Once replicating in the host, virions and virus infected cells become the target of antibody mediated complement dependent cytolysis. The glycoproteins can serve to evade this arm of the immune response by interfering with vital steps in its partway. Viral glycoproteins have been shown to bind both immunoglobulin Fc domain (the domain involved in binding the first component of complement C1q) and the C3b component of complement (essential in the cascade which leads to cytolysis). Early mapping studies suggested that gE bound to Fc and that this was comparable between HSV-1 and HSV-2 (Para et al, 1982). It has now been shown that a complex formed between gE-1 and gI-1 mediates the Fc binding observed with HSV-1 (Johnson et al, 1988). C3b binding is mediated by gC-1 and gC-2 and can provide protection against complement mediated neutralization of viral infectivity (McNearney et al, 1987). When individually expressed on the cell surface both gC-1 and gC-2 can bind C3b; however the C3b binding activity of gC-2 appears to be blocked in HSV-2 infected cells (Seidel-Dugan et al, 1988).
This is presumed to be due to interference by other viral glycoproteins.

1.5.) GLYCOPROTEIN B.

1.5.1.) A family of related proteins.

Glycoprotein B is worthy of particular note as it has recently been found that proteins quite closely related to gB-1 occur in many herpesviruses from different subfamilies. An open reading frame, labelled BALF 4, was detected during nucleotide sequence analysis of the EBV genome, which showed significant homology with the gB of HSV-1 at the level of predicted protein sequence and secondary structure (Pellet et al., 1985b). This open reading frame was subsequently related to a gp110 (Gong et al., 1987) identified in EBV infected cells. This protein showed structural similarities to gB-1, but had a significantly different distribution in the infected cell and did not appear on the cell membrane. There is evidence of serological cross reactivity between the BALF 4 gene product and HSV-1 gB (W.L. Chan, personal communication). There is also evidence of serological cross reactivity between an EBV protein, of molecular weight 125kDa, HSV-1 gB and VZV (gp63K) gpII (Emini et al., 1987), and between gB-2 specific sera and proteins from HSV-1, CMV and EBV (Balachandran et al., 1987). The cross reactivity between gB-1 and a gp63K of VZV had been previously noted (Kitamura et al., 1986).

Confirmation of a CMV analogue of gB has been provided by sequence analysis of a CMV gene and its expression in a recombinant vaccinia virus (Cranage et al., 1986). It shows extensive homology with the predicted amino acid sequence and secondary structure of HSV-1 gB and EBV BALF 4. The
signal sequences, transmembrane sequences, and the number and location of cysteine residues and potential N-linked glycosylation sites are all quite highly conserved. The predicted amino acid sequence of gII, a VZV glycoprotein also had significant homology with gB-1 in particular with regard to the cysteine residues and a number of potential N-linked glycosylation sites (Keller et al, 1986).

Antigenic homologues of gB also appear to occur in herpesviruses causing animal disease, such as bovine mammilitis virus (BMV) and equine herpes virus type 1 (EHV-1) (Snowden et al, 1985). The determination of nucleotide sequences of glycoprotein genes of animal herpesviruses has revealed gB homologues in pseudorabies virus (Robbins et al, 1987), bovine herpesvirus type 1 (Misra et al, 1988), equine herpesvirus type 1 (Whalley, et al, 1989) and Marek's disease virus (Ross et al, 1989).

These data indicate that the gB gene has been conserved to varying extents in the evolution of all these viruses. The antigenic cross reactivity and homology at the amino acid level may reflect structural conservation required for the glycoprotein's function or functions. This suggests that gB plays an indispensable role during the reproductive cycle common to all herpesviruses regardless of species/tissue specificity.

1.5.2.) Identification of gB-1 and gB-2 and the genes encoding them.

The viral proteins VP7 and VP8 were initially characterized biochemically (Spear & Roizman, 1972) and later designated gA and gB (Spear, 1976). Subsequently they were shown to be forms of the same protein by antigenic analysis and use of monoclonal antibodies (Eberle & Courtney, 1980;
Pereira et al., 1981). Genetic studies based on a HSV-1 virus strain tsB5, derived from strain HFEM by bromodeoxyuridine mutagenesis, helped define the gB-1 locus. tsB5 contained two genetic lesions. The first, syn3, represented a loss of the ability of the parental strain to cause syncytia formation. The second was a temperature sensitive lesion which at the non-permissive temperature caused a failure to produce the glycoprotein gB (Manservigi et al., 1977; Sarmiento et al., 1979). These two lesions were mapped to the same region of the genome, between 0.30 and 0.42MU, by marker rescue of the mutations, and by comparison of the electrophoretic mobilities of viral glycoproteins of HSV-1 x HSV-2 intertypic recombinants (Ruyechan et al., 1979; Honess et al., 1980). An earlier study by Marsden et al. (1978) had mapped a 117kDa glycoprotein between 0.35 and 0.40 MU using recombinant viruses. These studies not only defined the position of the genetic locus for gB but also indicated that the genes were colinear between HSV-1 and HSV-2. Though the genes were similarly located the comparative electrophoretic mobilities indicated differences between the two. The colinearity of many viral genes, including glycoprotein genes, suggested by analysis of HSV-1 x HSV-2 recombinant viruses (Morse et al., 1978; Preston et al., 1978), has been confirmed as more and more gene sequences have become available for HSV-1 and HSV-2.

The antigenic relatedness of gB-1 and gB-2 was demonstrated by the use of monoclonal antibodies (Pereira et al., 1981 and 1982b). Differential processing of gB of HSV-1 and HSV-2 produced in Vero and HEp-2 cells was shown to affect the binding of only one of 24 monoclonal antibodies specific to gB; the antibody affected was the only type-specific monoclonal, the rest were all type common. This demonstrated that the two proteins were very closely related antigenically, but that there were
detectable differences affecting antigenicity. Examination of intertypic recombinants with these antibodies, by these authors, showed that part of the gB genes comapped to the region 0.37-0.38 MU.

High resolution marker rescue experiments mapped the syn3 locus and the ts lesion between 0.345-0.355 MU and 0.360-0.368 MU respectively (DeLuca et al., 1982). The position of the tsB5 lesion was confirmed (Holland et al., 1983) and mutations, directing antigenic variants resistant to gB specific monoclonal antibodies, mapped to 0.350 to 0.360. Sequence analysis of this region of the HSV-1 KOS genome revealed genetic information, such as promoter sequences (TATA boxes), an initiation codon, termination codon, and polyadenylation signal, around an open reading frame of 862 amino acids (903 before removal of the putative signal sequence), to give a calculated molecular weight of 96kDa for the non-glycosylated protein (Bzik et al., 1984).

The polypeptide forms of the glycoproteins produced in infected cells treated with tunicamycin have been characterized (Pizer et al., 1980; Norrild & Pederson, 1982), and the size of the gB-1 polypeptide estimated as 85-95kDa. The discrepancy in size may be due to proteolytic cleavage which occurs in vivo in some cell types (Pereira et al., 1982b; Zezulak and Spear, 1984b).

Transcriptional analysis of the HSV-1 genome revealed a 3.3kb RNA which mapped between 0.29 and 0.45 map units. This mRNA although defined as β class (discussed in Section 1.4.2.) showed increased expression with viral DNA synthesis and it was expressed until late in infection (Weinheimer and McKnight, 1987). The mRNA was shown to encode gB-1 by in vitro translation of this transcript selected by
hybridization to DNA from 0.343-0.361 MU, and immune precipitation of the translation product with gB specific polyclonal antisera (Rafeild and Knipe, 1985). The products identified were 102kDa and 85kDa. The difference in their size to those previously reported may have been due to the in vitro translation system used or the acrylamide gel system used to resolve the products.

The gB-2 gene was initially characterized by expression of an HSV-2 serologically reactive polypeptide fragment in E. coli from a HSV-2 DNA fragment parallel with the gB-1 locus (Person et al, 1985). This was followed by nucleotide sequencing of the complete locus in HSV-2, which revealed a gene showing 89% homology with gB-1, subsequently designated gB-2 (Bzik et al, 1986).

1.5.3.) B cell epitope structure of gB-1.

Many monoclonal antibodies have been raised against gB-1. A panel of 16 have been used to identify monoclonal antibody resistant (mar) mutant forms of gB-1 and define five distinct antigenic sites, I to V (Marlin et al, 1986). Similar antigenic site mapping has been carried out for gC-1 (Marlin et al, 1985) and gD-1 and gD-2 (Eisenberg et al, 1985; Isola et al, 1989).

It was subsequently shown that antibodies which recognised sites I, II and IV required complement for neutralizing activity, whereas an antibody which recognised site III was able to block viral penetration (Highlander et al, 1988). Deletion mutants of gB-1 were used to map the physical locations of these antigenic sites. Site I (residues 381-441), site III (residues 283-380) and site IV (residues 241-282) all mapped to the amino-terminal half of the molecule; site II (residues 596-737) mapped close to the
transmembrane domain (Highlander et al., 1988). In work characterizing epitopes on native and denatured gB over half the monoclonal antibodies generated were directed against discontinuous epitopes (Chapsal and Periera, 1988). The mar mutants have been characterized at the nucleotide level thus identifying amino acid changes which alter antigenic structure and affect virus penetration (Highlander et al., 1989). This study also showed that sites II and III were type common and that sites I and IV were type specific. The deletion mutants have also been used to investigate virus penetration and cell fusion mediated by gB (Cai et al., 1988).

1.6.) IMMUNOLOGY OF HSV INFECTION.

There are two arms to immunity against infectious agents, i) natural resistance, mediated by macrophages, natural killer (NK) cells and interferon (IFN), and ii) adaptive immunity, controlled by the humoral response (B lymphocytes) and the cell mediated response (T lymphocytes). These two arms of the immune response interact in a complex manner to provide protection against infectious agents.

1.6.1.) Natural Resistance.

Natural resistance mechanisms are thought to be active in the earliest stages of an HSV infection (both primary or recurrent) and to play an important role in preventing the dissemination of infection. Understanding of natural resistance has come from observations of i) patients with primary or secondary immunodeficiencies, looking for an association between specific deficiencies and the nature and severity of disease; ii) examination of disease in animals
suppressed in one or other aspect of immune response, iii) examination of differences in response of genetically resistant or susceptible strains of animal. Mice are very commonly used in immunological studies, since inbred strains are genetically well defined allowing cell transfer experiments and genetic crossing. Fortunately, despite the species specificity of many herpesviruses, HSV-1 and HSV-2 will infect mice and show a similar though not identical progression of disease. The differences, however, indicate that care has to be taken in inference of the mechanisms controlling the diseases in different organisms.

Laboratory strains of mice show varying degrees of resistance to HSV infection. For example C57BL/6 mice may survive $10^6$ PFU/mouse of HSV-1 thus being classified as resistant, whereas A/J mice have a 50% lethal dose ($LD_{50}$) in the range $10^{-10^2}$ PFU/mouse and are classified as susceptible. BALB/c mice have an $LD_{50}$ in the range $10^3-10^4$ PFU/mouse and are considered moderately susceptible. Genetic crossing suggests that resistance is a dominant trait with two independently segregating loci that are not H-2 linked (Lopez, 1980). Moderately susceptible BALB/c mice are a common model since they can mount an immune response to infection which can be modified by prior immunization.

When mature macrophages of different strains of mice are infected they display viral antigen at the cell surface but do not produce infectious virion. A significant difference was observed between the inhibition of viral macromolecular synthesis in macrophages from resistant strains of mice compared with those from susceptible strains (Sarmiento, 1988). Macrophages of resistant strains appeared to produce more interferon (IFN). IFN production has shown a correlation with resistance and susceptibility to infection both in vivo and in vitro (Lopez, 1985). Recently Seid et al
(1986) demonstrated that IFN produced by T cells significantly enhanced the antiviral activity of macrophages. Other lymphokines may also be involved such as tumour necrosis factor alpha (TNFα) which has been shown to kill HSV infected but not uninfected cells in culture (Koff and Fann, 1986). There is some evidence to suggest that reduction of the density of the macrophage-like Langerhans cells of the epidermis, which function as antigen presenting cells, increases the severity of clinical symptoms of infection (Sprecker and Becker, 1987). A similar effect was observed when U.V. irradiation impaired the antigen presenting function of these Ia+, macrophage-like, cells (Hayashi and Aurelian, 1986). This observation may correlate with the triggering of recurrent lesions by exposure to U.V. light.

NK cells also play a role in natural resistance and have been shown to lyse HSV infected fibroblasts. In humans NK cell are large granular lymphocytes, which lack the general T cell marker (Leu 4-) and are in general Leu 11+, Leu 7+, though there is heterogeneity in the functions and markers. These cells have been shown to mediate a significant reduction in the titre of infectious virus produce by infected fibroblasts in vitro (Fitzgerald et al., 1985). HSV glycoproteins have been shown to be the apparent targets for NK recognition and lysis of infected fibroblasts (Bishop et al., 1986). However, NK killing of HCMV infected fibroblasts was shown to occur with cells expressing only IE and E antigens (Borysiewicz et al., 1985). Since uninfected fibroblasts and tumour cells may also be lysed it was suggested that HCMV infection caused alteration of a cellular antigen which was the target for recognition by NK cells. More recently use of HSV infected fibroblasts treated with metabolic inhibitors has suggested that early gene expression was required, but as with HCMV observed NK
killing may be related to alteration of a cellular antigen (Lopez-Guerrero et al., 1988). Genetic resistance in mice appeared to correlate with good NK cell responses (Lopez, 1985). Further support for a role for NK cells in protection against HSV infection was provided by evidence of the reduced antiviral capacity of immune spleen cells in adoptive transfer experiments from HSV primed "beige" mice, which are NK cell deficient (Rager-Zisman et al., 1987).

Mechanisms of natural resistance are very important as a primary response to infection to prevent uncontrolled replication and dissemination of the virus before the adaptive immune response has a chance to control and clear infectious virus from the host. They will also certainly play an important role in controlling the establishment of latency and the early response to recurrent infection.

1.6.2.) Humoral Response.

Antibody response to HSV infection in humans and the mouse model are readily demonstrated by virus neutralization, immunofluorescence, complement fixation, immunoprecipitation and immune staining of blotted ICPs, or more sensitively by radio-immunoassay (RIA) and enzyme linked immunosorbent assay (ELISA). These methods can identify responses to virus encoded proteins that are located on the cell membrane or intracellularly.

The antibodies present after primary infection will immunoprecipitate, or stain in blots, the known virus encoded glycoproteins gB, gC, gD, gE, gG, gH and gI. It is predominantly gB and gD that are recognised by these techniques. The level and profile of antibodies remains constant for over two years, according to one study, even in the event of clinical or subclinical recurrent infection.
The profile of proteins precipitated by human serum varies between individuals and it is interesting that only a few of the known non-glycosylated infected cell proteins are identified in this way. However most HSV encoded proteins are able to elicit an antibody response experimentally (Norrild, 1985).

Many experiments have shown that some monoclonal antibodies raised against specific HSV glycoproteins can afford protection against a challenge with a lethal dose of virus, by passive transfer of antibody to naive mice (Lukic et al., 1985; Kumel et al., 1985). These data do not, however, correlate with the ability of the glycoproteins to immunize mice against lethal challenge. A 110kDa glycoprotein of HSV-1 (gB) was able to protect mice completely against lethal challenge, even though the monoclonal antibody specific to it and polyclonal serum raised against it were unable to confer any protection by passive transfer (Lukic et al., 1985).

Experiments which compared mice depleted of B cells from birth with untreated littermates for their ability to clear virus from the ear pinna and prevent spread of virus to the peripheral and central nervous tissues showed two important points. First, the B cell depleted mice were still quite able to clear infectious virus over the same time course. Second, the B cell depleted mice displayed a more extensive infection of the peripheral and central nervous system, with the consequence that they also had a higher rate of establishment of latent infection (Kapoor et al., 1982a). This suggests that, although not essential to recovery from infection, the antibody response is important in restricting the spread of virus to the CNS, and therefore in the establishment of latency.
Most recent studies to the humoral response relate to the ability of individual glycoproteins to induce HSV specific antibody production when used as immunogens (Section 1.6.7.). These studies do not address the relevance of this response to the frequently observed concomitant protective immunity.

1.6.3.) Cytotoxic T Lymphocytes.

Much attention has been focused on cytotoxic T lymphocytes (CTLs) in response to viral infection since they display the ability to recognise and lyse virus infected cells, often from an early stage of infection and prior to release of infectious virion, with fine specificity. The CTL response to influenza virus has been well characterized, its specificity being demonstrated as directed against individual gene products i.e. the hemagglutinin (Townsend et al., 1984a) and the nucleoprotein (Townsend et al., 1984b; Townsend and Skehel, 1984). Antigen recognition by these CTLs has been shown to be specific to fragments of the nucleoprotein defined by short synthetic peptides (Townsend et al., 1985, 1986).

Similar work has detected that CTL responses to vesicular stomatitis virus (VSV) glycoprotein and nucleocapsid (Yewdell et al., 1986), Sendai virus (Kast et al., 1986), lymphocytic choriomeningitis virus (LCMV) (Moskophidis et al., 1987), rabies virus (Cho et al., 1987), CMV (Reddehase et al., 1986; Lindsley et al., 1986; Koszinowski et al., 1987) and HCMV (Borysiewicz et al., 1983, 1989).

CTLs were originally shown to be functionally restricted in their killing to cells expressing autologous MHC antigens usually class I (Zinkernagel and Doherty, 1979). MHC restricted cytotoxic response to HSV has been demonstrated
demonstrated (Sethi et al., 1980; Pfizenmaier et al., 1977). However in humans MHC class II restricted T clones, specific for viral glycoproteins, have been shown to have cytotoxic activity (Yasukawa and Zarling, 1984a, 1984b). In mice HSV, specific CTLs can be induced by injection of live virus intravenously (i.v.), intraperitoneally (i.p.) or subcutaneously (s.c.) (Nash et al., 1980b). They are detectable between days 4-14 post inoculation with maximum activity at days 6-7. These CTLs require in vitro culture for 2-3 days before they show detectable activity unless they are prepared from mice pretreated with cyclophosphamide. It has been shown that in vitro culture is required before CTL activity can be expressed (Nash et al., 1985). It was suggested that this culturing caused the loss of suppressor cells, which are thought to be sensitive to low doses of cyclophosphamide (Liew and Howard, 1980).

CTL precursors in mice have the Lyt 1+ 2,3+ phenotype, becoming Lyt 1- 2,3+ when mature and active (in humans CD4- CD8+ phenotype), and are restricted to lysis of cells expressing class I MHC antigens. HSV specific CTLs show this restriction though, as described above, some human CTL clones have been identified as having the CD4+ CD8- (T helper) phenotype and are restricted to the lysis of cells expressing class II MHC antigens (Yasukawa and Zarling, 1984a, 1984b). HSV specific CTLs show both type common and type specific responses (Nash and Ashford, 1982; Eberle et al., 1981).

There was evidence to suggest that the CTL response to HSV was specific to the viral glycoproteins (Carter et al., 1981) and that of these one in particular, gC-1 was immunodominant (Glorioso et al., 1985; Rosenthal et al., 1987). This has been confirmed in one respect by the very low level of detectable CTL activity directed against syngeneic cells.
expressing recombinant gB-1 and no detectable activity against syngeneic cells expressing recombinant gD-1 (Blacklaws et al., 1987; Martin et al. 1987). This is quite different to findings with influenza virus and HCMV in which CTL are predominantly directed against non-structural proteins i.e. 'flu NP (Townsend et al., 1984b) and HCMV IEA (Borysiewicz et al., 1988a, 1988b). However in both cases some CTL response to structural glycoproteins is observed.

Experiments involving in vivo depletion in mice of specific T cell subsets, by treatment with anti-Lyt 2 serum, with proven abolition of CTL activity, showed that the mice were unaffected in their ability to clear infectious virus from the site of inoculation. These mice did however suffer a more severe and sustained infection of the nervous system. Mice depleted of L3T4+ T lymphocytes showed a slower clearance of virus from the site of subcutaneous inoculation. This suggested that CTLs may be more important in control of infection in the nervous system and T helper cells more important in control of infection in the skin (Nash et al., 1987). The relevance of this to the immune response in control of human disease has yet to be clarified.

1.6.4. Helper T Lymphocytes.

The first clear indication of a role for T helper (T\textsubscript{h}) cells in the response to HSV was the failure of athymic mice to produce anti-HSV antibodies (Burns et al., 1975). Development of techniques which allowed the propagation and maintenance of T cells in vitro has led to the identification of T cell clones specific to HSV. A T cell clone with an Lyt 1+ 2,3- phenotype (indicative of T\textsubscript{h} cells) was shown i) to proliferate in response to HSV, ii) provide help in adoptive transfer for B cells to produce anti-HSV
antibody and iii) confer protection from infection (Leung et al., 1984). This clone did not show any ability to mediate a delayed-type hypersensitivity response. Further characterization of this clone demonstrated that interaction, mediated by IFN gamma, with macrophages could enhance their antiviral activity (Seid et al., 1986).

Th cells, with the Lyt 1+ 2,3- phenotype, have also been reported to provide help for CTLs, and their frequency has been shown to be equivalent to that of CTL precursors (Schmid and Rouse, 1983; Prymowicz et al., 1985). More recent evidence suggests that Th for CTLs in the in vivo response to HSV is not required and may in some way be counter productive, down regulating CTLs in vivo (Nash et al., 1985).

As described above (Section 1.6.3.), human CD4+ CD8- T cell clones can act as class II restricted CTL. Recently human CD4+ class II restricted CTL clones have been shown to act as Th and provide help for antibody production by autologous B cells (Yasukawa et al., 1988). This is an interesting observation since it suggests the HSV responsive T cells may be multifunctional.

1.6.5.) Delayed-type Hypersensitivity T lymphocytes.

The delayed-type hypersensitivity (DTH) response induced by HSV in mice was characterized by Nash et al. (1980a), using subcutaneous or intradermal inoculation and subsequent elicitation of the characteristic swelling by subcutaneous injection of antigen in the ear pinna. It was found to be transferable with T lymphocyte populations, which also mediated antiviral immunity, from primed donor mice to naive recipient mice, and was restricted by MHC compatibility (Nash et al., 1981a). The membrane phenotype of these T delayed-type hypersensitivity (T-DH) cells was later
demonstrated to be Lyt 1+ 2,3-. This model was used to demonstrated DTH response to purified glycoproteins gC and gD of HSV-1. The response was type common for gD but type specific for gC (Schrier et al., 1983a). A specific DTH response was also demonstrated to purified gB-1 (Chan et al., 1985). The HSV-1 glycoproteins gB and gD constitutively expressed on mouse L cells were found to elicit a DTH response when injected into the ear pinna of HSV-1 primed mice (Blacklaws et al., 1987). The glycoprotein gD-1 expressed in a recombinant vaccinia virus was able to induce an HSV specific DTH response and stimulate L3T4+ cells which could mediate clearance of virus (10^4 PFU) from the ear pinna (Martin and Rouse, 1987). However tolerization of the DTH response by i.v. inoculation did not affect induced viral clearance, suggesting that the T-DH cells were not involved.

DTH responses have also been identified in other viral systems and, by the use of footpad swelling, show similar characteristics. This model has been used to demonstrate responses by T-DH specific for the viral hemagglutinin of reovirus (Weiner et al., 1980; Greene and Weiner, 1980), and by T-DH specific for hemagglutinin of influenza virus (Liew et al., 1979). In the influenza system this response was not associated with antiviral activity.

1.6.6.) Suppressor T Lymphocytes.

T cell populations that are capable of suppressing specific DTH response are induced by intravenous (i.v.) injection of live or U.V. inactivated HSV (Nash et al., 1981b). These T suppressor cells (Ts) can be of two types, i) those that suppress induction of DTH - afferent (Ts-eff) and, ii) those that suppress expression of an induced DTH - efferent (Ts-eff) (Schrier et al., 1983b). Further investigation demonstrated that these cells were
specific for the suppression of DTH and that the responses of T lymphocytes capable of specific cytotoxic activity and in vitro proliferation were not affected (Nash and Ashford, 1982). The membrane phenotype of these Tₜ cells was found to be Lyt 1+ 2,3- around seven days after inoculation but the suppressor population became increasingly Lyt 1- 2,3+ in character at times thereafter. Both early and later phase Tₜ were I-J positive (Nash and Gell, 1983). Analogous systems were identified with the i.v. route of inoculation for DTH response to reovirus (Greene and Weiner, 1980) and influenza virus (Liew and Russell, 1980).

1.6.7.) Immunization studies with individual HSV antigens.

Many of the HSV glycoproteins have now been analysed in isolation from the whole virus by purification of native antigen (Schrier et al., 1983; Chan et al., 1985; Dix and Mills, 1985), expression of individual genes in recombinant cell lines (Blacklaws et al., 1987; Rosenthal et al., 1987) or, more frequently, by expression of individual genes in recombinant vaccinia virus (r-vac).

Immunization of mice with a recombinant vaccinia virus expressing HSV-1 gB (gB-vac) induced an HSV specific antibody response and provided significant protection against lethal HSV challenge (Cantin et al., 1987). Further investigation revealed that immunization with live gB-vac induced an HSV specific class I restricted CTL response (McLaughlin-Taylor et al., 1988). This correlated with a gB directed CTL response induced by immunization of mice with syngeneic cells expressing gB-1 (Blacklaws et al., 1987). However Rosenthal et al. (1987) did not observe specific lysis of syngeneic cells expressing gB-1 by effector cells induced by HSV infection of mice, and this correlated with similar observations by Blacklaws et al. (1987). It may be
that the gB induced CTLs (Blacklaws et al., 1987; McLaughlin et al., 1988) occur as a result of induction of immune response in the absence of synergistic/antagonistic effects from lymphocytes responding to other HSV components. The significance of these observed CTL responses remains unclear.

Immunization of mice with a recombinant vaccinia virus expressing gC-1 (gC-vac) induced HSV-1 specific neutralizing antibodies and 90% protection against lethal challenge, versus 20-40% non-specific survival (Weir et al., 1989). In addition HSV-1 specific lymphoproliferation was observed. A recombinant vaccinia virus expressing gD-1 (gD-vac) has been used to immunize mice and provide significant protection against lethal challenge (Cremer et al., 1985). Immunization with gD-vac has been shown to induce an HSV-1 specific lymphoproliferative response in mice that appears to be mediated mainly (80%) by Lyt 2.1+ lymphocytes (Martin et al., 1987). However there is no class I restricted CTL activity, only a weak class II restricted CTL activity, possibly reflecting in the mouse the human class II restricted CTL activity observed by Yasukawa and Zarling (1984a, 1984b). Cells were induced in response to gD-vac, which appeared to inhibit HSV specific CTL induction. Though the phenotype of the suppressor cells was not demonstrated it was suggested to be Lyt 2.1+ (Martin et al., 1987). Both gG-1 and gI-1 have also been expressed in recombinant vaccinia viruses and have been shown to induce specific humoral responses (Sullivan and Smith, 1987, 1988).

The recombinant vaccinia virus system is a useful tool since it allows infection of animals or cells with a virus that will express a defined heterologous viral gene on the infected cell surface and in the virus envelope. However results from the use of these as immunogens must be
interpreted in the knowledge that synergy between anti-viral lymphocyte subsets, responding to the recombinant vaccinia, will be different from synergy occurring in response to the virus of interest (HSV). The availability of r-vac expressing the various HSV glycoproteins will allow definition of targets and types of response induced by HSV both in animals and in man.

1.7.) AIMS OF THE THESIS.

The observations of Chan (1985) and Lukic et al (1985) showed that the glycoprotein gB-1 evoked a strong T cell response which could confer protection against lethal HSV-1 infection in adoptive transfer experiments in mice. The overall aim of the work described here was to characterize the homologous gB-2 antigen of HSV-2, with particular interest in identifying the "T cell antigenic" domains of the protein, employing recombinant DNA technology to manipulate the gene product and in vitro and in vivo immunological systems to characterize the recombinant products.

This was approached in three stages.
1) The first aim was to locate accurately the gB-2 gene and identify the transcript encoding the polypeptide, since at the outset of this work the gene had not been characterized or sequenced.
2) The next aim was to express fragments of the gene product as polypeptide fusions to β-galactosidase in E.coli, to provide defined regions of the polypeptide to examine the antigenic domains of the protein.
3) The final aim was to analyse the immune response in mice to defined fusion proteins and the whole glycoprotein gB-2, with particular reference to the T cell response and the protective effects of immunization with these antigens.
2. GENERAL MATERIALS AND METHODS.

2.1. Cells.

Baby hamster kidney cells (BHK C13) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 60μg/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine, 0.01% sodium bicarbonate and 10% new born calf serum. They were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide in air. Cultures were seeded at between $10^5$ and $10^6$ cell per 500ml glass tissue culture flask and grown to confluence. Once confluent they were trypsinised and passaged, to maintain a working stock. For infections cells were grown in 75mm$^2$ plastic tissue culture flasks (Falcon).

African green monkey kidney cells (Vero) were grown in RPMI 1640 medium supplemented with 60μg/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine, 0.01% sodium bicarbonate and 10% foetal calf serum. They were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide in air. Working stocks were also maintained by serial passage.

Monolayers of cells were harvested for passage by trypsinization with 0.01% trypsin (w/v), 0.2% EDTA in PBS. Cells were washed by centrifugation (2000g for 10 minutes at 4°C) and resuspension in growth medium. They were counted and plated out at an appropriate dilution. Viable cells were identified by exclusion of 0.2% trypan blue in PBS and counted in a 0.1mm x 1/400 mm$^2$ haemocytometer (Weber).

2.2. Virus.

HSV-2 strain BRY was used throughout this work unless otherwise stated. Virus was prepared by infecting confluent
monolayers of BHK cells at a multiplicity of infection (m.o.i.) of 0.1 plaque forming units (PFU)/cell. Infected cells were cultured as described above, until the majority of cells in the monolayer showed cytopathic effect (for approximately 48 hours). Cytopathic effect (c.p.e.) was characterized initially by a change in morphology of the cell (rounding up) then by loss of adherence to the plastic. Infected cells were harvested from the plate, centrifuged (2000g for 10 minutes at 4°C), washed with ice cold PBS, centrifuged and resuspended in a small volume (0.5ml/10^7 cells) of DMEM. The infected cell suspension was then sonicated 3 x 15 seconds, using an MSE 100W Ultrasonicator (setting 20 = 6μm peak to peak). The infected cell sonicate was centrifuged at 4000g for 15 minutes at 4°C. The supernatant containing infectious virus was aliquoted and stored at -70°C. Unless otherwise stated samples referred to as virus are from dilutions of this material.

2.3.) Purification of virus.

BHK cells were grown to confluence and infected with HSV-2 BRY at an m.o.i. of 0.1. After 48 hours incubation the medium was harvested and cells removed from it by centrifugation at 2500g for 10 minutes at 4°C. Virus extruded from infected cells into the media, following maturation and egress, was pelleted from the supernatant by centrifugation at 27,000g for 20 minutes at 4°C. The virus pellet was resuspended in a small volume (2-3ml) of PBS and carefully layered onto a 40ml discontinuous sucrose gradient; 10ml each of 15, 30, 45 and 60% sucrose in PBS. The gradient was spun at 112,000g for 1 hour at 4°C. The opaque band between the 45% and 60% steps representing the intact enveloped virion was removed using a hypodermic needle (21G gauge) and 5ml syringe by puncturing the side wall of the centrifuge tube. The extracted band was diluted.
in PBS and the virus recovered by centrifugation at 27,000g for 20 minutes at 4°C. The pellet was resuspended in PBS. An aliquot was diluted, the O.D.280nm read and the protein concentration calculated. Suitable aliquots were dispensed and stored at -20°C.

2.4.) Virus plaque assay.

In order to estimate the amount of infectious virus present in a sample of virus preparation, it was diluted and titrated onto monolayers of Vero cells in 24 well tissue culture plates (Flow). After adsorption of the virus to the cells for 1 hour the supernatant was drawn off and the cultures overlayed with supplemented RPMI medium containing 1% carboxy-methylcellulose. After 48-72 hours of culture the overlay was removed and the cells stained with 0.1% crystal violet (w/v), 20% methanol (v/v), 10% formalin (v/v). Titrations were 5 fold and in quadruplicate. The concentration of virus in PFU/ml was calculated from the average of two dilutions (in the range 20-100 and 5-20 plaques per well). A typical virus preparation gave 5ml of 3x10^7 PFU/ml from 10^8 infected cells.

2.5.) Growth of hybridomas.

Hybridomas (TI57; Chan, 1983) were removed from liquid nitrogen storage, quickly thawed to 37°C and immediately transferred to a large volume of CM medium to wash out DMSO in the freezing buffer. The cells were centrifuged (2000g for 10 minutes at 4°C) and resuspended at 5x10^5 cells/ml in CM medium (RPMI supplemented with 60μg penicillin, 100μg streptomycin, 2mM L-glutamine, 0.01% sodium bicarbonate, 0.1mM sodium pyruvate and 10% foetal calf serum). They were incubated in a humidified atmosphere of 5% carbon dioxide in air. The culture was observed daily and the media
supplemented as necessary according to the increase in cell density. When sufficient cell numbers had accumulated they were harvested by centrifugation (2000g for 10 minutes at 4°C). The supernatant containing secreted monoclonal antibody was kept for use to immuno-stain Western blots. The cells were resuspended in a small volume of growth medium to give 10^7 cells/ml, and used to prepare ascitic fluid.

2.6.) Preparation of ascitic fluid.

Mice syngeneic with the strain used to produce the hybridomas (BALB/c) were primed intra peritoneally (i.p.) with 0.5ml of pristane. 5 days later they were administered i.p. with 5x10^6 hybridoma cells in 0.5ml of growth medium, introduced slowly through a 21 gauge hypodermic needle. After 10-15 days the mice displayed considerable distension of the abdomen. The mice were sacrificed and the ascitic fluid accumulated in the peritoneal cavity drawn off. The yield of ascitic fluid was between 1.5-2.0ml/mouse. The ascitic fluid was centrifuged in an Eppendorf microfuge at 4°C for 10 minutes to remove cell and solid debris. The supernatant was stored at -20°C or used to prepare purified monoclonal antibody.

2.7.) Preparation of purified monoclonal antibody.

Monoclonal antibody was purified from ascitic fluid by affinity chromatography on a 5ml protein A-Sepharose column. The column was cleaned with 5 column volumes of the elution buffer, 0.1M sodium citrate pH 3.0, then equilibrated with 10-20 column volumes of 0.1M sodium phosphate pH8.5. The ascites fluid sample was adjusted to pH 8.5 by the addition of 0.2M disodium hydrogen phosphate (∼ pH 9.0). This sample was loaded on to the column and equilibrated for 1 hour under stopped flow conditions. It was allowed to flow and
the eluate collected, re-applied to the column and once again equilibrated for 1 hour. The column was then washed extensively with 20 column volumes of PBS. The specifically bound immunoglobulin was eluted with 4-5 column volumes of 0.1M sodium citrate pH 3.0. This was collected into a vessel containing 1M Tris.HCl pH 9.6 at 0.2 of the final volume eluted. The neutralized eluate was then dialysed extensively against several changes of 0.1M sodium carbonate pH 9.6, prior to coupling to activated Sepharose CL4B.

2.8.) Polyacrylamide gel analysis of proteins.

Proteins were analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS PAGE). The buffer system used was that described by Laemmli (1970). The stacking gel (0.125M Tris.HCl pH 6.8, 3% [w/v] acrylamide* and 0.1% [w/v] SDS) and the resolving gel (0.3M Tris.HCl pH 8.8, 7.5% [w/v] acrylamide* and 0.1% SDS) were polymerized by addition of 0.005 volumes of 10% ammonium persulphate and 0.001 volumes of TEMED (* the acrylamide contained 8/300 parts of methylene bis acrylamide, cross-linking reagent). All samples were prepared for electrophoresis by addition of an equal volume of sample buffer (0.125M Tris.HCl pH 6.8, 4% SDS, 20% [v/v] glycerol, 0.2M DTT and 0.1% [w/v] bromophenol blue). Gels were run in a buffer of 0.025M Tris. 0.192M glycine and 1% SDS pH 8.3 at 30mA for approximately 2 hours. Gels were fixed and stained with 0.1% Coomassie brilliant blue R-250, 4.5% methanol, 1% acetic acid, and then destained to reveal bands with 25% methanol, 0.1% acetic acid. Alternatively proteins were transferred to nitrocellulose by electrophoresis (see below).
2.9.) **Electrophoretic transfer of proteins to nitrocellulose**."Western blotting".

After resolution by SDS PAGE proteins were transferred to a nitrocellulose membrane as described by Towbin *et al* (1979). Briefly, a nitrocellulose membrane (cut to size and pre-wetted in electrolysis buffer) was placed in direct contact (all air excluded) with the acrylamide resolving gel, supported on either side by a piece of Whatman 3MM filter paper, a Scotch-Brite pad and a plastic grid. This was subjected to electrophoresis in a buffer 25mM Tris., 192mM glycine, 20% methanol (v/v), pH 8.3, such that the nitrocellulose membrane faced the anode. A voltage of 10V/cm was applied for 18 hours. The membrane was removed and washed with TSB (see below). The acrylamide gel was stained with Coomassie brilliant blue to confirm the loss of proteins due to transfer. The membrane was prepared for immuno-staining. This technique is subsequently referred to as Western blotting.

2.10.) **Immuno-staining of Western blot.**

Proteins were transferred to nitrocellulose membranes to facilitate identification of specific proteins with specific antibodies. The membrane carrying the transferred proteins was incubated for 1 hour in 10% dried skimmed milk (DSM) in TSB (10mM Tris.HCl pH7.4, 0.9% NaCl w/v) at 37°C, in order to saturate remaining protein binding sites. The filter was rinsed in TSB then incubated with the specific appropriate antibody diluted in 10% DSM in TSB for 3 hour at room temperature. The membrane was washed three times with 0.05% NP40 (v/v) in TSB and twice with TSB alone over 30 minutes. It was then incubated with a second (indicator) layer of 125I-rabbit anti-(mouse immunoglobulin) or 125I-Protein A.
(Amersham) appropriately diluted \(10^6\) cpm/ml using 20ml/100cm\(^2\) of membrane) in 10% DSM in TSB for 3 hour at room temperature. The membrane was washed, dried thoroughly and exposed to Kodak X-Omat R film with an intensifying screen at -70°C.

2.11.) Large scale preparation of DNA.

Plasmid DNA was prepared by the method of Birnbiom and Doly (1979) as described in Maniatis et al (1982, pp366). A fresh bacterial colony harbouring the plasmid of interest was transferred to 10ml of L-broth containing 100\(\mu\)g/ml of ampicillin (Sigma). After 6 hours incubation at 37°C the culture was transferred to 500ml of the L-broth with ampicillin. After overnight incubation at 37°C the bacteria were harvested by centrifugation at 4000g at 4°C. The bacterial pellet was resuspended in 10ml of a solution of 50mM glucose, 25mM Tris.HCl(pH 8.0), 10mM EDTA, 5mg/ml lysozyme, and incubated at room temperature for 5 minutes. This was mixed gently with 20ml of a freshly made solution of 0.2M NaOH, 1% SDS and let stand on ice for 10 minutes. Then 15ml of an ice cold solution of 3M potassium acetate-acetic acid (pH 4.8) was added and the covered tube sharply inverted several times before standing on ice for 10 minutes. Bacterial debris was removed by centrifugation in a Beckman SW27 at 20,000 rpm for 20 minutes at 4°C. DNA was recovered from the supernatant by precipitation with 0.8 volumes of isopropanol at room temperature for 15 minutes followed by centrifugation at 12,000g for 30 minutes at room temperature. The DNA pellet was washed with 70% ethanol, dried in a vacuum desiccator and resuspended in 8ml of TE (pH 8.0) (10mM Tris.HCl pH 8.0, 1mM EDTA).

The plasmid DNA was purified by centrifugation to equilibrium on a caesium chloride-ethidium bromide gradient
as described by Maniatis et al (1982, pp 93). The DNA solution was made up to 1g/ml caesium chloride and 600μg/ml ethidium bromide, transferred to a Beckman Quick-seal tube, topped up with caesium chloride-ethidium bromide in TE and the tube sealed. This was centrifuged at 45,000 rpm for 36 hours at 20°C in a Beckman ultracentrifuge. Under U.V. illumination two bands were visible near the middle of the tube the lower of which was closed circular plasmid DNA. This was removed with a hypodermic needle and syringe. The ethidium bromide was removed by repeated extraction with water saturated butan-1-ol. The DNA was then dialysed against several changes of TE (pH 8.0), precipitated with 0.1 volumes 1M NaCl and 2.2 volumes ethanol, washed with 70% ethanol, dried and resuspended in an appropriate volume (1-2ml) of TE (pH 8.0). DNA concentration was determined by monitoring optical density at 260nm (for DNA at 50μg/ml O.D.260nm = 1.0). An indication of the purity of the sample was obtained by monitoring the O.D.280nm; for pure DNA the ratio of O.D.260nm/280nm = 2.0.

2.12.) Mini preparations of DNA.

When subcloning specific fragments of DNA or undefined mixtures of DNA fragments it was often necessary to screen the DNAs of a large number of transformants for inserts. This was done by analysing mini-prep DNA, which was prepared essentially by the method of Birnboim and Doly (1979) as described in Maniatis et al (1982, pp 366). Briefly it was scaled down from the large scale preparation using 5ml of an overnight culture of the transformant in L-broth plus ampicillin. The method was the same up to the addition of the potassium acetate, subsequently the supernatant was extracted and precipitated as described below (Section 2.16). The pellet was washed with 70% ethanol, dried and resuspended in 50μl of TE (pH 8.0).
2.13.) Restriction analysis of DNA.

Restriction analysis of DNAs was carried out by digestion of the DNA with specific restriction endonucleases. An appropriate amount of DNA was diluted in a reaction buffer to which an appropriate number of units of the restriction enzyme was added. The sample was mixed gently and incubated at 37°C (or temperature recommended by the manufacturer if not 37°C) for 1 hour, before addition of DNA sample loading buffer and gel electrophoresis. In most cases the reaction buffer was 50mM Tris.HCl (pH 7.5), 10mM MgCl$_2$, 6mM 2-mercaptoethanol, plus an appropriate amount of 1M NaCl to provide the optimum salt concentration for a specific enzyme's activity; low salt - 10mM NaCl e.g. KpnI, medium salt - 50mM NaCl e.g. BamHI, high salt - 100mM NaCl e.g. EcoRI. For certain restriction enzymes such as SmaI special condition were required e.g. 20mM KCl, 10mM Tris.HCl (pH 8.0), 10mM MgCl$_2$, 1mM DTT and incubation at 30°C. Digestion of DNA with more than one enzyme was often carried out simultaneously in the same buffer or by sequentially adding more salt with the second or third enzyme (provided that the final salt concentration did not cause abnormal activity in any of the enzymes present). Where this was not possible the DNA was ethanol precipitated, washed, dried and resuspended in TE between digestions.

For analysis of transformants or newly subcloned material (and for Southern blotting) approximately 1μg of DNA was digested in a final volume between 10-20μl. When preparing particular fragments as probes or for subcloning, between 10-50μg of DNA were digested in 20-100μl.
2.14.) Agarose gel electrophoresis of DNA.

2.14.1.) Analytical gels.

In order to determine i) restriction fragment lengths, ii) presence of insert DNA in transformants, iii) purity of probes or fragments for subcloning, and iv) to resolve bands for Southern blotting, digested DNA was electrophoresed in horizontal agarose slab gels. Electrophoretic grade agarose was prepared at between 0.8-1.2% in TBE (89mM Tris.base, 89mM boric acid, 8mM EDTA), melted by heating (without boiling), cooled to about 50°C when ethidium bromide (to stain the DNA) was added to 0.5μg/ml, then poured into a mould with comb to produce wells of the desired volume. When cooled and set, the gel was immersed in a tank containing TBE with 0.5μg/ml ethidium bromide. The DNA samples were mixed with 0.2 volumes of a 5x DNA sample buffer (0.25% bromophenol blue w/v, 0.25% xylene cyanol w/v, 15% Ficoll 400 w/v) and loaded into the wells (at the cathode end of the gel). Appropriate size standards were used in all gel runs including lambda HindIII 23kbp-516bp, 1Kb ladder (BRL) 12kbp-249bp, or 123bp ladder (BRL) 1230bp-123bp (detailed in the appendix). Gels were run at 4V/cm for between 1-4 hours depending on the size range being resolved. Migration of DNA was indicated by the migration of the dye fronts (in a 1% agarose gel bromophenol blue runs at a position approximately equal to 300-500bp and xylene cyanol to 1500-2000bp) and by observation of the bands by U.V. transillumination at 360nm on a Chromato-Vue transilluminator. When resolution was complete gels were transilluminated and photographed using Polariod 4x5 Land Film type 55, on a Polaroid MP-4 Land camera, fitted with a Kodak orange (22A Wratten) filter.
2.14.2.) Preparative gels.

When a particular DNA fragment was required for further treatment, such as subcloning, radioactive labelling or further digestion, it was fractionated by gel electrophoresis and subsequently electroeluted. Fractionation was carried out by running a standard analytical gel in which a number of wells were joined to provide a sample trough, the length of which was dependent on the total amount of DNA and the size and number of bands expected. The percentage of agarose in the gel was varied (0.8%-1.2%) to optimize the separation of bands in the range desired (Maniatis et al., 1982, pp150).

2.15.) Electroelution of DNA fragments.

This was performed using the procedure described by Maniatis et al. (1982, pp 168). DNA was electrophoresed on a preparative gel until the band of interest was clearly separated from all other bands (by visual checking on a U.V. transilluminator). At this point electrophoresis was interrupted and under U.V. transillumination two incisions were made in the gel, each slightly longer than the band, one immediately on the anode side and one immediately on the cathode side. A piece of dialysis membrane the length of the incision and just slightly deeper than the gel, was inserted on either side of the band. In addition, on the anode side a piece of Whatman 3MM the same size as the dialysis membrane, was placed in between the membrane and the band. Electrophoresis was continued until the band had migrated completely into the 3MM paper and onto the dialysis membrane. The DNA was retrieved from the paper and membrane by washing off with elution buffer (200mM NaCl, 50mM Tris.HCl [pH 7.6], 0.1% SDS, 1 mM EDTA). The DNA was extracted and precipitated as described below.
2.16.) Extraction and precipitation of DNA.

Samples of DNA were vortexed vigorously with an equal volume of 25:25:1 phenol/chloroform/isoamylalcohol, to remove (by differential partitioning from aqueous to organic phase) any restriction enzymes or DNA modifying enzymes or impurities from electroelution. After centrifugation at 5000g for 2 minutes to separate the two phases the aqueous phase was re-extracted. This was repeated with two volumes of chloroform (to remove the small amount of phenol which may have entered the aqueous phase - this would otherwise inhibit subsequent enzyme reactions). The sodium salt concentration of the sample was then increased to 0.1M by the addition of the appropriate amount of 1M NaCl. 2.2 volumes of absolute ethanol were added and the sample incubated at -20°C for >2 hours. The DNA was then recovered by centrifugation at 10,000g for 15 minutes, dried and resuspended in an appropriate volume of buffer (usually TE pH 8.0).

2.17.) Subcloning of DNA fragments.

This was carried out essentially as described by Maniatis et al (1982, pp 390). Reactions were carried out in small volumes (5-20μl) with insert DNA and vector DNA, 0.1 volumes of ligation buffer (0.6M Tris.HCl pH 6.5, 0.1M MgCl₂, 10mM spermidine, 0.66mM ATP, 0.5mg/ml BSA) and 1 unit of T4 DNA ligase (New England Biolabs). Reactions involving cohesive ends were incubated for 4-18 hours at 15°C, those involving blunt ended DNA fragments were incubated at 4°C for 20 hours. The ratio of insert DNA to vector DNA was varied according to the ratio of size of insert and vector (in base pairs) such that the molar concentration of available ends of each species of DNA was equal.
2.18.) Transformation of E.coli with plasmid DNA.

*E.coli* cells were prepared and transformed as described by Maniatis *et al* (1982, pp250). A single colony of *E.coli* HB101 (F", *hsdS20* (r^b^, r^m^), recA13, ara-14, proA2, lacY1, galK2, rpsL20 (Sm^R^), xyl-5, mtl-1, supE44) was cultured overnight in L-broth at 37°C. 1ml was subcultured in 100ml of L-broth in a 500ml flask at 37°C and grown until the turbidity of the broth reached an O.D.550nm of 0.4. The culture was then chilled on ice for 10 minutes. The cells were harvested by centrifugation at 4000g for 5 minutes at 4°C. The supernatant was discarded and the cells resuspended in half the original culture volume of an ice cold sterile solution of 50mM CaCl\(_2\), 10mM Tris.HCl pH8.0. The cells were incubated on ice for 15 minutes then harvested by centrifugation at 4000g for 5 minutes at 4°C. The supernatant was discarded and the cells resuspended in 1/15 of the original volume of an ice cold sterile solution of 50mM CaCl\(_2\), 10mM Tris.HCl pH8.0. The cells were dispensed into 200μl aliquots and stored at 4°C for 12 hours. DNA (approximately 50ng in ligation buffer or TE) was added to an aliquot of cells and the mixture incubated on ice for 30 minutes. The cells were then heat shocked by incubation in a water bath at 42°C for 90 seconds, then returned to 4°C for 2 minutes. They were incubated with 800μl of L-broth for 1 hour then various dilutions were plated on L-agar containing the appropriate antibiotics (e.g. 100μg/ml ampicillin or 15μg/ml tetracycline).

89
3. IDENTIFICATION OF THE gB-2 GENE.

3.1. INTRODUCTION.

A number of glycosylated proteins have been identified which are specific to cells infected by HSV and encoded by the virus. Investigation of primary translation products of the genes encoding the glycoproteins and the availability of monoclonal antibodies with unique specificities has shown that a single gene product may result in a number of glycosylated forms with different electrophoretic mobilities. Furthermore, the size of a single glycoprotein can vary between host cells due to proteolytic cleavage (Pereira et al., 1982b; Zezulak and Spear, 1984). The number, genetic location and definitive size of HSV glycoproteins have therefore required careful assessment. The successful identification of the glycoproteins has been achieved by gene mapping, using recombinant and mutagenized viruses, transcriptional analysis of regions of the genome and the use of monoclonal antibodies, as described in Section 1.5.6.

The work described in this section aimed to clarify the location of the gB-2 gene, which analysis of HSV-1 x HSV-2 recombinant viruses (Ruyechan et al., 1979) had indicated was colinear with the gB-1 gene. The gB-1 gene location, between 0.343-0.386 MU, was contained within a ≈17kbp fragment of the HSV-2 genome, represented by the clone pGR93 (Reyes, 1982; Reyes et al., 1982), shown in Figure 3.4. [The clone pGR93 was supplied by Dr. A. Minson.]

In order to identify the primary product of the gB-2 gene it was first necessary to determine whether the HSV type common gB specific monoclonal antibody TI57 (Chan, 1983) bound to the polypeptide of the glycoprotein in the
absence of its carbohydrate component. This would confirm its usefulness as a tool for identifying in vitro translation products from HSV-2 mRNAs. Subclones and fragments of pGR93 were used to identify and select by hybridization infected cell mRNA transcribed from the region of the genome corresponding to 0.35-0.38 MU.

A subclone representing the gB-2 locus was restriction mapped to allow further in vitro manipulation of the DNA and to provide data for comparison with publications, regarding gB-2, appearing during the course of and after this work (Person et al., 1985; Bzik et al., 1986).
3.2.) MATERIALS AND METHODS.

3.2.1.) Isolation of HSV-2 infected cell RNA.

Confluent monolayers of BHK C13 cells were infected at an m.o.i. of 1.0. At 14 hours post infection the cells, beginning to show cytopathic effect, were harvested by scraping from the plastic gently with a latex blade. They were washed several time in PBS; on the final wash the pellet volume was determined by comparison of the total minus the supernatant volumes. The RNA was extracted using a method modified from Maniatis et al (1980). The cell pellet was solubilized by vortexing in 5 volumes of a buffer containing 8M guanidinium hydrochloride, 5mM sodium acetate pH 5.0, 0.7M 2-mercaptoethanol. After incubation with vigorous shaking for 4 hours at room temperature 1g of caesium chloride was added per 2.5ml of homogenate. This was gently layered onto a cushion of 1.2ml of 5.7M caesium chloride, 1mM EDTA pH 7.5 in a Beckman SW50.1 polyallomer tube, and topped up to within 2mm of the top of the tube with solubilizing buffer plus caesium chloride. The RNA was pelleted by centrifugation at 35,000 rpm for 12 hours at 20°C. The supernatant, containing protein and DNA, was removed by swift inversion of the tube and the walls of the tube dried thoroughly. The RNA pellet was dissolved in a buffer containing 10mM Tris.HCl pH 7.4, 5mM EDTA, 1% SDS. This was extracted with an equal volume of 4:1 chloroform/butan-1-ol, the organic phase being re-extracted with the Tris.HCl buffer and the two aqueous phase pooled. The RNA was recovered by the addition of 0.1 volumes of 3M sodium acetate (pH 5.2) and 2.2 volumes of ethanol, incubation at -20°C overnight and centrifugation at 10,000g for 30 minutes at 4°C. The RNA pellet was redissolved in 1ml diethylpyrocarbonate treated autoclaved ddH2O. A small
aliquot was taken to determine the concentration and purity, the remainder was reprecipitated with ethanol and stored in 70% ethanol at -70°C. Great care was taken to avoid RNase contamination of any equipment or solution coming into contact with the sample. RNA concentration was determined by O.D.260nm.

For RNA at 40μg/ml O.D.260nm = 1.0

RNA purity was assessed by the ratio of O.D.260nm to O.D.280nm, which for pure RNA is 2.0.

3.2.2.) Preparation of messenger RNA.

Eukaryotic messenger RNAs have a cap structure at their 5' end and are poly-adenylated (poly A) at their 3' end. HSV messenger RNAs also have these structures. The infected cell RNA preparation was enriched for messenger RNA, utilizing the poly-adenylated tail, by chromatography on a column of oligodeoxythymidylic acid (oligo dT) on a cellulose matrix, using a method modified from Edmonds et al (1971).

The RNA sample was heated to 65°C for 5 minutes and cooled to room temperature and applied to a pre-equilibrated 1ml oligo-dT column in a loading/equilibration buffer of 20mM Tris.HCl (pH 7.6), 0.5M NaCl, 1mM EDTA, 0.1% SDS, and allowed to bind. After 15 minutes one column void volume (approx. 400μl) was collected, re-heated and re-applied. After 15 minutes the column was washed with 10 column volumes of equilibration buffer followed by 4 column volumes of equilibration buffer containing 0.1M NaCl. Poly A+ RNA was eluted with 2-3 column volumes of 10mM Tris.HCl (pH 7.5), 1mM EDTA, 0.05% SDS. The sample was made up to 0.5M NaCl and reloaded on to the column. The wash and elution procedures were carried out once again. The poly A+ RNA was recovered by ethanol precipitation and centrifugation as previously described. RNA content of eluates was monitored.
by taking O.D. 260nm readings.

3.2.3. Agarose gel electrophoresis of RNA.

mRNA is single stranded and as such has strong secondary structure. In order to obtain good resolution of individual species and promote retention by the transfer membrane the RNA was fractionated in a denatured state. This was achieved by glyoxalation of the RNA which was subsequently resolved by horizontal agarose gel electrophoresis (as described by McMaster and Carmichael, 1977).

An aliquot of glyoxal was deionized with Amberlite mixed bed ion exchange resin (BDH). Each RNA sample was prepared as indicated below.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% glyoxal</td>
<td>5.8 μl</td>
</tr>
<tr>
<td>dimethylsulphoxide (DMSO)</td>
<td>20 μl</td>
</tr>
<tr>
<td>0.1M sodium phosphate pH 7.0</td>
<td>4 μl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.8 μl</td>
</tr>
<tr>
<td>RNA (2 μg) + ddH2O</td>
<td>9.4 μl</td>
</tr>
<tr>
<td>Total volume</td>
<td>40 μl</td>
</tr>
</tbody>
</table>

This was incubated at 50°C for 60 minutes. As a standard size marker 1kb ladder DNA (BRL) was glyoxalated in the same manner. A 1% agarose gel was prepared by melting the agarose in ddH2O, adding sodium phosphate pH 7.0 to 0.01M final concentration when cool. Samples of 1 μg of size marker or 2 μg of RNA were cooled, each mixed with 10 μl of 5x loading buffer (15% Ficoll type 400, 0.25% Bromophenolblue) and loaded onto the gel. The gel was run at 30V/cm for ≈ 4 hours in 0.01M sodium phosphate pH 7.0, with the running buffer recirculating from the anode to cathode end of the tank via a peristaltic pump, to prevent pH gradient formation during electrophoresis (glyoxal dissociates from nucleic acids at pH >8.0). To visualize RNA the gel was soaked in ethidium bromide (0.5 μg/ml in 0.01M sodium phosphate pH 7.0) and
transilluminated at 360nm. RNA for Northern blotting was not stained.

3.2.4.) Northern transfer.

This was achieved using an apparatus described for Southern blotting by Maniatis et al. (1982, pp201 and 382). A pre-wetted wick consisting of a sheet of Whatman 3MM filter paper was supported on a plastic tray above a reservoir containing 20x SSC (3.0M sodium chloride, 0.3M sodium citrate, pH 7.0). The RNA gel, not stained and not treated with alkali (Thomas, 1980), was placed on the filter paper/plastic support and surrounded by cling film, to ensure that the blot buffer passed through and not around the gel. The gel surface was covered with a Pall Biodyne transfer membrane, which had been cut to size and pre-wetted with 20x SSC. Trapped air bubbles were removed by rolling a pipette over the membrane. A wet sheet of filter paper was placed over the membrane, this was topped with a 75mm stack of water absorbent paper towels, a plastic tray and a 1kg weight. The blot buffer was left to pass up through the gel overnight (minimum of 12 hours), after which the assembly was dismantled and the membrane removed. The membrane was rinsed briefly with 10x SSC then baked at 80 °C for 1 hour to increase the binding of the transferred RNA.

3.2.5.) Hybridization of 32p-labelled probes to Northern blots.

To examine Northern blots the single stranded mRNAs, separated on the agarose gel, transferred and bound to a Pall Biodyne membrane was exposed to radio-labelled denatured (single stranded) DNA in conditions that promote the formation of hybrid double stranded nucleic acid (RNA/DNA) along regions of sequence homology.
The manufacturers recommended method was used. The following solutions were prepared.

formamide - deionized with Amberlite MB1 mixed bed ion exchange resin

50x Denhardt's - 1% (w/v) Ficoll type 400

1% (w/v) polyvinylpyldone in ddH₂O

1% (w/v) BSA (Pentax Fraction V)

20x SSPE - 1 litre contains 174g NaCl, 27.6g NaH₂PO₄·H₂O,

7.4g EDTA, pH to 7.4 with NaOH.

single stranded salmon sperm DNA - 1mg/ml sheared by serial passage through 25G hypodermic syringe needle, then boiled, aliquots stored at -20°C.

Hybridization solution containing 50%(v/v) formamide, 5x Denhardt’s, 5x SSPE, and 250μg/ml nonhomologous DNA (from salmon sperm) was boiled for 10 minutes and cooled. 4ml were added per 100cm² of transfer membrane. This was incubated in a sealed plastic bag, covered in an outer bag containing damp paper towels as weight, immersed in a water bath at 42°C for 6 hours. The prehybridization solution was removed and replaced with fresh hybridization solution containing the radioactive probe (the mixture again boiled and cooled prior to addition). This was incubated in double bags in a water bath at 42°C overnight, with gentle shaking.

When the hybridization was complete the membrane was briefly rinsed in 2x SSC, 0.1% SDS. This was followed by then three washes of 15 minutes at room temperature, with moderate agitation, in 2.5ml per cm² membrane area of the same buffer. It was then washed twice in 2.5 ml per cm² of 0.1x SSC and 0.1% SDS for 15 minutes in a water bath at 50°C. The membrane was dried and autoradiographed (using Kodak X-Omat film with intensifying screens).
Hybridization selection of RNA.

Preparation of DNA solid phase.

DNA was coupled to a solid matrix (cellulose) as a substrate for hybridization selection of specific mRNA. Preparation of activated cellulose is described below. The chemicals used are extremely hazardous, all procedures were carried out in a fume cupboard with suitable precautions particularly in disposal of wastes.

1g of microgranular cellulose (Sigma) was incubated with 10.5ml of 0.5M NaOH and 4.5ml of 1,4-butandiol diglycidyl ether (carcinogenic!) overnight at room temperature, then washed (by centrifugation and resuspension) three times in 50% ethanol. It was then incubated with 25ml of 2% aminothiophenol in ethanol plus 25ml of 0.5M NaOH at room temperature for 4 hours. The resulting aminophenylthioether (APT)-cellulose was washed three times with 50% ethanol, twice with 0.1M HCl, three times with 100% ethanol, then dried under vacuum.

20mg of APT-cellulose was activated by incubation in 2ml of 0.027% sodium nitrite(w/v), 1.2M HCl for 15 minutes at room temperature then washed twice in 10ml of ddH₂O, twice in 10ml 0.2M sodium acetate pH 5.5 and pelleted. 100mg of the DNA selected for coupling was dissolved in 80μl of 25mM sodium phosphate pH 6.5. This was boiled for 10 minutes then cooled on ice. 320μl of DMSO were added and the sample heated to 50°C for 5 minutes and cooled on ice. This DNA/DMSO solution was used to resuspend the activated APT-cellulose pellet. The mixture was incubated at room temperature for 10 minutes and washed five times with 1ml of ddH₂O. It was incubated for a further 15 minutes at 37°C
with 0.4M NaOH and finally washed five times with 1ml aliquots of ddH₂O.

To assess the binding, a small amount of ³²P-radiolabelled DNA was incorporated in a 100µg DNA sample and the counts followed by the Cherenkov counts present in the original sample, the washes and the coupled ATP-cellulose pellet. [82% of incorporated counts irreversibly bound to the APT-cellulose.]

3.2.6.2.) Hybridization.

The DNA-cellulose was prehybridized in 1.2ml of a solution of 50%(v/v) formamide (de-ionised), 15mM PIPES pH 6.4, 1mM EDTA, 0.6M NaCl, 0.2% SDS, 1mg/ml tRNA, for 30 minutes at 37°C in an Eppendorf tube (1.5ml). 50µg of poly A+ RNA in SDS, EDTA, and tRNA (in 200µl) was heated to 70°C for 5 minutes then cooled and added to the remaining components described above for a final hybridization of 600µl. After overnight incubation at 37°C, with gentle shaking, the solid phase was washed (by centrifugation and resuspension) with five times 1ml of hybridization buffer to remove unhybridized RNA. The bound mRNA was eluted by thorough mixing and incubation at 65°C for 3 minutes in 100µl of a buffer of 90%(v/v) formamide, 10mM PIPES pH 6.4, 5mM EDTA, 0.2% SDS and 20µg/ml tRNA. The elution was repeated and the two supernatants pooled with an equal volume of ddH₂O. The RNA was precipitated with 0.1 volumes 3M sodium acetate pH 5.5 and 2.2 volumes ethanol, for 1 hour at -70°C, recovered by centrifugation, washed twice with 80% ethanol and dried under vacuum. The RNA was resuspended in 10µl of ddH₂O.
3.2.7.) In vitro Translation.

In vitro translation was performed by a rabbit reticulocyte lysate system (BRL), using the manufacturers protocol. Briefly, the rabbit reticulocyte lysate (RRL) mixture (3x), containing 3.5mM MgCl₂, 0.05mM EDTA, 25mM KCl, 70mM NaCl, 0.5mM dithiothreitol, 25μM hemin, 50μg/ml creatine kinase, 1mM CaCl₂, 2mM [ethylene-bis(oxy-ethylene nitrilo)]tetraacetic acid (EGTA), the reaction mixture (10x), containing 250mM HEPES pH 7.2, 400mM KCl, 100mM creatine phosphate, 19 amino acids at 500μM each, the RNA sample and the radioactive tracing amino acid (³⁵S-methionine), were mixed as follows,

- Reticulocyte lysate mixture: 10μl
- Reaction mixture: 3μl
- Potassium acetate 2M pH 7.2: 1.3μl
- ³⁵S-methionine: 5μCl
- RNA and ddH₂O: 15.7μl
- Final volume: 30μl

This was incubated at 30°C for 1 hour, then 15 minutes following the addition of 3μl of 10mM cold methionine chase, after which samples were prepared for counting, immunoprecipitating or SDS PAGE analysis. Counting of incorporated ³⁵S-methionine was carried out by taking 2x2μl samples from each in vitro translation reaction adding 1.0ml ddH₂O, 0.5ml of a solution containing 1M NaOH, 1mg/ml methionine, 5%(v/v) H₂O₂ (to hydrolyse unincorporated radiolabelled amino-acylated tRNA and bleach the haemoglobin present). After 15 minutes incorporated label was precipitated with 1ml 25% (w/v) TCA for 10 minutes on ice. Precipitate was collected by slow filtering through a Whatman GF/C filter disc and washed with 8% (w/v) TCA followed by ethanol. The dried filter discs were counted in
liquid scintillant (Optiscint, BDH) on a Beckman multi-channel scintillation counter.

Samples for SDS PAGE analysis (4μl) were diluted with 4 volumes of SDS PAGE load buffer (because of the large amount of haemoglobin present), and the 14C labelled protein markers (myosin 200kDa, phosphorylase b 92.5kDa, BSA 69kDa, ovalbumin 46kDa, carbonic anhydrase 30kDa and lysozyme 14.3kDa, Amersham) were loaded and run with an equal volume (4μl) of unlabelled RRL, for optimum reproducibility of migration between samples on a gel.

3.2.8.) Immunoprecipitation of in vitro translation products.

The remaining sample of in vitro translation product (22μl) was diluted 5 fold with 50mM Tris.HCl pH 8.0, 5mM EDTA, 0.5%(v/v) NP40, 1mg/ml BSA, 0.5M NaCl (TENS). This was incubated with 1μl of rabbit anti-mouse antisera (RaMIg, [kindly provided by Mr. M. Smith, Wellcome, Beckenham]) for 30 minutes on ice. 40μl of a suspension of Protein A-Sepharose (Pharmacia) was added and incubation continued for 15 minutes on ice. This was spun in an Eppendorf centrifuge for 5 minutes at room temperature and the supernatant (now depleted for non-specific binding) collected. 1μl of monoclonal antibody TI57 (hybridoma culture supernatant) was added to the supernatant and incubated for 1 hour on ice. Addition and incubation of RaMIg and Protein A-Sepharose was repeated as described above. The product/antibody/Protein A-Sepharose complex was pelleted by centrifugation in an Eppendorf centrifuge for 5 minutes at room temperature. The pellet was washed three times in 0.5ml of TENS buffer and twice in 0.5ml of 50mM Tris.HCl, 5mM EDTA, 0.5% (v/v) NP40. The final pellet was resuspended in 30μl of SDS PAGE loading buffer and heated to 100°C for 3 minutes. The sample was
pelleted again and the supernatant carefully collected. Half of the sample was mixed with 4μl of unlabelled RRL (to balance the salt/protein/haemoglobin concentration of the sample with respect to the other samples) and analysed with complete translation products by SDS PAGE.

3.2.9.) Radio-labelling of DNA probes.

A method modified from Feinberg and Vogelstein (1983) was used to label specific DNA samples. This involved the hybridization of random sequence oligodeoxyribonucleotides (hexamers) to a piece of DNA and the use of the Klenow fragment of DNA polymerase to synthesize new DNA from the 5' end of the hexamer to the 3' end of the next adjacent hexamer, incorporating a radio-labelled deoxynucleotide.

To label DNA, 0.1-0.05μg in 2μl was heated at 95°C for 2 minutes, rapidly cooled on ice and added to 12.5μl of a solution containing 100mM Tris.HCl pH 8.0, 1mM MgCl₂, 2mM 2-mercaptoethanol, 2mg/ml BSA, 0.4mg/ml random hexadeoxyribonucleotides (Amersham), 40μM each of dCTP, dGTP and dTTP. Then 5μCi of ³²P-dATP, 2.5units of Klenow fragment of DNA polymerase and ddH₂O were added to a final volume of 25μl. This was incubated for 3 hours at room temperature. The sample was fractionated on a Sephadex G50 column (in TE buffer) the first peak of radioactivity eluted being the labelled DNA separated from the unincorporated radio-nucleotide.

Probes prepared in this way gave specific activities in the range of 1-5x10⁸cpm/μg. Probes were heated to 100°C for 5-10 minutes before addition to a hybridization. 1-5x10⁷cpm of probe were used per 100cm² of membrane in a hybridization reaction.
3.3.) RESULTS.

3.3.1.) Identification of the gb polypeptides of HSV-1 and HSV-2.

Protein extracts, prepared from BHK cells infected with HSV-1 or HSV-2 in the presence or absence of 0.5μg/ml of tunicamycin, were analysed by Western blotting. The results of immunostaining these Western blot with the monoclonal antibody TI57 are shown in Figures 3.1 and 3.2.

As shown in Figure 3.1 tunicamycin untreated HSV-1 infected cells (lane 1) showed two forms of the glycoprotein gb-1 with molecular weights estimated as 110 and 115kDa. In contrast tunicamycin treated HSV-1 infected cells (lane 2) showed only one resolvable form of the polypeptide, with a molecular weight estimated as 93kDa. No polypeptides were specifically stained by TI57 in uninfected BHK cell extracts (lane 3).

In Figure 3.2 a doublet band was also observed in extracts from HSV-2 infected cells untreated with tunicamycin (lane 3), with molecular weights estimated as 110 and 115kDa. This doublet was also stained in tunicamycin treated HSV-2 infected cell extracts (lane 4) together with a single band with an estimated molecular weight of 94kDa. In both infected cell extracts (lanes 3 and 4) a small number of products, with molecular weights estimated as 65kDa, 55kDa, 50kDa and 45kDa were stained. There was no staining of proteins extracted from uninfected BHK cells either treated (lane 2) or untreated (lane 1) with tunicamycin. The 93kDa polypeptide detected in HSV-1 infected cells and the 94kDa polypeptide detected in HSV-2 infected cells are likely to be non-glycosylated forms of
gB. This result suggests that TI57 is capable of binding to unglycosylated gB.

Figure 3.1. Uninfected and HSV-1 strain KOS infected BHK cell lysates were resolved by SDS PAGE then Western blotted to nitrocellulose and probed with the gB specific monoclonal antibody TI57. A second layer of rabbit anti-mouse immunoglobulin antibody conjugated to horseradish peroxidase substrate was used to visualize bound monoclonal antibody following treatment with a chromogenic substrate (4-chloronapthol). Infected cell were grown in the absence Tu- (lane 1) or presence Tu+ (lane 2) of tunicamycin at 0.5µg/ml, to show the effect of inhibition of glycosylation by the drug. Lane 3 phosphorylase b (Sigma) 92.5 kDa.
Figure 3.2. Uninfected and HSV-2 strain BRY infected BHK cell lysates were resolved by SDS PAGE then Western blotted to nitrocellulose and probed with the gB specific monoclonal antibody TI57. A second layer of ¹²⁵I-rabbit anti-mouse immunoglobulin was used to visualize bound monoclonal antibody. Uninfected cells were grown in the absence Tu- (lane 1) or presence Tu+ (lane 2) of tunicamycin at 0.5μg/ml. Infected cells were grown in the absence Tu- (lane 3) or presence Tu+ (lane 4) of tunicamycin at 0.5μg/ml.
3.3.2 DNA subcloning to isolate the \( gB-2 \) gene.

3.3.2.1 Confirming restriction sites in the HSV-2 genomic clone pGR93.

Since the plasmid pGR93 had been obtained from another laboratory it was necessary to confirm the identity and expected structure of the plasmid. To this end plasmid pGR93 (Figure 3.3) was examined by digestion with the restriction enzymes \( \text{BamHI}, \text{BglII}, \text{KpnI} \) and \( \text{HindIII} \). The observed restriction fragment sizes were compared to those predicted from a reported restriction map of the HSV-2 genome (Reyes, 1982, Reyes et al., 1982), from 0.291-0.401 MU (shown in Figure 3.3).

![Restriction map of the HSV-2 genomic clone pGR93](image)

**Figure 3.3.** Restriction map of the HSV-2 genomic clone pGR93 indicating especially \( \text{BamHI}, \text{BglII} \) and \( \text{KpnI} \). Also indicated are the relative location of HSV-1 \( gB \) at 0.348-0.370 MU and the 7kbp \( \text{BamHI-BglII} \) fragment at 0.345-0.387 MU, which was subcloned into pBR322 to produce pBH10.

105
The results in Figure 3.4 and Table 3.1 showed that, within the limits of resolution, the banding patterns created by the various digestions were as expected.

Figure 3.4. Restriction digest of pGR93 with HindIII lane 1; BglII lane 2; BamHI lane 3; BamHI,BglII lane 4; BamHI,BglII,KpnI lane 5; HindIII,KpnI lane 6; undigested lane 7. Size standards were lambda HindIII (H) and the 1 kbp ladder (1kb).
Table 3.1 Comparison of observed restriction digestion pattern of pGR93 with a published restriction map of the region of the HSV-2 genome represented in the plasmid.

<table>
<thead>
<tr>
<th>Digestion</th>
<th>Predicted Fragments</th>
<th>Experimental Fragments</th>
<th>Lane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Sizes</td>
<td>No.</td>
</tr>
<tr>
<td>Hind III</td>
<td>2</td>
<td>~ 17</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>Bgl II</td>
<td>2</td>
<td>~ 10.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>~ 10.1</td>
<td></td>
</tr>
<tr>
<td>Bam HI</td>
<td>6</td>
<td>~ 10</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.3*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.6</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Bam HI/Bgl II</td>
<td>8</td>
<td>~ 7</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.3†</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>3.0</td>
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<td></td>
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<td>0.6</td>
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<tr>
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<td>0.5</td>
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<td></td>
<td></td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Kpn I/Hind III</td>
<td>5</td>
<td>~ 7</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.0</td>
<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
<td></td>
<td>2.2</td>
<td></td>
</tr>
</tbody>
</table>

*band is a doublet  † band is a triplet
3.3.2.2.) Subcloning the region of the genome approximately 0.345-0.390 MU.

To localize the region of pGR93 thought to encode gB-2, smaller DNA fragments were subcloned from the plasmid. The 7kbp fragment of the BamHI/BglII digestion of pGR93 (Figure 3.4 lane 4, uppermost band) should represent the region 0.345-0.390 MU, the portion of the HSV-2 genome colinear with the HSV-1 gB gene, 0.343-0.386 MU (Figure 3.3). This was confirmed by digestion with KpnI, Fig.3.4 lane 5. This 7kbp fragment was subcloned into the BamHI site of pBR322 (BRL). The resulting clone, pBH10, was initially identified because it conferred ampicillin resistance and tetracycline sensitivity on E.coli cells harbouring it. Plasmid DNA was prepared from cells harbouring this clone and its structure was confirmed by restriction endonuclease mapping. The banding patterns following digestion of pBH10 DNA (Figure 3.5) allowed the orientation of the insert in the vector to be determined. The positions of PvuII and BstEII sites in the clone were as shown in Figure 3.6.

3.3.2.3.) Preparation of probes.

Smaller fragments of the clone pBH10 were prepared as probes to analyse HSV-2 mRNAs transcribed from the region of the HSV-2 genome 0.345-0.390 MU. The 4.7kbp fragment from the BamHI/KpnI digestion of pBH10, representing 0.345-0.376 MU, was prepared as a $^{32}$P-radionucleotide labelled DNA probe (described in Section 3.2.9.). Digestion of pBH10 with BamHI/KpnI/PvuII yielded a 3.8kbp fragment containing the remaining 2.2kbp of the HSV-2 insert, representing 0.376-0.390 MU, and 1.6kbp of vector (see Figure 3.6). This was also prepared as a radio-labelled probe.
Figure 3.5. Restriction digests of pBH10 (7kbp BamHI-BglII fragment in pBR322) with PvuII,KpnI lane 1; BstEII,KpnI lane 2; BamHI,KpnI lanes 3 and 4. Size standards were lambda HindIII (H) and the 1 kbp ladder (1kb).
Figure 3.6. Restriction map of pBH10 showing the interpreted locations of the PvuII, KpnI and BstEII sites. Also shown are the two probes prepared from this clone, a 4.7kbp BamHI-KpnI fragment (later subcloned into pUC18) and a 3.8kbp KpnI-PvuII fragment (containing a portion of vector shaded region).
3.3.3.) Northern blot analysis.

In order to identify and characterise mRNAs transcribed from the region of the HSV-2 genome represented in the clone, pBH10 total RNA was prepared from BHK cells infected with HSV-2. Samples of RNA were subsequently purified by oligo dT cellulose chromatography to enrich for mRNA (poly A+ RNA).

A typical total RNA preparation from 30 tissue culture flasks (75cm²), approximately 3x10⁸ cells, yielded 1ml of RNA in solution with an O.D.260nm of 0.605 at a dilution of 1:200.

\[
40\mu g/ml \text{ of RNA} = \text{O.D.260nm of 1.0}
\]

\[
\text{Concentration of RNA preparation} = 0.605 \times 40 \times 200
\]

\[
= 4.84\text{mg/ml}
\]

\[
\text{Total volume of preparation} = 1.0\text{ml}
\]

\[
\text{Total RNA in preparation} = 4.84\text{mg}
\]

The ratio of O.D.260nm/O.D.280nm for RNA is 2.0 and for DNA is 1.8. For this preparation of RNA,

\[
\text{O.D.260nm} = 0.605
\]

\[
\text{O.D.280nm} = 0.288
\]

\[
\text{Ratio of O.D.260nm/O.D.280nm} = 2.1
\]

The value of O.D.260/O.D.280nm ratio of 2.1 indicated that the preparation was essentially free of contamination with protein/phenol.
The preparation was then enriched for mRNA (poly A+ RNA) by oligo-dT column chromatography.

- RNA loaded onto 1ml oligo-dT column = 4.84mg
- RNA eluted in 1st column volume of 1st application = 3.28mg
- RNA eluted in 1st column volume of 2nd application = 0.204mg
- O.D.260nm of recovered poly A+ RNA (1:100 diln) = 0.695
- O.D.280nm = 0.317
- O.D.260nm/O.D.280nm of poly A+ RNA = 2.19
- Total poly A+ RNA recovered (in 1ml) = 0.695 x 40 x 100 = 278μg

Poly A+ RNA as percentage of total = 278/4,840 x 100/1 = 5.7%

The value of 5.7% of poly A+ RNA recovered from enrichment of total infected cell RNA indicated that this was a good preparation. Two sets of samples of poly A+ and poly A− RNA were fractionated on a glyoxal gel and transferred to nitrocellulose. Each of the two sets of samples was hybridized separately with one of the radio labeled probes described above (Section 3.3.2.3.).

There was no hybridization in tracks containing poly A− RNA (Figure 3.7 lanes 1 and 3). The 3.8kbp probe did not hybridize with the poly A+ RNA (Fig. 3.7 lane 2). The 4.7kbp probe hybridized to a discrete RNA species (Fig. 3.7 lane 4) of approximately 3.2kb, compared to the migration of single stranded DNA. These hybridization results implied that the 3.2kb transcript, possibly encoding gB-2, originated from within the region of the genome represented by the 4.7kbp BamHI-KpnI fragment (0.345-0.376 MU).
Figure 3.7. Northern blots of HSV-2 BRY infected BHK cell RNA showing oligo dT column selected poly A⁺ (lanes 2 and 4) and flow through (lanes 1 and 3) probed with the 3.8kbp KpnI-PvuII fragment 0.376-0.390 MU (lanes 1 and 2) and with the 4.7kbp BamHI-KpnI fragment 0.345-0.376 MU (lanes 3 and 4). Size markers indicated the migration of glyoxalated single stranded 1kbp ladder DNA.
3.3.4.) Subcloning of the 4.7kbp BamHI-KpnI fragment representing 0.345-0.376 MU.

The 4.7kbp BamHI-KpnI fragment was subcloned into BamHI and KpnI digested pUC19 (BRL). The resulting clone, pUH10, facilitated more detailed restriction mapping of the region, and provided a complementary DNA with which to hybridization select the 3.2kb transcript for in vitro translation.

3.3.5.) In vitro translation.

50μg of the poly A+ messenger enriched RNA was hybridized to 100μg of linearized, immobilized pUH10 DNA. RNA specifically hybridizing to the DNA/cellulose matrix was eluted and the total sample translated in vitro in a rabbit reticulocyte lysate system (BRL). Samples of translates and TI57 immunoprecipitated translates of total poly A+ and hybrid selected RNAs were analysed by SDS PAGE.

The first experiment detected a polypeptide band of 45kDa, with a minor band at 50kDa, in the immunoprecipitate of translated poly A+ RNA (Fig.3.8 lane 2), and bands at 60kDa, 50kDa, 47kDa, 43kDa and 30kDa in the translate of hybrid-selected RNA (lane 3). There was little high molecular weight material in the poly A+ translate, the majority was in the range 30-60kDa (Figure 3.8 lane 1).

The hybridization selection of messenger RNA with pUH10 was repeated. Prior to the second experiment the RNA samples were heated to 65°C for five minutes then chilled on ice before translation. Under these conditions a faint but detectable band was seen at 92kDa in both the hybrid-selected translate and the immunoprecipitate of poly A+ translate, together with a major band at 45kDa (Figure 3.9 lanes 2 and 3 respectively). These were accompanied by minor
bands at 63/62kDa, 54kDa and 28kDa in the immunoprecipitate of poly A+ translate. There was however no band detectable in the immunoprecipitate of the hybrid-selected translation (lane 4), possibly due to the low amounts of protein present in that sample.

**Figure 3.8.** In vitro translation and immunoprecipitation of HSV-2 infected BHK cell poly A+ and hybrid-selected RNA. Total poly A+ RNA translations, lanes 1 and 2, pUH10 hybridization selected poly A+ RNA translations, lanes 3 and 4. Immunoprecipitations using the monoclonal antibody TI57, RaMIG and protein A sepharose lane 2 and 4.
Having identified the region of the genome contained in pUH10 as the origin of the gB-2 transcript it was now necessary to characterise the DNA in more detail, initially by restriction mapping and later by cloning for expression and limited nucleotide sequencing.

**Figure 3.9.** In vitro translation and immunoprecipitation of HSV-2 infected BHK cell poly A+ and hybrid-selected RNA. Total poly A+ RNA translations, lanes 1 and 3, hybridization selected poly A+ RNA translations, lanes 2 and 4. All RNA samples were heat denatured prior to translation. Immunoprecipitations with TI57 lanes 3 and 4.
3.3.6.) Restriction mapping of pUH10.

A number of restriction sites were located using the method of Smith and Bernstiel (1976). The BamHI site of pUH10 was cut and radiolabelled then the 4.7kbp insert released by KpnI digestion.

**Figure 3.10.** The use of partial digestion of single end radiolabelled pUH10 DNA to construct a restriction map of the pUH10 insert. Restriction enzymes used were PvuII (VII), SphI (S), PstI (P), DraI (D), PvuI (VI), RsaI (R) and BstEII (B). Fragment sizes were determined from a semi-logarithmic plot of the migration of 1kbp ladder and 123bp ladder DNA size standards run on the same gel.
Aliquots of the end radiolabelled DNA fragment were treated individually by controlled partial digestion with specific restriction enzymes. A number of restriction sites were positioned in relation to the BamHI site (Figure 3.10). Subsequent analysis was performed by single and double digestion of the clone pUH10 with restriction enzymes which have unique sites in the vector (pUC19): SmaI (M), SacI (C), PstI (P), SphI (S), SalI (L), XhoI (X). The restriction digestion patterns and the fragment sizes are shown in Figures 3.11 and 3.12. The positions of the restriction enzyme sites were calculated (in conjunction with the position of sites located by partial digestion of end labelled DNA) and assigned as shown in Figure 3.13.
Figure 3.11. Various single and double digests of pUH10 with the restriction enzymes Smal (M), SacI (C), XhoI (X), PstI (P), SphI (S), SalI (L), EcoRI (E), and HindIII (H); size standards were lambda HindIII (λ) and the 123bp ladder (123). Each lane in (a) represented 500ng of digested DNA electrophoresed through a 1% agarose gel at 100V, visualized after i) 45min, and ii) 2 hrs. b) represents the sizes (in base pairs) determined for each fragment, interpreted from semi-logarithmic plot of the migration of the size standards. P.T.O.
Figure 3.11. cont d  
b) Estimated fragment sizes from digestion of pUH10. () indicates a product derived from incomplete digestion; [] indicates a product not clearly visible on these photographic plates; most are inferred from Fig.3.10 or calculated from change in migration of larger fragments; * indicates a doublet band.
Figure 3.12. Further single and double digests of pUH10 with the restriction enzymes described in Fig.3.11. a) represents migration after i) 45min and ii) 2 hrs through a 1% agarose gel at 100V. b) represents the size (in base pairs) determined for each of the fragments.
Figure 3.12. contd  

b) Estimated fragment sizes from digestion of pUH10. () indicates a product derived from incomplete digestion; [] indicates a product not clearly visible on these photographic plates; most are inferred from Fig. 3.10 or calculated from change in migration of larger fragments; * indicates a doublet band.
Figure 3.13. A restriction map of the insert of pUH10 derived from interpretation of the data shown in Figures 3.10, 3.11 and 3.12.
3.4.) DISCUSSION.

3.4.1.) Monoclonal antibody recognition of the gB-1 and gB-2 polypeptides.

The results shown in Figure 3.1 demonstrated that the gB-1 specific monoclonal antibody TI57 bound to the polypeptide portion of gB-1 and not the carbohydrate component. It also illustrated that the two glycoproteins gB and pgB (formerly gA/gB) originated from different degrees of post translational modification of the same polypeptide as described by Eberle and Courtney (1980). The polypeptide, expressed in BHK cells infected with HSV-1 strain KOS, identified by TI57, was determined to have a molecular weight of 93kDa.

The results shown in Figure 3.2 confirmed that the monoclonal antibody was type common, as published (Chan, 1983), and recognised both the glycosylated and unglycosylated forms of the gB-2 polypeptide. The gB-2 polypeptide (produced in BHK cells infected with HSV-2 strain BRY) identified by TI57 was determined to have a molecular weight of 94kDa. This confirmed that the monoclonal antibody would be a useful reagent for analysis of in vitro translation products. It was noted that in HSV-2 infected BHK cell extracts a number of lower molecular weight products were immunostained by TI57. These polypeptides may be generated by proteolytic cleavage of the full length gB-2 polypeptide or glycoprotein (Pereira et al., 1982b; Zezulak and Spear, 1984b), though evidence from in vitro translation (discussed below) suggests that there may also be some early termination of translation of gB-2, which may account for the occurrence of these products.
3.4.2.) Northern blot analysis of HSV-2 infected cell mRNA.

The results of probing Northern blots of HSV-2 infected cell mRNA with two probes, derived from the clone pBH10, representing 0.345-0.376 MU and 0.376-0.390 MU, showed hybridization between the 4.7kbp BamHI-KpnI fragment (0.345-0.376 MU, pUH10) and a single discrete mRNA of 3.2kb (Figure 3.3). This was colinear with the map location of a 3.3kb transcript of HSV-1, 0.343-0.361 MU, encoding gB-1 identified by Rafield and Knipe (1984) and the gB-1 gene identified by Bzik et al (1984). The implication of a colinear location for the gB-2 transcript was consistent with evidence from analysis of the protein profiles of recombinant HSV-1 x HSV-2 viruses (Ruyechan et al, 1979; Honess et al, 1980; and Pereira et al, 1982a&b) which also indicated that the gB gene was approximately co-linear between the HSV-1 and HSV-2 genomes.

3.4.3.) In vitro translation of hybridization selected 3.2kb mRNA.

Confirmation that the 3.2kb mRNA encoded the gB-2 polypeptide was provided by the co-migration of the high molecular weight polypeptides (approximately 92kDa) produced by in vitro translation of hybrid-selected mRNA and immunoprecipitate of total poly A+ in vitro translation by the gB-specific monoclonal antibody (TI57) (Figure 3.9). A major product of these in vitro translations was a truncated product of approximately 45kDa. Formation of this polypeptide may have been due to the presence of strong secondary structure in the mRNA. This structure would be approximately 1.2kbp into the coding sequence in order to generate a 45kDa product. Computer analysis of the nucleotide sequences using algorithms that identify hairpin and long range loops (Staden and McLachlan, 1984) did not
identify any significant structures. Heat treatment of the RNA prior to translation did, however, increase the sizes of products obtained by translation of the same preparations of mRNA, and allowed the identification of the 92kDa product. This suggested that there was involvement of secondary structure in production of the truncated products. Reports of the in vitro translation of hybrid-select gB-1 mRNA (Rafield and Knipe, 1984) also described products in this size range which immunoprecipitated with anti-HSV-1 antisera, and a larger 83kDa product immunoprecipitated with anti-gB antisera. Pereira et al (1981) reported small polypeptides of 41kDa, 37kDa and 27kDa associated with immunoprecipitation of gB-1 from Vero and HEp-2 cells. Analysis of recombinant gB-1 expression in mouse L cells by Blacklaws et al (1987) detected gB related polypeptides of 49kDa, 43kDa and 27kDa from immunoprecipitation. These observations may represent in vivo the phenomenon observed here in vitro. Though these truncated products could have arisen by post-translational cleavage of full length gB, this type of cleavage would not be expected in an in vitro translate, which should be free of protease activity. The poly A+ and hybrid-select RNAs both originated from the same preparation as that probed in the Northern blot in which a single 3.2kb transcript was identified, so it is unlikely that the smaller products arose from truncated transcripts. Perhaps the most plausible explanation for the generation of these products is internal initiation of translation, as has been observed with the HSV thymidine kinase gene (Marsden et al, 1983). This is discussed in more detail in the General Discussion (Section 6.1.).

The fact that the monoclonal antibody TI57 failed to precipitate anything from the hybrid-select translation may have been due to the low quantity and insufficient concentration of products present in the hybrid-select
translation prior to immunoprecipitation (cf. protein content of lane 2 and 4 in Figure 3.9). It may also have been due to a lower affinity of the antibody for the gB polypeptide (compared with the glycoprotein) or for gB-2, in particular, in immunoprecipitation.

3.4.4.) Restriction analysis of pUH10.

The restriction map produced for pUH10 spanning the region encoding the 3.2kb gB-2 transcript was compared with a computer analysis of the published sequence of HSV-1 KOS gB (Bzik et al, 1984), shown in Figure 3.14. This revealed that the relative positions of a number of sites (PvuII, PstI, Smal, SalI and SacI) were retained, although other sites recognized by these and other enzymes (PvuI, XhoI, BstEII and SphI) were positioned differently. This would be expected of DNAs encoding closely related but not identical genes. One nucleotide change in the six base recognition sequence can result in the loss of a common restriction site; thus two sequences could be 83% homologous but not share a restriction site.

During the analysis of pUH10 a report by Person et al (1985) demonstrated HSV antigenic material in recombinant E.coli expressing DNA from 0.354-0.363MU of HSV-2. A limited amount of sequence data published by these authors showed that at the 3’ and 5’ ends, this DNA had 86% homology at the DNA level and 89% at the amino acid level to HSV-1 KOS gB, indicating that this represented the HSV-2 gB gene. Comparison of the XhoI and SalI sites published by Person et al (1985) to those located in pUH10 (Figure 3.15) provided further confirmation that this clone contained the gB-2 coding sequences. Subsequently comparison of the pUH10 map with a map generated by computer analysis of the complete nucleotide sequence for gB-2 strain HG52 published by Bzik
et al (1986) showed good correlation of restriction sites (Figure 3.15). The only notable difference was the location of a PvuI site at map position 0.364 and an SphI site at map position 0.369.

**Figure 3.14.** A comparison highlighting the similarities between the restriction map of pUH10 and a restriction map showing recognition sites for the same enzymes derived by computer analysis of the nucleotide sequence of gB-1 published by Bzik et al (1984). Restriction enzymes SmaI (M), SacI (C), XhoI (X), PstI (P), SphI (S), SalI (L), EcoRI (E), HindIII (H), BstEII (B), PvuI (VI) and PvuII (VII).
Figure 3.15. A comparison of the restriction map of pUH10 with a map published by Person et al (1985) describing the location of the gB-2 gene (i), and a restriction map derived from computer analysis of the nucleotide sequence of gB-2 published by Bzik et al (1986) (ii).
3.4.5.) Summary.

The 4.7kbp BamHI-KpnI fragment in pUH10 (0.345-0.376 MU) was shown to encode a 3.2kb transcript which translated to a polypeptide that comigrates on SDS PAGE with immunoprecipitated gB-2 polypeptide. The restriction map of the insert in pUH10 was similar to a map of the gB-1 gene and nearly identical to the map of the gB-2 gene published later.
4.) EXPRESSION OF FRAGMENTS OF THE gB-2 POLYPEPTIDE IN ESCHERICHIA COLI.

4.1.) INTRODUCTION.

The aim of the work reported in this section was to express regions of the polypeptide of glycoprotein gB-2 and to characterize and purify the expressed gB-2 peptides, providing material for immunological study. Initially the total 4.7kbp insert of pUHIO was used but in the early stages of this work data was published by Person et al. (1985) describing a similar experimental approach which successfully defined the location of the gB-2 gene within the fragment (discussed in Section 3.4.4.). Subsequent to obtaining this additional information, efforts to express fragments of gB-2 were focused on a 2.6kbp XhoI fragment containing the extracellular domain of the protein (See also discussion in Section 4.4.1.). Expression of HSV-2 related antigen from a fragment of this DNA, coupled with sequencing data, confirmed this as the location for the gB-2 gene.

Early approaches used to examine the T cell antigenic domains of proteins had involved the generation of peptides by the specific cleavage of fragments from purified proteins. Cleavage was achieved using either a site specific endopeptidase such as trypsin or chemical cleavage at methionine residues using cyanogen bromide (CNBr). These methods were used by Sercarz et al. (1978) to examine the antigenicity of β-galactosidase (GZ), and by Adorini et al. (1979) to examine hen egg lysozyme. The peptide fragments generated required HPLC purification and extensive characterization. These procedures also required a large amount of purified starting material. Since milligram quantities of gB-1 could be purified using the TI57
immunoaffinity column this approach was an option. However the application of these procedures to gB-1 was impractical due to problems arising from quantity, quality and reproducibility of yields (K. Smith personal communication).

An alternative approach utilizing recombinant DNA technology was decided upon, since it offered options to improve yields and reproducibility at the level of purification, ease of characterization of products and the possibility of further manipulations. Eukaryotic cell based expression systems would have the advantage of yielding a glycosylated product whereas prokaryotic expression systems would yield an unglycosylated polypeptide. Since T cell responses are known to be specific to peptide determinants (discussed in Section 5.1.3.), lack of glycosylation should be less important in this study. We therefore decided to use an E.coli based expression system. A fusion protein approach was chosen since a carrier would provide a common factor as a target for purification and an internal control antigen for immunological assays.

At the time of initiation of this work a number of systems were available for the expression of foreign genes as fusion proteins in E.coli. Foreign gene sequences have been fused to either terminus of β-galactosidase (GZ). Examples of vectors which produced carboxy terminal fusions to GZ were phage vector lambda gt11 (Young and Davis, 1983), controlled by modified P\text{lac} promoters, and the plasmid vector pEX (Stanley and Luzio, 1984) (controlled by lambda P\text{R} promoter). Carboxy-terminal GZ fusion proteins were however frequently reported to be insoluble. Other vectors produced fusions to the carboxy-terminus of the trpE gene but this system also frequently yielded insoluble products (Dr. A. Makov personal communication).
The system chosen for the gB-2 expression work was an open reading frame (ORF) expression plasmid (Weinstock et al., 1983) developed at the Wellcome Research Laboratories, Beckenham by Dr. M. Winther and Dr. P. Talbot, which produced polypeptide fusions to the amino-terminus of GZ (Winther et al., 1986). The expression of fusion proteins from the plasmid, named pXY460 (Figure 4.1), was under the control of a powerful Ptac hybrid promoter, composed of the -35 region of the trp operon and the -10 region of the lac operon (Aman et al., 1983). This promoter is inducible with the lactose analogue isopropyl-β-D-thiogalactopyranoside (IPTG). Translation initiates at the vector’s own initiation codon, therefore foreign DNAs were not required to be expressed from their own initiation codon. This vector was designed to receive random fragments of DNA from genes, in the absence of determined nucleotide sequence and coding frames. Its GZ gene is out of frame at the amino terminus, but insertion in the cloning site of an open reading frame fused in frame will reconstitute GZ expression. This provides a simple selection procedure and yields a fusion protein product. Small protein fusions to the amino-terminus of GZ (≈100-200 amino acids) using this vector were reported to be soluble (M. Winther, D. Parker, personal communications).

A companion vector pXY461 (provided by Dr. M. Winther) was derived from pXY460 by insertion of an EcoRI-BamHI linker which removed the cloning site and reconstituted the reading frame of GZ. E.coli cells transformed with pXY461 were used to prepare recombinant GZ purified using the methods described in this section (designated Ag461), providing material as negative control for experiments in Section 5.
4.2.) MATERIALS AND METHODS.

4.2.1.) The vector pXY460 and bacterial host.

The vector pXY460 is described in Figure 4.1. This vector had been shown to maintain insertions in the range 50-500bp (maximum 1000bp). This constraint was based on observations of the poor stability of constructs containing larger DNA inserts and the inability of the bacteria to maintain enhanced expression of very large fusion proteins (Dr. M. Winther, personal communication). Insertion of random DNA fragments generated from a coding sequence into the expression site on pXY460 should yield an expressed product from that gene in 1:18 constructs (1:2 for the correct orientation, 1:3 for the correct reading frame at the 5' end, and 1:3 for the correct reading frame at the 3' end). This is reduced to 1:6 by screening for GZ activity which requires a 3n-1 insert, i.e. the reading frame at the 3' end is fixed by the reading frame at the 5' end. This probability is reduced further by the frequent occurrence of stop codons in non-coding frames.

The constructs were transformed into E. coli host TG1 (lac- proAB lon- (F' traD proAB lacIqZDM15)) a strain derived from JM101 which is deleted in the lon protease gene. This bacterium also harbours a deletion in its own lacZ gene which renders it uninducible, truncated and enzymatically non-functional. This host bacterium also harbours an F' episome which carries the constitutively expressed lac operon repressor lacIq, which regulates expression from the P_{tat} promoter. This allowed an additional step to be incorporated into primary screening of recombinants. Recombinants were selected for ampicillin resistance and by the use of 5-bromo-4-chloro-3-indolyl-β-
Figure 4.1. The plasmid pXY460 (6378bp) is based on the pAT153 replicon with a selectable ampicillin resistance marker (β-lactamase). It has three unique cloning sites (EcoRI, SmaI and BamHI) at the 5' (N-terminal) end of the β-galactosidase (GZ) gene. The gene product is not translated from the plasmid due to a frame shift between the third codon (Ser) and the fifth codon (Gly) of the recombinant GZ due to the cytosine residue indicated in the SmaI site. The vector was designed to accept random blunt ended DNA fragments into the SmaI site which would be translated as polypeptides fused to the N-terminus of GZ. The insert must be 3n-1 nucleotides long and in frame (with no stop codons) with the ATG initiation codon. 11 nucleotides upstream of the ATG is a Shine-Dalgarno sequence (AGGA) at the ribosome binding site. Transcription through the cloning site and GZ is controlled by the Ptac promoter (hybrid of the −35Ptrp region and the −10 Plac region), which is strongly inducible with the lactose analogue IPTG. pXY461 contains the EcoRI-BamHI linker GAATTTGGCGATCC and produces in frame GZ.
D-galactopyranoside (X-gal; Sigma) which produces a blue pigment when digested by GZ. In order to be enzymatically active GZ must form tetramers. In general amino-terminal fusions to GZ do not interfere with tetramer formation or with the active site. Therefore transformants giving blue colonies on agar plates containing X-gal and ampicillin were expected to contain ORF fusions to GZ.

4.2.2.) Preparation of DNA for cloning.

Gel purified electroeluted DNA fragments from pUH10 were prepared for cloning into pXY460 by a "shotgun" method designed to produce a random array of sub-fragments of various lengths representing all parts of the parent fragment. The method chosen was to carry out a controlled partial digestion of the DNA with frequent cutting restriction enzymes which produced blunt ends suitable for direct cloning into pXY460. The three enzymes used were expected to cut 69% G+C DNA with the following frequency: Rsal (GT/AC) once every 349bp, AluI (AG/CT) once every 349bp, and Thai [FnuDII] (CG/CG) once every 70bp. The cumulative frequency would be a cut every 50bp.

1μg of eluted DNA was digested with 1 unit of enzyme for 1 hour at 37°C in a medium salt buffer (Section 2.13.). The products were analysed by agarose gel electrophoresis to confirm complete digestion. For multiple partial digestion 5μg of eluted DNA was digested with 1 unit of Rsal, 1 unit of AluI and 1 unit of Thai simultaneously in medium salt buffer at 37°C. Aliquots of 0.1 volumes were removed after 1, 2, 3, 4, 5, 10, 15, 20, 25, and 30 minutes, and immediately added to a buffer containing 10mM EDTA on ice, to stop the digestion. An aliquot of the pooled final sample was analysed by agarose gel electrophoresis. The final sample was extracted and precipitated. An aliquot of this
material was ligated with pXY460 at an equimolar ratio calculated in terms of free ends, using an average of the observed size range of fragments.

4.2.3.) **Selection of clones expressing GZ activity.**

Bacteria transformed with vector ligated to gB-2 ORF DNA were grown on selective solid medium. L-agar (L-broth plus 1.4% w/v bacto-agar) was used, supplemented with 100 \( \mu \)g/ml ampicillin, 60 \( \mu \)g/ml IPTG, and 100 g/ml X-gal. Blue colonies were picked, restreaked on selective solid medium (see above) and grown in L-broth containing 100 \( \mu \)g/ml ampicillin at 37 °C, overnight in a shaking incubator. Resultant blue ampicillin resistant transformants were purified and glycerol stocks were prepared by addition of sterile glycerol to give 20% final concentration and stored at -20 °C.

4.2.4.) **Preparation of protein from clones.**

Overnight liquid cultures grown from individual colonies were inoculated into 5ml (for screening) or 500ml (for affinity purification) of fresh L-broth/ampicillin (100 \( \mu \)g/ml) and grown for 30 minutes at 37 °C in an orbital shaker. When the bacteria reached log phase growth (O.D.560nm = 0.3) they were induced with 0.001 volumes of 60mg/ml IPTG. Cultures were grown for another 4 hours and then harvested by centrifugation at 5,000g for 10 minutes at 4 °C. The bacterial pellet was resuspended in 25mM Tris.HCl pH8.0, 1mM EDTA, 0.2% NP40, 5mg/ml lysozyme, 1mM phenylmethylsulphonyl fluoride (PMSF), at 20 \( \mu \)l/ml culture and incubated overnight at 4 °C. Then 0.2 volumes of 0.1M MgCl\(_2\) and 0.04 volumes of DNase at 1mg/ml were added and the lysate incubated at 4 °C for 1 hour. The supernatant was precleared by centrifugation at 10,000g for 10 minutes at 4 °C.
before chromatographic purification. Clones were screened for fusion protein by SDS PAGE analysis of 10µl of crude lysate.

4.2.5.) Immuno-affinity chromatography of GZ fusion proteins.

An anti-GZ monoclonal antibody, BG79, coupled to cyanogen bromide activated Sepharose CL4B was used to immunopurify the fusion proteins. The matrix was a gift from Mr. M. Ali, Wellcome Diagnostics, Beckenham. A 5ml column was pre-equilibrated at room temperature with 50mM NaCl, 50mM triethanolamine pH7.6 (ST) at a flow rate of 20ml/hr. A 0.05 sample volume of 1M NaCl was added to the bacterial lysate supernatant which was then loaded onto the column at a flow rate of 5-10ml/hour. The column was washed at 20ml/hour with approximately 10 column volumes of ST buffer until the O.D.280nm of the eluate was <0.020. The bound protein was eluted with 0.1M sodium borate, 1M xylitol, 1mM EDTA, pH10.5 at a flow rate of 15-20ml/hour. Fractions generated during loading, washing and elution were collected and a sample from each fraction analysed for protein content and for GZ activity, by the lac Z microtitre assay (Section 4.2.7.).

Positive fractions were pooled, dialysed extensively against 2mM sodium phosphate pH7.4, lyophilized and reconstituted in a small volume of distilled deionized water.

4.2.6.) Enzyme substrate affinity chromatography.

This method was based on the fact that these fusion proteins retained their enzymic activity. Purification of β-galactosidase has been reported from bacterial lysates of E.coli and Bacillus megaterium (Steers et al, 1971) and
Streptococcus pneumoniae (Glasgow et al., 1977) utilizing the enzymic activity. The method employed a compound, p-aminophenyl-β,D-thiogalactopyranoside (TPEG) which acted as a competitive inhibitor of enzymic activity. TPEG covalently attached to an agarose solid phase (TPEG-agarose, Sigma, Steers et al., 1971), at an optimised spacing of 21Å was used as an affinity matrix. These authors described elution of the enzyme with high pH buffers. They found the product to be free of detectable impurities.

The method used was essentially as described by the authors above. Protein was precipitated from the pre-cleared supernatant of the bacterial lysate, overnight at 4°C, by addition of 231 mg of ammonium sulphate per ml, followed by 1 ml 1 M NaOH for each 10 g ammonium sulphate added. Protein was recovered by centrifugation at 10,000g for 10 minutes at 4°C. The protein pellet was resuspended in 0.1 volume of 20 mM Tris.HCl pH 7.4, 10 mM MgCl₂, 0.1 M NaCl and 10 mM 2-mercaptoethanol (TMSM). The sample was dialysed extensively against this buffer at 4°C. The sample was pre-cleared by centrifugation at 10,000g for 10 minutes at 4°C before it was applied to the TPEG-agarose column. A 10 ml column was pre-equilibrated with TMSM buffer at a flow rate of 20 ml/hour. All steps were carried out at 4°C. The sample was applied to the column and run through at 10 ml/hour. It was washed with 5 column volumes of TMSM buffer or until the O.D.280 nm of the eluate was <0.020. The bound protein was eluted with 0.1 M sodium borate pH 10.0, 10 mM 2-mercaptoethanol. Fractions during loading, washing and elution were collected and a sample from each fraction analysed for GZ activity, by the lac Z microtitre assay (see below), and for protein content by O.D.280 nm. Samples of eluate showing GZ activity were pooled, dialysed extensively against 2 mM sodium phosphate pH 7.4, lyophilized and reconstituted in a small volume of ddH₂O.
4.2.7.) lac Z microtitre assay.

A 5μl sample from an eluted fraction was placed in the well of a flat-bottomed 96 well microtitre plate (Falcon). 100μl of Z buffer (0.1M sodium phosphate pH7.0, 10mM KCl, 1mM MgSO₄, 50mM 2-mercaptoethanol) was added followed by 20μl of ONPG solution 4mg/ml ortho-nitrophenyl-galactopyranoside (ONPG), in 0.1M sodium phosphate pH7.0, to start the reaction. After incubation for 10 minutes at 30°C the reaction was stopped with 50μl of 1M sodium carbonate. The enzymic activity was determined by the generation of free ONP which was recorded as O.D.420nm readings using a Titertec automatic plate reader. The arbitrary units of GZ activity determined in this assay were obtained by O.D.420nm/total sample volume (in ml).

4.2.8.) DNA sequencing.

This was carried out using the dideoxynucleotide chain termination method of Sanger and Coulson (1976), by primer extension on single stranded DNA template of denatured plasmid DNA.

Two synthetic 19mer oligonucleotides complimentary to sequences flanking the cloning site of pXY460 (Figure 4.2) facilitated dideoxynucleotide sequencing of insert DNA directly in the expression construct. These were synthesised and supplied by Mr.H.Spence, Wellcome Biotech, Beckenham. The 19mer TGTGTGGAATTGTGAGCGG (forward primer) hybridized to sequences 40 base pairs upstream of the SmaI site and promoted sequencing through the ATG initiation codon into the "directed" reading frame of the foreign DNA. The 19mer CGATTAAGTTGGGTAACGG (reverse primer) hybridized to a position 43 base pairs downstream of the SmaI site and
promoted sequencing through the reading frame of GZ into the reading frame of the foreign DNA.

Sequence surrounding the cloning site of pXY460.

\[
\begin{align*}
\text{TGTGTGGAAATTGTGAGCGG} & \rightarrow \text{SD} \\
\ldots\text{TGTGTGGAAATTG.GAGCGGATAACAATTTCACACAGGAAACAGAAT} \\
\ldots\text{ACACACCTAACAACGCTGCTATTGTTAAAGTGTGTCCTTTGTCTTA} \\
\text{M N S} & \text{ G D P V} \rightarrow \text{GZ} \\
\text{ATTATGAAATTCCC} & \text{GGGATCCCCGTCTGTTTTACAACGTCGACTGGGAA} \\
\text{TAATACCTTAAAGG} & \text{CCCCTAGGGCAGCAAAATCTTGCAGCACTGACCCTT} \\
\text{EcoRI Smal BamHI} & \\
\text{AACCCTGGCGTTACCGATATCGCTTGCGACACATCCC} & \ldots \\
\text{TTGGGACCAGCTATGCTTTGGAATTACGCGGAACGTCGTGTAGGGGG} & \ldots \\
& \text{<--- CGCAATGGGGTTGAATAGC}
\end{align*}
\]

Figure 4.2. Nucleotide sequences surrounding the cloning site of pXY460 showing the location of the sequencing primers. The forward primer and the reverse primer were synthesised at Wellcome Biotech and supplied as a gift by Mr. H. Spence.

2.5μg of caesium chloride gradient purified plasmid DNA in 16μl of ddH2O was denatured by addition of 4μl of 1M NaOH, 1mM EDTA. The alkali was neutralized by addition of 2μl of 2M ammonium acetate pH4.5 and the single stranded DNA immediately precipitated with 2.2 volumes of ice cold ethanol. After incubation on ice for 10 minutes the DNA was recovered by centrifugation at 12,000g for 30 minutes at 4°C. The DNA pellet was washed with 80% ethanol and dried on a rotary vacuum desiccator.
The denatured plasmid DNA was resuspended in 9.5 μl of ddH₂O. 1 μl of 5 μM primer, 1.5 μl of 70 mM Tris.HCl pH 7.5, 70 mM MgCl₂, 50 mM 2-mercaptoethanol, 1 mM EDTA and 4 μl ³⁵S-dATP (>600 Ci/mmol, Amersham) were added to the DNA sample. This was incubated at 37°C for 15 minutes to allow the primer to anneal. 1 μl containing 2 units of Klenow fragment of DNA polymerase I was added and the samples then dispensed 3 μl into each of four 0.5 ml eppendorf tubes labelled G*, A*, T* and C* respectively. 2.5 μl of the appropriate dNTP:ddNTP mix was spotted on the upper wall of the tube. The respective dNTP:ddNTP mixtures were as follows:

<table>
<thead>
<tr>
<th>dNTP mixes (N*)</th>
<th>dGTP</th>
<th>dCTP</th>
<th>dTTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>G'</td>
<td>17.5 μM</td>
<td>175 μM</td>
<td>175 μM</td>
</tr>
<tr>
<td>A'</td>
<td>75 μM</td>
<td>75 μM</td>
<td>75 μM</td>
</tr>
<tr>
<td>C'</td>
<td>175 μM</td>
<td>17.5 μM</td>
<td>175 μM</td>
</tr>
<tr>
<td>T'</td>
<td>175 μM</td>
<td>175 μM</td>
<td>17.5 μM</td>
</tr>
</tbody>
</table>

ddNTP solutions

ddGTP = 1 mM, ddATP = 25 μM, ddCTP = 1 mM, ddTTP = 2 mM

dNTP:ddNTP mixes (N*)

G' = G' : ddGTP = 4:6
A' = A' : ddATP = 1:1
C' = C' : ddCTP = 5:6
T' = T' : ddTTP = 4:6

The reaction was started by spinning the tubes briefly in a microfuge. They were incubated at room temperature for 20 minutes, then 2 μl of chase (2 mM dATP, 2 mM dGTP, 2 mM dCTP, 2 mM dTTP) was added and started by microfuging the tubes. This was incubated for 15 minutes at room temperature. 4 μl of stop solution/gel load buffer (0.05% bromophenol blue, 0.05% xylene cyanol, 10 mM EDTA, 95% deionized formamide)
was added to each tube. These samples were heated to 95 °C for 3 minutes and 4 μl of each loaded per track on a resolving gel.

The resolving gel system was 8% polyacrylamide (38:2 acrylamide to bis-acrylamide ratio) with 8M urea and 1x TBE (see Section 2.13.). Standard gels were 400mm x 300mm and 0.25mm thick. Samples were run on the gels at 40mA for approximately 1 hour (additional longer runs were frequently used to extend the number of bases that could be read). Gels were fixed in 10% acetic acid for 10 minutes at room temperature, rinsed several times with water then dried down onto filter paper and exposed to Kodak X-Omat R film.

Occasional problems of compression of banding on sequencing gels, due to secondary structure caused by the high G+C content of the templates, were overcome by running gels containing 8% acrylamide, 40% deionised formamide, 7M urea and 1x TBE.

4.2.9.) DNA sequence analysis.

Sequence data was analysed using the "Microgenie" DNA analysis software (Beckman) on an IBM XT personal-computer, in order to align cloned fragments with the known sequence of gB-2 (Bzik et al., 1986).
4.3.) RESULTS.

4.3.1.) Cloning of fragments from the 2.6kbp XhoI fragment of pUH10.

Digestion of the 2.6kbp XhoI fragment of pUH10 with RsaI, AluI and ThaI produced between 7 and 10 discrete bands in each track (Figure 4.3), ranging in size from 100-1000bp.

![Figure 4.3. Restriction enzyme digestion pattern of the 2.6kbp XhoI fragment of pUH10 with ThaI (T), RsaI (R) and AluI (A). Size standards were lambda HindIII (H) and the 123bp ladder (123).](image-url)
Multiple partial digestion of the 2.6kbp DNA with these enzymes produced a heterogeneous mixture of fragments migrating as a smear of DNA, ranging in size from 200bp-1000bp (Figure 4.4).

Figure 4.4. Multiple partial restriction of the 2.6kbp XhoI fragment of pUH10 with ThaI, RsaI and AluI (MP). Size standards were lambda HindIII (H) and the 123bp ladder (123).
Ligation of this material into pXY460 and transformation into E.coli TGI yielded >100 blue transformants. These were examined for the production of induced proteins with molecular weights greater than 116kDa (i.e. GZ), by SDS PAGE analysis of induced culture lysates.

Figure 4.5. SDS PAGE of IPTG induced culture lysates of pXY461 and clones pXH02-85 derived from the 2.6kbp XhoI fragment of pUH10. Two gels were run simultaneously. Gel a) was stained with Coomassie blue; molecular weight standards were 200kDa myosin, 116kDa GZ, 97kDa phosphorylase b, 66kDa bovine albumin, 45kDa egg albumin, 29kDa carbonic anhydrase (Sigma). Gel b) was Western blotted and stained with rabbit polyclonal anti-HSV-2 antisera and 125I-protein A. The lane marked HSV-2 contains lysate from HSV-2 infected BHK cells.
Induced culture lysates from clones identified as expressing fusion proteins were run in duplicate on SDS PAGE for Coomassie blue staining and for Western blotting. The results (Figure 4.5a) showed the variation in electrophoretic mobility and intensity of staining with Coomassie blue between fusion proteins. The result of probing the Western blot of these samples with a polyclonal anti-HSV-2 antisera (Figure 4.5b) revealed a number of clones which showed HSV-2 specific immunostaining. The fusion proteins produced by these particular clones, identified as pXH 53, 55, 59, 65, 71, 73 and 85, showed varying degrees of staining with the antisera.

Digestion of mini-preparation DNA from a number of fusion protein positive clones with EcoRI and BamHI (Figure 4.6) showed an average insert size of 446bp (range 155-1150bp). The average insert size for clones showing HSV-2 specific immunostaining was smaller ≈ 248bp (range 155-350bp). The insert size of individual clones is summarized in Table T4.2, at the end of Section 4.3.3.
Figure 4.6. Analysis of insert size in a number of pXH clones. Agarose gel electrophoresis of EcoRI-BamHI digested mini-preparation DNA. 123bp and 1kbp DNA ladders were used as size markers.
4.3.2.) DNA sequencing.

The nucleotide sequence data (example shown in Figure 4.7) generated from analysis of these clones were summarized in Figures 4.8 a,b,c,d,e and f. Of seven clones analysed only three could be positively identified as having sequences of gB-2 in frame with GZ. Clones pXH 59 and 65 were shown to be in frame at the 5' and 3' ends and, by homology to the gB-2 sequence, to contain DNA of approximately the size indicated by restriction digests. Clone pXH 71 contained sequences from nucleotides 2135 to 2386 which were out of frame (by +1) attached to sequences from 1854 to 1918 (in a frame -1 to the preceding sequences) which was in frame with the distal ATG, and the proximal GZ sequences.

Sequence data from the other clones also showed homologies to gB-2, but no other constructs were shown to have regions of gB-2 in frame with GZ, despite the fact that pXH 53, 55 and 73 were shown to have immunostaining fusion proteins. Nucleotide sequence of the 3' end of pXH 55 and for either end of pXH 53 and 73 did not align with the HSV-2 sequence.

In order to interpret future results it was important for the antigens to be characterised unequivocally. For this reason work was only continued with clones pXH 59, pXH 65 and pXH 71, which had all been characterised at both the 5' and 3' ends.
Figure 4.7. An example of nucleotide sequence analysis of pXH clones showing the confirmation of reading frame of the insert with respect to the ATG initiation codon of the vector. The example shown is pXH65.
Figure 4.8. Summary of sequence data generated for clones pXH53-85. Upper sequences are those of the published sequence for gB-2 (Bzik et al, 1986) including the nucleotide number, (n), and the codon number, [n]. Nucleotides are shown grouped in the codon triplets of gB-2 in the upper sequences. The lower sequence are those generated from the particular clone and the nucleotides are grouped in the codon triplets expressed from the vector ATG. Nucleotides illustrated as lower case letters (a, g, c, or t) were ambiguous and X represents and undetermined nucleotide.

a) pXH55 sequences originated from the 5' flanking sequences of the gB-2 gene (hence no codon number), the 3' end of pXH55 showed no identifiable homology.

b) pXH59 contained a fragment from nucleotide 1566 to 1732 including codons 339-394.

c) pXH65 contained a fragment from nucleotide 1818 to 1999 including codons 423-484.

d) pXH71 contained a fragment from nucleotide 2135 to 2386 out of frame, then another fragment from nucleotides 1854 to 1918 including codons 436-457, in frame with GZ.

e) pXH84 contained a short unidentified fragment of 27 nucleotides then a fragment from 1831 to 2021 which was out of frame with GZ.

f) pXH85 contained 3 fragments, the first an unidentified 13 nucleotides then a fragment from nucleotides 1807 to 1858 including codons 420-437 in frame, then a fragment of unidentified origin to the 3' junction with GZ.

NNN........NNN represents contiguous sequence (intermediate sequence not determined).

NNN- sequence continued below
NNN-
a) **pXH55**

(243)

\[ \text{gB-2} \]

ATTCGCCG AGC TCA TTA ATC GCC ACC ACA ATC-TATT ATG AAT TCC CG tGC TCA TTA ATC GCC ACC ACA ATC- 

pXY460

(304)

TTT GCG TCG GTC GTG CGG GGA GCT TGA GTT...

TTT GCG TCG GTC GTG CGG GGA GCT TGA GTT...

b) **pXH59**

(1566)

\[ \text{gB-2} \]

AAC CTG CTG ACG ACC CCC AAG TTT ACC GTG GCC- 

ATG AAT TCC C:TG CTG ACG ACC CCC aaG TTT AcC GTg GCC- 

pXY460

(1606) | (1688)

TGG GTG CCG AAG.........................TTC TCC TCC GAC- 

TgG GTg CCG aaG.........................cTC TaC TCX GXC- 

(1732)

[392] \[ \text{gB-2} \]

GCC ATC TCG ACC ACC TTC ACC ACC AAC CTG ACC GAG 

GCC ATC TgG gCC AcC gTC ACC AaC ACC CTc AcC:GGG GAT 

pXY460
c) pXH65

\[ (1818) \]
\[ \text{gB-2} \quad [424] \]
\[ \text{CGC AAG TAC ACC GCC ACG CAC ATC AAG GTG-} \]
\[ \text{TATT ATG AAT TCC C:AG TAC AAC GCC ACG CAC ATC AAG GTG-} \]
\[ \text{pXY460} \]

\[ GGC CAG CCG CAG TAC TAC CAG GCC ACG GGG GGC TTC CTC- \]
\[ GGC CAG CCG CAG TAC TAC CAG GCC ACG GGG GGC TTC CTC- \]

\[ (1897) \quad (1955) \]

\[ \text{ATC GCG TAC CCC...............AAG CCC CGG-} \]
\[ \text{ATC GCG TAC CCC...............cAG CCC CGG-} \]

\[ (1999) \]
\[ \text{[483] \; \text{gB-2} \; 153} \]
\[ \text{AAT GCC ACG CCC GCG CCA CTG CGG GAG GCG CCC AGC GGC ACG CCC GGC TTC CTC-} \]

\[ \text{d) pXH71} \]

\[ (2135) \]
\[ \text{gB-2} \quad [529] \]
\[ \text{GAG CTG CAG AAC CAC GAG CTG ACT CTC TCG AAG GAG-} \]
\[ \text{ATG AAT TCC C:CT GCA GAA CCA cGA GCT GAC TCT CTg GAA CGA-} \]
\[ \text{pXY460} \]

\[ (2203) \]

\[ \text{GCC CGC AAG CTC AAC CCC AAC GCC TCC GCC ACC GTC...............} \]
\[ \text{GTC CCG CAA GCT CAA CCC CAA CgA gTC gGC CtC XgX...............} \]

\[ (2328) \]

\[ \text{...............C CTG GTC AGC TTT CGG TAC GAA GAC-} \]
\[ \text{...............cCTG GTC cTT CtG GTA CGA AGA-} \]

\[ (2386 :1854) \]
\[ \text{[610] \; [438]} \]
\[ \text{CAG GGC CCG CTG ATC GAG GGG CAG :AC TAC CAG GCC ACG GGG GGC TTC-} \]
\[ \text{CCA GGG CCC GCT GAT CGA GGG GCA G:AC TAC CtG GCC AcG GGG GGC TTC-} \]

\[ (1918) \]
\[ \text{[456] \; \text{gB-2} \; 153} \]
\[ \text{CTC ATC GCG TAC CAG CCC CTC CTC AGC AAC ACG CTC GGC GAG CTG TAC} \]
\[ \text{CTC ATC GCG TAC CAG CCC CTC CTC tGC AAC ACG CTC GGC GAG:GGG GAT} \]
\[ \text{pXY460} \]
e) pXH84

(1831)

$$\text{gB-2}$$

$$\text{ACG CAC ATC-}$$

TATT ATG AAT TCC C:GA AAG CGC GCA AGT ACA CCG CCA CGC T:CA TCA-

pXY460

AAG GTG GCC CAG CCG GAC TAC TAC CAG GCC ACG GGG GCC TTC CTC-

AgG TGg GCC AGC CTG ACT ACT ACC tGG CCA CGG GGG GCT TCC TCA-

(1899) (1972)

ATC GCG TAC C..............................CC ACG CCC GCG-

TCG CGT ACC.............................CCA CGC CCG CGC-

(2022)

CCA CTG CGG GAG CCC AGC GCC AAC GCG TCC GTG GAG CGC ATC AAG

CAC TGC GGG AGC CCA GCG CCA ACG CGT CCG TGG AGC GCA:GGG GAT

pXY460

f) pXH85

(1806)

$$\text{gB-2}$$

$$\text{ATT ATG AAT TCC C:GG CCG GGG TGC CCG TTT GCG CGC AAG TAC AAC AAC-}$$

pXY460

(1858)

[435]$$\text{GCC ATC AAG GTG GCC CAG CCG CAG TAC TAC CAG GCC CAG}$$

GCC ATC AaG GTg GCC CAG CCG tAG TAC TAC:TAC gTG GCC AcG G...
Table 4.1

<table>
<thead>
<tr>
<th>Clone</th>
<th>DNA insert (bp)</th>
<th>Protein (kDa)</th>
<th>In frame</th>
<th>Immuno-</th>
<th>gB-2 codons</th>
</tr>
</thead>
<tbody>
<tr>
<td>pXH</td>
<td>Expt. Sequ.</td>
<td>Expt. Sequ.</td>
<td>gB-2 staining</td>
<td>represented</td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>190</td>
<td>ND</td>
<td>≈5</td>
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<tr>
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<td>350</td>
<td>ND</td>
<td>≈15</td>
<td>ND</td>
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</tbody>
</table>

Values for DNA insert and protein (additional to GZ 116kDa) were determined by gel electrophoresis (Expt.) or by interpretation from sequencing data (Sequ.). ND - not determined.
4.3.3.) Protein Purification.

4.3.3.1.) Enzyme substrate affinity chromatography.

A typical wash and elution profile for purification of GZ from bacterial lysate of clone pXY461 (referred to as Ag461) is shown in Figure 4.9. SDS PAGE analysis of the fractions collected shows that a certain amount of intact GZ is consistently eluted with other bacterial proteins in the unbound fraction (shown in Figure 4.10). Intact GZ was also detected in the wash fractions, accounting for the low activity detected in those washes. This suggested that the enzyme was not tightly bound to the column and that only a fraction could be specifically eluted. The total enzyme activity eluted with borate buffer (1020 units) represented 13% of the total GZ activity loaded (7801 units). Since the yield was low various adjustments were made to the loading conditions such as i) loading and running at room temperature, ii) loading and recirculating over the column for a number of hours, and iii) loading and leaving to equilibrate and bind to the column overnight. None of these conditions increased the amount of enzyme activity or protein specifically eluted. Adjusting the ionic strength or pH of the elution buffer or adding ethanediol at high molarity also had no positive effect on the yield from the column. Increasing the total enzyme activity added to the column did not decrease the yield, nor did reducing the activity added increase the yield.

This method was used to prepare a batch of purified protein from each of the clones pXH59, pXH65, and pXH71 and pXY461, providing Ag59, Ag65, Ag71 and Ag461 for use in the first immunological assay. The yields obtained from 500ml cultures prepared in parallel for Ag59, Ag65, Ag71 and Ag461 are summarized in Table 4.2.
Figure 4.9. Elution profile of enzyme substrate-analogue affinity chromatography of GZ from bacterial lysate of pXY461. After binding of the lysate the column (10ml bed volume TPEG-agarose, Sigma) was washed (fractions 1-12) then eluted (fractions 12-16) as described in Section 4.2.3. 5ml fractions were collected and assayed for GZ activity using the lac Z microtitre assay •---• (Section 4.2.5.) and protein content by O.D.280nm ••.
Figure 4.10. SDS PAGE analysis of fractions collected during elution of GZ from bacterial lysate of pXY461 from the enzyme substrate affinity column (Fig. 4.11.). Lane numbers correspond to the fraction numbers from the elution profile. Lane 17 is a sample of the cleared lysate before loading.
4.3.3.2.) Monoclonal antibody affinity chromatography.

To increase the yield of GZ, pXY461 culture lysate was purified on an anti-GZ immunoaffinity column (5ml column volume). The profile of protein and GZ activity, eluted from the column (Figure 4.11), showed that the majority of the protein was present in the first 3-4 column volumes of flow through and wash. However, unlike the TPEG column only a small amount of GZ activity was detected in the flow through, and there was no detectable GZ in subsequent washes. The column was eluted with the high pH buffer causing specific release of protein and concomitant GZ activity, which was eluted within 3 column volumes. SDS PAGE analysis of samples from the column eluates (Figure 4.12) showed that only a small amount of GZ was present in the sample flow through (lanes 2-4), which otherwise retained the full complement of bacterial proteins. The washes contained little or no protein (non-specific or GZ). The sample from the pooled high pH eluate (Figure 4.12, lane 5) confirmed that the protein was intact GZ. Similar profiles for BG79 column runs of bacterial lysates from pXH 59, 65 and 71, showed that the N terminal fused polypeptides did not affect the binding to or elution from the column of the GZ moiety.

This method was used to prepare batches of purified protein from 500ml induced cultures of each of the clones pXH 59, 65, 71 and pXY461. The results in Table T4.1 showed that this was a much more efficient method of recovery of the fusion proteins and GZ than the substrate analogue affinity column, and yielded products with a good degree of purity (assessed by SDS PAGE, Figure 4.13).
Figure 4.11. Elution profile of BG79 immuno-affinity chromatography of GZ from bacterial lysate from pXY461. The column (5ml bed volume, of BG79 monoclonal antibody coupled to Sepharose CL4B), after binding of the lysate, was washed (fractions 1-10) then eluted (fractions 11-14) as described in Section 4.2.4. 5ml fractions were collected and assayed for GZ activity (Section 4.2.5.) and protein content.
Figure 4.12. SDS PAGE analysis of fractions collected during elution of the immuno-affinity column (Fig. 4.11.). Lane 1 was a sample of cleared lysate before loading, lane 2 represented column fraction 1, lane 3 represented column fraction 3, lane 4 represented column fraction 5, lane 5 represented column fraction 12.
Figure 4.13. SDS PAGE analysis of immunopurified samples of Ag461, Ag59, Ag65 and Ag71. Molecular weight standards were 200kDa myosin, 116kDa GZ, 97kDa phosphorylase b, 66kDa bovine albumin, 45kDa egg albumin, 29kDa carbonic anhydrase (Sigma).
Table 4.2.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Yield from 500ml culture</th>
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<tr>
<td></td>
<td><strong>Enzyme affinity</strong></td>
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<tr>
<td>Ag 461</td>
<td>872µg</td>
</tr>
<tr>
<td>Ag 59</td>
<td>702µg</td>
</tr>
<tr>
<td>Ag 65</td>
<td>658µg</td>
</tr>
<tr>
<td>Ag 71</td>
<td>581µg</td>
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Comparison of the recovery of recombinant proteins from bacterial cell lysate.
4.4.) DISCUSSION.

4.4.1.) Cloning for expression of gB-2 fusion proteins.

In the first instance the attempt to express DNA sequences from within the pUH10 insert was carried out prior to the publication of a nucleotide sequence or specific location for the gB-2 gene. An approach to the cloning for expression of gB-2 polypeptides using Bal31 nuclease to produce random 5' and 3' ends on specific restriction fragments from various locations within the pUH10 sequence (results not shown) was unsuccessful, due to technical difficulties involving failure of treated DNA to ligate. A parallel approach using random fragments of the 2.6kbp XhoI fragment of pUH10, generated by multiple partial digestion with frequent cutting restriction enzymes, did yield a number of clones expressing fusion proteins. However, some of these clones showed no reactivity with the polyclonal anti-HSV-2 antiserum, including some with relatively large inserts (Fig.4.6; pXH 02, 05 and 17, 500bp-1kbp).

Recombinant plasmids directing the expression of non-gB-2 fusion proteins may have been isolated for several reasons. Firstly, fragments originating from regions of non-coding DNA flanking the gene, although expected to contain numerous stop codons, may have contained untranslated open reading frames which it would be possible to express in this system. In addition, analysis of the published sequence of the gB-2 gene (Bzik et al., 1986) showed that there were a number of extensive open reading frames in two of the non-coding frames of the gene (one in each strand - see Figure 4.14), so that even when cloning from the 2.6kbp XhoI fragment it would have been possible to produce non-gB-2 polypeptide fusions.
Figure 4.14. Plot of stop codons within the gB-2 sequence in the three reading frames of each orientation. The true coding frame is indicated as a blocked box.

gB-2 (HG52) stop codons (positive sense).

100bp

Secondly it may have been due to the potentially toxic effects of expressing particular protein sequences, such as cytoplasmic anchor (CA) and transmembrane (TM) structures, on the host bacteria. The CA and TM sequences from gD of HSV-1 have been shown to be toxic to the host bacteria when expressed in E. coli (Watson et al, 1984; Steinberg et al, 1986). Although expression of HSV-1 gD signal sequences was possible, expression of a VSV glycoprotein fusion protein containing signal sequence proved lethal (Rose and Shafferman, 1981) as did a rabies virus glycoprotein (Yelverton et al, 1983). It is also known that some sequences representing structural, non-signal, non-transmembrane
portions of a protein can also prove toxic to the host bacteria, for example HSV-1 gC (Amann et al., 1984) and human fibroblast interferon (Remaut et al., 1983).

Subcloning from the 2.6kbp XhoI fragment of pUH10, representing the extracellular domain of gB-2 (Person et al., 1985), was performed to avoid specific domains (CA and TM) likely to be toxic to the host bacteria. However, expression of gB-2 oligopeptides from within the extracellular domain may also have proven toxic to the host, and constructs containing such sequences would not have been represented as viable transformants for subsequent screening.

4.4.2.) Sequence analysis of clones.

Sequence analysis of these clones (summarized in Figure 4.8) showed that only two could be identified as having continuous inserts in frame for gB-2 and GZ. These were clones pXH59, with fifty five amino acids representing codons 339-394 of gB-2, and pXH65, with sixty three amino acids representing codons 421-484 of gB-2. Another clone, pXH71, contained 21 amino acids of gB-2 originating from part of the sequence of pXH65, representing codons 436-457 of gB-2, between nonsense polypeptide and GZ. A number of other clones, including pXH55 and pXH85 showed the same characteristic of pXH71 in that the expression vector contained a hybrid insert originating from two (or possibly more) positions in the gB-2 sequence. This may have been due to the presence of small insert sequences (around 50bp) which, if present in the ligation in a molar excess to vector, could lead to a situation in which two insert molecules may have been able to ligate one to each end of the vector, or to each other, before a ligation which recircularized the molecule. An alternative explanation was
that two separate regions of DNA were brought together by deletion of the sequences between them. However, this does not explain the occurrence, in the correct orientation, of the gB-2 sequence 1854-1918 downstream of the sequence 2135-2386 in pXH71.

The occurrence of such hybrid inserts could be reduced with the following precautions. Firstly, the partially digested DNA could be fractionated by agarose gel electrophoresis or column chromatography to separate very small fragments (as found in pXH71 and pXH85) and very large fragments from those of the optimum length for cloning. Secondly, the ligation reaction could be carried out using a lower concentration of DNA (than the 20μg/ml used here) to reduce the incidence of intermolecular ligations and prevent multiple inserts. Thirdly, ligation between random fragments could be prevented by treatment with phosphatase prior to insertion into the vector.

Sequence analysis at the two ends of the insert in clones pXH53 and pXH55 (sequence in Figure 4.8) showed that no gB-2 sequences were in frame in these regions, yet they showed HSV-2 serological reactivity (Figure 4.5). Since the entire insert has not been sequenced this may be explained if they contained at some internal position in their insert a portion of gB-2 which is ultimately in frame with GZ, such as pXH85 with a short 17 codon region that is in frame. In addition, the sequences determined at the insertion junctions of those two clones did not show homology to the gB-2 sequence (with the exception of the 5' end of pXH55). This may have been due to their originating from the portion of 2.6kbp XhoI 5' of the determined sequence. Alternatively they may have originated from fragments of the vector which co-migrated with the electroeluted 2.6kbp band. Characteristics of these clones could have been further
investigated by subcloning the inserts into M13 vectors and determining their entire sequence.

4.4.3. Recombinant protein purification.

The method of purification which utilized the GZ enzyme activity was very efficient in respect to purifying the GZ as a single band on SDS PAGE (Figures 4.9 and 4.10), but much less efficient in respect to providing a high yield of protein (Table 4.2). One of the reasons for employing bacterial expression of the gB-2 gene product, rather than producing trypsic cleavage peptides to isolate regions of the protein, was to increase the amount of product for further investigation. These results were comparable to the relatively small amounts of glycoprotein which could be immunopurified using TI57 from HSV-2 infected BHK cell cultures. These amounts were in the range of 1mg-500μg, and were even less for purified trypsin generated peptides. Despite the fact that the E.coli were over-producing the fusion proteins yields of protein were no higher than might be expected from mammalian tissue culture and immuno-affinity chromatography.

When the monoclonal antibody BG79 was used, GZ was bound with high affinity so that the column could be extensively washed without significant non-specific elution (Figures 4.11 and 4.12). In addition amino-terminal substitutions of GZ, such as these fusion proteins, did not interfere with binding as assessed by comparative yields (Table 4.2). This method provides a simple and efficient way of producing high yields (Table 4.2) of each of the fusion proteins and GZ with good purity (Figures 4.12 and 4.13) using identical, reproducible conditions. This was essential to provide fusion proteins and GZ for comparative assessment in subsequent immunological experiments.
5.) STUDIES OF THE IMMUNE RESPONSE TO qB-2 AND THE RECOMBINANT ANTIGENS.

5.1.) INTRODUCTION.

5.1.1.) T lymphocyte responses to HSV.

The potential importance of the T lymphocyte response to HSV in protection and recovery from infection has been discussed (Section 1.4.). Lyt 1+ 2- T cells (Th and T-DTH) were shown to mediate clearance of virus from the site of infection (Nash and Gell, 1983) whilst Lyt 1- 2+ T cells (CTL) were shown to mediate long term memory to HSV infection (Larson et al., 1984). Lyt 1- 2+ cells also control the spread of the virus in the nervous system (Nash et al., 1987). T lymphocyte responsiveness was determined in vitro by cytotoxicity using specific $^{51}$Cr release and lymphocyte proliferation using incorporation of $^{3}$H-thymidine into dividing cells, and in vivo by the delayed-type hypersensitivity (DTH) response using skin swelling. The lymphoproliferative response to HSV, first demonstrated by Lopez and O'Reilly (1977), was subsequently shown to be attributable 60% and 40% to T cells of the Lyt 1+ 2- phenotype and the Lyt 1- 2+ phenotype respectively (Horohov et al., 1985).

5.1.2.) The mouse model.

The mouse model has provided an excellent system for analysing T cell mediated responses to various HSV gene products, in particular the glycoproteins. Various methods have been applied to the determination of the roles of the glycoproteins in the cell mediated immune response. Specific DTH response was induced by purified gC-1 and gD-1. gC-1 induced a type specific response which coincided with
protective immunity, whereas gD-1 induced a type common response and no protection (Schrier et al, 1983a)). Comparison of cytotoxic response to mutant viruses defective in the production of gB-1 and gC-1, in a limiting dilution assay, suggested that gC was the major target of HSV-1 specific memory CTL (Glorioso et al, 1985). Responses to gB-1 were undetectable. This was supported by observation that HSV primed mouse CTLs recognised gC but not gB, gD or gE, when individually expressed on the surface of recombinant syngeneic mouse target cells (Rosenthal et al, 1987).

115kDa and 96kDa glycoproteins representing gB-1 and gD-1 were shown to protect mice against challenge with HSV-1 but not HSV-2, when immunized and challenged by the intraperitoneal (i.p.) route (Chan, 1983). Similarly when mice immunized i.p. independently with immunopurified gB-1, gB-2, gD-1 and gD-2, were challenged i.p. with HSV-1 or HSV-2, gD-1 and gD-2 provided protection against both homologous and heterologous virus whilst gB-1 or gB-2 provided protection only against HSV-1 (Dix and Mills, 1985). gB-1 was shown to induce an antibody response, a DTH response, and a lymphoproliferative response whilst gD-1 induced only a lymphoproliferative response (Chan et al, 1985). The DTH response induced by gB-1 did not however seem to correlate with protective immunity.

Expression of gB-1 and gD-1 on the surface of mouse L cells provided an excellent tool for examining the responses to individual glycoproteins (Blacklaws et al, 1987). Antibody and DTH response were detected to both glycoproteins but the CTL response was low. As a protective immunogen, gD-1 caused a lower incidence of establishment of latency and a higher clearance of virus from site of infection than gB-1, though gB-1 gave significant results compared with controls (Blacklaws et al, 1987).
5.1.3.) In humans.

The information on T cell responses in humans to specific HSV antigens is somewhat limited in comparison to the mouse model. The use of HSV type specific human CTL clones in conjunction with intertypic recombinant viruses has helped to elucidate involvement of the glycoproteins in the CTL response to the virus (Yasukawa and Zarling, 1985). Cloned and purified gB-1 and gD-1 were shown to stimulate T cells from human peripheral blood lymphocyte preparations to proliferate in vitro (Zarling et al., 1986). Lymphoproliferation has also been shown in peripheral blood lymphocytes from twenty two individuals suffering from recurrent herpes labialis when stimulated with native immunopurified gB-1, gC-1, gD-1 and recombinant purified gD-1 (Torseth et al., 1987). Vaccination of HSV-2 seronegative volunteers with purified HSV-2 glycoproteins (Ashely et al., 1987) showed that, in the humoral response in humans, the most rapid seroconversion and highest titre was induced by gB-2.

5.1.4.) Aims.

gB has been shown to elicit a significant portion of the immune response to HSV in humans. In the mouse model it has been shown to confer protective immunity. The most significant characterised response to this antigen appears to be that of the Lyt 1+ 2- T cells measured by lymphoproliferation and DTH. The aims of the work in this section were to use the mouse model to examine the T cell responses to the recombinant antigens Ag59, Ag65 and Ag71, as compared with gB-2 and HSV-2 in terms of lymphoproliferation, DTH response and ability to confer protective immunity. Antibody responses in the protection experiment were also determined.
5.2.) MATERIALS AND METHODS.

5.2.1) Mice.

The mice used in this work unless otherwise stated were 8 to 10 week old BALB/c females. Occasionally males were used when females were not available. In all cases all the animals used in any one experiment were of the same age, sex and strain. Animals were obtained from the breeding centre at the Department of Experimental Immunobiology, Wellcome Research Laboratories, Beckenham.

5.2.2) Collection of blood samples.

a) Collection of blood samples from mice within an experiment was carried out by tail bleeding. Individuals were warmed under an infrared lamp until the tail vein was clearly visible then the tip of the tail was nicked with a sharp scalpel blade. The tail was then drawn towards the end and the blood collected in capillary tubing and transferred to a 1.5ml microfuge tube.

b) Anti-HSV-2 antisera for radio-immunoassay (RIA) and normal mouse serum (NMS) for use as negative controls in RIA or as media supplements were obtained from anaesthetized mice by cardiac puncture using a 25 gauge needle attached to a 1ml syringe.

c) All serum samples were allowed to coagulate overnight at 4°C then spun in a microfuge for 15 minutes to pellet the clot and erythrocytes. Cleared serum was carefully removed and either stored at 4°C with 0.05% sodium azide, or frozen at -20°C. NMS for use in media was heat inactivated at 56°C for 45 minutes before use.
5.2.3.) Inactivation of virus.

In order to prime and elicit responses to virus in vivo or in vitro without causing infection, samples of inactivated virus were prepared. Two methods of inactivation were used. Heat inactivation (HI) was carried out by incubating the virus preparation at 56 °C for 45 minutes. U.V. inactivation was carried out by incubating the virus preparation at 4 °C at a distance of 70mm from a 320nm U.V. source for 15 minutes. Samples of virus preparation containing 3x10^7 PFU/ml inactivated by both of these methods showed a reduction in infectious virus to <10^2 PFU/ml.

5.2.4.) Extraction of infected cell proteins.

Monolayers of BHK cells, typically five 75cm^2 flasks, approximately 10^7 cell/flask, were infected at an m.o.i. of 1.0. At 48 hours post infection the cells were harvested by agitation of the flask and media causing the cells to slough off from the surface of the flask. The free cells were separated from the medium by centrifugation at 2000g for 10 minutes at 4 °C. The infected cells were washed twice by resuspension in and pelleting from PBS at 4 °C. The washed cell pellet was resuspended in 1.0ml of extraction buffer (150mM NaCl, 25mM Tris.HCl pH8.0, 2mM EDTA, 2mM EGTA, 1% NP40) containing protease inhibitors (1% aprotinin, 1mM hydroxy-mercuric-benzoate, 1mM PMSF, 1mM N-[[p-tosyl-L-lysine chloromethyl ketone [TLCK]) per 10^7 cells. The suspension was sonicated, using a micro-probe on an MSE 100kW Sonicator on setting 6 μm, 3x 15 seconds on ice, then mixed on a rotary mixer at 4 °C for 1 hour. The mixture was centrifuged at 100,000g for 1 hour at 4 °C to remove particulate debris. The supernatant was then applied to the anti-gB immuno-affinity column (described below), or dispensed into suitable aliquots and stored at -20 °C.

173
5.2.5.) Preparation of anti-gB immuno-affinity column.

An anti-gB immuno-affinity column was prepared by coupling the gB specific monoclonal antibody TI57 (Chan, 1983) to Sepharose CL4B. 10g of Sepharose CL4B was washed with 200ml of 0.9% w/v saline solution and dried by suction through a sintered glass filter funnel. The Sepharose was activated by incubation with 2ml of a freshly made solution of 100mg/ml sodium metaperiodate per gram of gel. This was gently mixed on a rotary mixer for 4 hours at room temperature (20 °C). The Sepharose was rapidly washed with 200ml of saline in a sintered glass funnel under vacuum. This was followed by a slow wash under gravity with 2ml of 10% ethanediol per gram of Sepharose used. It was then washed quickly with a large amount of 0.1M sodium carbonate pH9.5 and immediately added to a solution containing affinity purified monoclonal antibody (Section 2.6.) in 0.1M sodium carbonate pH9.5 at a concentration of 0.3mg/ml. This was incubated on a rotary mixer for 18 hours at 4 °C. The antibody solution was removed by suction through a sintered glass funnel and the coupled Sepharose washed extensively with ~500ml of saline. It was then mixed with a fresh solution of 5mg/ml sodium borohydride, at a ratio of 1ml/g of gel, at room temperature for 2 hours with gentle mixing. The coupled Sepharose was finally washed extensively with saline and then packed in a 10ml syringe.

The amount of antibody coupled was determined by a comparison of the O.D.280nm value of the supernatant before and after coupling. The column used to prepare the glycoprotein in this work was 3mg of TI57 antibody coupled to 10g of gel making a 10ml bed column.
5.2.6.) Purification of gB-2.

Purification was carried out at 4°C as described by Chan (1983). The column was pre-washed with 5 column volumes of elution buffer (see below) followed by 10 column volumes of PBS. The sample of 5-10ml of infected cell protein extract (Section 5.2.4.) was loaded onto the column and when fully run into the matrix the flow was stopped and the sample left to equilibrate for 1 hour. The column was washed extensively using 50-100 column volumes of PBS overnight (18-20 hours). It was then washed successively with 5 column volumes each of the following buffers at 15ml/hour : i) 25mM Tris.HCl pH8.0, 50mM NaCl, 0.5% (v/v) NP40, 0.5% (w/v) sodium deoxycholate, ii) 50mM Tris,HCl pH6.8, iii) 9M ethanediol, 0.34M NaCl, 3.75mM sodium phosphate pH7.4. The glycoprotein was eluted in 5 column volumes of an elution buffer of 9M ethanediol, 0.3M NaCl, 0.05M citric acid pH3.0. The eluate was continuously neutralized to pH 7.5 by addition of 1M Tris.HCl pH9.6. The eluate was then extensively dialysed against 2mM sodium phosphate pH7.4, then lyophilized and reconstituted in a small volume of ddH2O. The typical yield from 5x10^7 infected cell was 0.5-1.0mg.

5.2.7.) Lymphocyte proliferation assay.

5.2.7.1.) Priming.

Immune cells were obtained from mice injected subcutaneously with antigen and adjuvant in the hind footpads and the base of the tail. Antigens were mixed vigorously with an equal volume of Freund's Complete Adjuvant (FCA) until a thick white emulsion was formed. As indicated in some experiments Alhydrogel (aluminium hydroxide colloid suspension; a gift from Dr.R.Bomford, Wellcome Biotech) was used as the adjuvant. In these cases
antigen was precipitated with an equal volume of 2mg/ml Alhydrogel for 1 hour on ice before use.

Immune cells were obtained by injecting mice with 20μg of antigen or 10^6 PFU HI HSV-2 in adjuvant, or with PBS plus adjuvant as control.

5.2.7.2.) Preparation of draining lymph node cells.

The draining lymph nodes (DLN) prepared from mice immunized in the hind footpads and the base of the tail were the popliteal, inguinal and the para-aortic. Immune mice were sacrificed and the lymph nodes removed by dissection and placed in ice cold RPMI medium. The lymph nodes from all individuals in a group were treated together and homogenized carefully with a syringe plunger through a sterile tea strainer, sterilized by boiling, into the RPMI. The single cell suspension was recovered by centrifugation at 1500rpm for 5 minutes, in a refrigerated bench top centrifuge. The cells were washed and counted and finally resuspended at an appropriate cell density in Click's medium + 0.5% NMS + penicillin (60μg/ml) and streptomycin (100μg/ml) + glutamine (2mM) + 2-mercapto-ethanol (0.1mM).

5.2.7.3.) Plating out of DLN cells.

Cells were dispensed at 4x10^5 viable cells per well in 100μl of medium into a 96 well flat bottomed tissue culture plate (LinBro). Antigen was added in a further 100μl of medium. Each stimulating antigen was plated in triplicate with both antigen primed cells and negative control cells, and one set of wells for each cell type received medium only to act as a control for unstimulated proliferation. The cells were then incubated at 37°C in humidified air + 5% carbon dioxide for 4 days.
5.2.7.4.) Measurement of $^3$H-thymidine uptake.

a) Pulsing. After 4 days incubation proliferation of the cells was measured by following their incorporation of $^3$H-thymidine, on the basis that proliferating cells are dividing and therefore synthesizing DNA. $20\mu l$ of supplemented Click's medium containing 1/20 diluted $^3$H-thymidine (40-60Ci/mmol; Amersham) was added to each well and incubated for a further 4 hours.

b) Harvesting. After the pulse the cells were harvested onto glass fibre filter paper and washed extensively for 90 seconds in a continuous flow of ddH$_2$O using a Dynatec 2000 cell harvester and dried.

c) Counting. The dried filter discs containing cells from individual wells were transferred to separate vials and 0.5ml of Optiscint "HiSafe" (LKB) scintillant was added. The vials were capped and the contents of each counted on a Beckman multichannel scintillation counter.

5.2.7.5.) Calculation.

For each set of counts in triplicate a mean plus or minus standard error was calculated. These values were then used to calculate the stimulation index of an antigen for a particular set of primed cells. The calculation compared directly proliferation of primed cells in the presence or absence of specific antigen stimulation. The specificity of stimulation was determined by comparing the stimulation index (S.I.) of control primed cells (saline or non-HSV related antigen). Further control of specificity is determined from the S.I. of cells primed with non-HSV related antigen and stimulated with the antigen in question.
Stimulation Index (SI) = \frac{\text{mean cpm (primed cells + Ag)}}{\text{mean cpm (primed cells - Ag)}}

In the case of unstimulated cells there was a basal level of $^3$H-thymidine incorporation at between 1000-2000 cpm. All results illustrated fell into this category.

5.2.8.) Delayed-type hypersensitivity assay.

DTH responses to HSV-1 have been studied in mice using infectious virus inoculated into the ear pinna (Nash et al., 1980). In this study infectious virus was not used as either the priming or eliciting inoculum. Therefore DTH was investigated by inoculation of the footpad, a method that has been successfully used to examine DTH responses to non-infectious components of influenza virus (Liew and Russel, 1982) and reovirus (Greene and Weiner, 1980).

5.2.8.1.) Priming (inducing).

Priming for DTH response was carried out by intradermal (i.d.) injection of the mice at two points on the flank with a total of 20 µg of antigen or HI purified HSV-2 per mouse. This was carried out under anaesthesia and the area of skin to be injected being plucked so that it was free of fur. In all experiments four 10 week old BALB/c female mice were used per group. Negative control mice were primed with PBS or a non- HSV related antigen (e.g. GZ)

5.2.8.2.) Eliciting.

This was performed by s.c. injection of the eliciting antigen diluted in 50 µl PBS into the right hind footpad, and as an internal control s.c. injection of 50 µl PBS into the left hind footpad. The antigen dose used was 10 µg/footpad of...
protein antigen or $2 \times 10^5$ PFU HI HSV-2.

5.2.8.3.) Measurement of footpad swelling.

The animal was held at the tail by the little finger and by the fore finger and thumb just below the nape of the neck so that it involuntarily spreads its hind limbs. In this position its hind limb movement was restricted and its digits spread wide open. A reverse spring caliper gauge was used to measure the thickness of the middle of the footpad. The gauge was allowed to settle for 10 seconds before the reading was taken. Right and left hind footpads were measured for each individual. Readings were taken to the nearest 0.5 of a graduation equivalent to 0.05mm.

5.2.8.4.) Calculation.

The values for footpad swelling or DTH response recorded are based on difference between right and left hind footpad thickness per mouse. The results of footpad swelling represent the mean value per group +/- the standard error. Significance was determined using Student’s t test.

5.2.8.5.) The use of cyclophosphamide.

Cyclophosphamide has been shown to significantly enhance the level of swelling observed in DTH response to components of influenza virus (Liew and Howard, 1980; Liew and Russel, 1980). It is thought that suppressor cells of DTH are more sensitive to cyclophosphamide toxicity than T-DTH lymphocytes (Liew, 1985). In the work reported here, where indicated, mice were treated with 500mg/kg body weight of cyclophosphamide by i.p. injection of 0.5ml of an appropriate aqueous dilution, 48 hours prior to priming with specific antigens.
5.2.9.) Protection studies.

5.2.9.1.) Priming.

Following discussion with Dr. Chan and Dr. R. Bomford, an immunisation regime was determined to give repeated exposure to antigen, in the presence of adjuvant, in order to maximise the potential for a strong immune response. Five week old female BALB/c mice were injected s.c. with a total of 20μg of antigen in FCA adjuvant at two site on the flank. They were subsequently boosted twice with 10μg of antigen (no adjuvant) in the flank at two weekly intervals.

5.2.9.2.) Challenge.

The immunized mice were challenged with a lethal dose containing 10x LD₅₀ of infectious virus into the hind footpad, 4-5 days after the last boost. Careful note was made to use the same batch of virus, and strain, sex and age of the mice at the time of challenge as those used to determine the LD₅₀. The virus dose used for challenge infection was determined in an LD₅₀ experiment. Briefly, 10 week old BALB/c female mice in groups of 5 were infected with a starting dose of 2x10⁶ PFU HSV-2 BRY in 20μl per mouse in the hind footpad, through four 5-fold dilutions. Disease onset and spread was monitored daily for 3 weeks and deaths recorded. The spread of infection to the sacral ganglia and lower spinal column was noted by the limb paralysis with a subsequent spread through the CNS or systemically, leading to death.

In this protection experiment 11 mice were used for each immunization group.
5.2.9.3.) Calculation.

For a particular dose of virus (X) a cumulative total of the mice killed (D) by a dose less than or equal to X, and a cumulative total of the mice surviving (S) a dose greater than or equal to X were calculated. The mortality ratio equal to D/(S+D) was calculated then expressed as a percentage. This is illustrated in the calculation shown in Table 5.1. (Section 5.3.3.1). The % mortality obtained for a number of doses were plotted and the graph used to determine the dose lethal to 50% of animals challenged, designated as the LD$_{50}$.

The results of the protection experiment were analysed using $2 \times 2$ contingency table by the $X^2$ test (However, n<20 so this is not a very accurate test). Since it was of interest to have values of significance within the time scale of the experiment, life tables were calculated for specific experimental and control groups to allow longitudinal analysis of the data (Colton, 1974).

5.2.10.) Preparation of $^{125}$I-rabbit anti-mouse immunoglobulin.

Rabbit anti-mouse immunoglobulin (RaMIg) was iodinated with $^{125}$I by the chloramine T method. The radio-iodinated protein was separated from unincorporated label by chromatography on a 5ml Pharmacia prepacked Sephadex G50 column in PBS buffer and collected. % coupled radio-iodine was determined by precipitation of labelled protein with 10% TCA and 10% serum protein (as carrier) for 30 minutes on ice. Counts per minute of radioactivity in the pellet (incorporated) and supernatant (unincorporated) were determined in a gamma counter.
5.2.11.) Radio-immunoassay (RIA).

A predetermined optimum concentration of viral antigen (infected cell sonicate) at 50μl/well was incubated in the wells of a 96 well flexible plastic plate, kept at 4°C overnight in a humidified sandwich box. The wells were washed out twice with 200μl/well PBS and incubated at 37°C for 1 hour with 200μl/well of a blocking solution of 10% DSM in PBS. The wells were washed twice with 200μl/well PBS before 100μl/well of the test or control preimmune sera were added, at an appropriate dilution in 10% DSM in PBS, and incubated at 37°C for 1 hour. The wells were washed twice with 200μl of 0.05% Tween 20 in PBS and three times with PBS alone. The 100μl/well of the second layer antibody 125I-rabbit anti-mouse immunoglobulin, diluted in 10% DSM in PBS to 10⁶ cpm/ml, was added. Care was taken when adding the radio-labelled antibody to minimize aerosol and contact of contaminated tips with the plate. After incubation at 37°C for 1 hour the wells were washed three times with each of 0.05% Tween 20 in PBS and PBS alone. The upper surfaces of the plate outside the wells were also washed extensively to remove any contamination. The plates were dried and the wells cut out and counted in a Gamma counter. All samples were tested in triplicate and the result expressed as a mean plus or minus standard error. The optimum concentration of virus antigen per well was determined by titration against a concentration of positive control (anti-HSV-2) serum. In subsequent assays the determined concentration of antigen was used, and test sera titred against it.
5.3.) RESULTS.

The immune response to the recombinant antigens, gB-2 and HSV-2 in mice was examined using three assays. The first involved in vitro stimulation of primed lymphocyte proliferation. The second involved priming and eliciting DTH responses (in vivo). The third examined the ability of mice immunised with HSV-2, gB-2 or the recombinant antigens to survive a challenge with a lethal dose of HSV-2. In addition anti-HSV-2 antibody levels in the serum of mice was assayed in the latter experiment prior to challenge.

5.3.1.) Lymphocyte proliferation assay.

5.3.1.1) Timecourse.

In order to establish the assay, a timecourse of response to HSV-2 and gB-2 at various times post priming was performed. As shown in Figure 5.1, the proliferation of HSV-2 primed lymphocytes to in vitro stimulation by the whole virus was already at a maximum value at day six post priming. The response to the whole virus then declined gradually towards day twenty four post priming. However, the response of these same immune lymphocytes to gB-2 stimulation in vitro increased from day six to a maximum around day twelve, slowly declining towards day twenty four, but much less marked than the decline in the response to the whole virus. The peak of response to gB-2 stimulation was approximately four times less than to the whole virus. Stimulation with gB-2 should always be lower than HSV-2, as HSV-2 is a "mixture" of viral proteins which will recruit a wide range of HSV-2 immune memory T cells. The response to higher doses of gB-2 was determined in a subsequent experiment. Twelve days post immunization was subsequently
used as the time point for studying the proliferative T lymphocyte response to gB-2 and to the fusion proteins containing fragments of gB-2.

![Graph](image)

**Figure 5.1.** Timecourse of the response of HSV-2 primed lymph node cells to in vitro stimulation with HSV-2 or gB-2. At various times post priming, DLN cells from mice immunized with $10^6$ HI PFU of HSV-2 in FCA were tested for their ability to proliferate in vitro in response to HI HSV-2 or immunopurified gB-2 stimulation. $4\times10^5$ cells/well were set up in triplicate. Stimulation index values were calculated from $^3$H-thymidine incorporation (see Section 5.2.9.). Stimulating antigens used were HSV-2 infected cell sonicate diluted 1:200 in medium (equivalent to $10^5$ HI PFU/well) and 1μg/well of immunopurified gB-2. All cultures were set up in triplicate.
5.3.1.2. Response to recombinant antigens.

The use of anti-HSV-2 sera to select for gB-specific recombinant antigens indicated that they all contained B cell determinants. However T and B cell determinants are not, in general, the same. The former are determined by primary structure while the latter often depend on tertiary structure. Therefore the recombinant antigens were examined for T cell immune reactivity, both as antigens and/or immunogens. In this initial experiment their antigenicity was tested by in vitro stimulation of HSV-2 strain 333 primed DLN cells with recombinant antigens, purified by enzyme affinity chromatography.

As shown in Figure 5.2 for each of the recombinant antigens used, DLN cells from mice primed with the higher dose of virus proliferated more than those from mice primed with the lower dose of virus. This higher dose was used on all subsequent occasions to induce populations of HSV-2 reactive lymphocytes for proliferation assay. It was also clear that for the same dose of antigen, used for in vitro stimulation, DLN cells from the same mice were stimulated to proliferate to different extents by Ag59, Ag65 and Ag71. Ag59 showed the highest degree of stimulation at both priming doses whereas Ag71 only showed a significant stimulation of DLN cells from mice primed with the higher dose. Ag65 showed a good stimulation of immune DLN cells at both doses.
Figure 5.2. Response of HSV-2 strain 333 primed lymph node cells to gB-2 or enzyme affinity purified recombinant antigens. Mice were primed with $10^6$ HI PFU (high) or $10^5$ HI PFU (low) of HSV-2 strain 333. 4x10^5 DLN cells were stimulated with $10^5$ HI PFU of HSV-2 BRY, 10µg of immunopurified gB-2 or 10µg of enzyme affinity purified recombinant antigen. All cultures were set up in triplicate.
5.3.1.3.) Effect of dose of antigen on in vitro T lymphocyte proliferation response.

HSV-2, gB-2 and the recombinant antigens purified by anti-β-gal immuno-affinity chromatography were next examined to see if they stimulated HSV-2 primed DLN cells to proliferate in a dose dependent manner (Figure 5.3). As shown in Figure 5.3a) the proliferation response of HSV-2 primed DLN cells to HI purified HSV-2 (pHSV-2) stimulation increased with antigen concentration. The range of antigen concentration used (0.02µg/well to 50µg/well) was not high enough to tolerize T cells, thereby reducing their proliferation. The values of stimulation indices for HSV-2 were much higher than those in the previous two experiments. The major difference was the use of purified virus at the stage of in vitro stimulation.

Figure 5.3. Response of HSV-2 primed DLN cells to varying doses of purified antigens. Mice were primed with $10^6$ PFU HI HSV-2 strain BRY in FCA or PBS in FCA as control s.c. in the hind footpad. At day 12 $4\times10^5$ DLN cells were stimulated with a) 0.02, 0.2, 2.0, 10 and 50µg of HI purified HSV-2 BRY, or with 0.2, 2.0, 10 and 50µg of immunopurified b) gB-2, c) Ag59, d) Ag65, e) Ag71 or f) Ag461. The recombinant antigens used were purified by BG79 immunoaffinity chromatography. All cultures were set up in triplicate. Control primed cells stimulated by each antigen are indicated by closed symbols.
The response of the HSV-2 primed DLN cells to the recombinant gB-2 fragments and native gB-2 (Figure 5.3b) showed a similar trend to that of the response to pHSV-2, though the S.I. values were all much lower; the maximum values for S.I. here being 9-11 compared to 68. Both gB-2 and Ag65 increased to a maximum value at 10 and 50μg/well respectively, but without signs of plateauing. For Ag59 the stimulation index reached a peak at 10μg/well and declined at 50μg/well. In this experiment there was no response to Ag71. Stimulation as with Ag461 remained below the SI value of 2 which is not considered significant, except at the highest dose (50μg/well) when Ag461 also rose slightly above 2.0. On a number of repeated occasions the original enzyme affinity purified batch and the immuno-affinity purified batch of Ag71 failed to stimulate proliferation of HSV-2 strain BRY primed lymphocytes.

5.3.1.4.) Immunogenicity of the Recombinant Antigens.

Since the recombinant antigens Ag59 and Ag65 could stimulate HSV-2 primed lymphocytes to proliferate in vitro the possibility that they might prime lymphocytes for a response to HSV-2 was examined. The results in Figure 5.4 showed that HSV-2 primed for proliferative T cell responses to HSV-2 viral antigens and gB-2 while control Ag461 (GZ) primed for a response only to itself. Ag59 primed for a proliferative response not only to Ag461 but also to HSV-2 viral antigens and to gB-2. Ag65 primed for a response to Ag461 but not, however, for a response to HSV-2 viral antigens or gB-2. Values for PBS primed cells responding to stimulation with each of the test antigens were less than an S.I. of 2.0 (as were values for all the antigen primed cells cultured in the absence of antigen stimulation).
Figure 5.4. Response of lymph node cells primed with different antigens to in vitro stimulation with HSV-2 strain BRY and gB-2. Groups of mice were primed s.c. in the hind footpad with antigens Ag59, Ag65, Ag461 and HI pHSV-2 at 20μg/mouse in 0.1mg of Alhydrogel. The groups were boosted with 10μg/mouse of the appropriate antigen 7 days later and assayed 5 days after the boost. 4x10⁵ DLN cells per well were stimulated with 10μg of immunopurified Ag461 (GZ), ovalbumin, HI HSV-2 BRY purified antigen or immunopurified gB-2. All cultures were set up in triplicate.
Similar results, which are shown in Figure 5.5, were obtained when HSV-2 strain 333 was used as a stimulating antigen to cells primed with the recombinant gB-2 antigens. The batch of gB-2 used in this experiment failed to stimulate any cells; values are therefore not included. Positive control values for HSV-2 strain 333 stimulating HSV-2 333 primed cells were high (SI of 60), as were those for ovalbumin stimulating ovalbumin primed cells (SI of 30). Neither Ag461 nor Ag65 primed for an in vitro response. Ag59, however, primed for a response to HSV-2 333 with an SI of 5.9.

![Figure 5.5](image)

**Figure 5.5.** Response of DLN cells primed s.c. in the hind footpad with 20μg/mouse of HSV-2 strain 333, antigen Ag 59 or Ag 65 or ovalbumin (Ova) in 0.1mg of Alhydrogel. The groups were boosted with 10μg/mouse of the appropriate antigen 7 days later and assayed 5 days after the boost. 4x10^5 DLN cells per well were stimulated with media alone (-Ag), 10μg of ovalbumin, or 10μg of HSV-2 strain 333 viral antigen. All cultures were set up in triplicate.
5.3.2.) Delayed-type hypersensitivity (DTH) response.

5.3.2.1.) Timecourse of response to HSV-2 antigens and the effect of cyclophosphamide treatment.

Cyclophosphamide pretreatment has been shown to drastically reduce suppressor T cell precursors (Howard and Shand, 1979; Shand and Liew, 1980) and hence bring about a significant increase in DTH response. Two groups of mice,  

![Footpad swelling (mm x 10^-2)](image)

**Figure 5.6.** Timecourse of the DTH response in the right hind footpad of mice primed with HSV-2 antigens from time of eliciting. 48 hrs before antigen priming the mice were divided into two groups; one treated with cyclophosphamide (150mg/kg each individual), the other untreated. All mice were primed with 20μg each of HI density gradient purified HSV-2 BRY by i.d. injection at two sites on the flank. The response was elicited in four mice from each group at day 7 post priming by s.c. injection of 2x10^5 PFU UVI of HSV-2 strain BRY into the centre of the right hind footpad. In each instance swelling was measured at 4, 8, 24, 32, 48, 72 and 96 hrs post eliciting.
one group pretreated with 150μg/g of cyclophosphamide, were
immunised with HSV-2 antigen and the subsequent DTH response
was measured using footpad swelling. Antigen challenge was
performed at various times post priming, and for each time
point of antigen challenge measurements of footpad swelling
were taken at 4, 8, 24, 32, 48, 72 and 96 hours post
eliciting. The maximum value in each case was around 24hrs
(illustrated in Figure 5.6) with a decline in the swelling
over the subsequent 24hrs and an almost complete resolution
of the swelling by 72hrs post-eliciting. Measurements were
taken on animals elicited 5, 7, 9 and 12 days post priming.
A representative example, day 7, is shown in Figure 5.6.

![Graph](image)

Figure 5.7. Timecourse of the footpad DTH response to
HSV-2 antigens in mice primed with HSV-2 antigens, and
the effect of cyclophosphamide on that response.
Summarising the results at 24 hrs post eliciting at day
5, day 7 (Fig.5.6), day 9 and day 12 post priming.
The results (Figure 5.7) of 24 hours footpad swelling showed that at day 5 there was already a clearly measurable DTH response to HSV-2. The response increased from day 7 to day 9, which gave the peak value, and by day 12 post priming the response was beginning to decline. Throughout the groups of mice and at all time points it was observed that pretreatment of the mice with cyclophosphamide caused a significant increase in the response, which was on average 34% greater.

5.3.2.2.) Priming with recombinant antigens.

![Graph showing footpad DTH response to HSV-2 viral antigens in cyclophosphamide pretreated mice primed with various antigens. Mice pretreated with cyclophosphamide (150μg/g) were primed by i.d. injection at two sites on the flank with 20μg HI density gradient purified HSV-2 BRY or 20μg immunopurified gB-2 or 20μg immunopurified recombinant antigen (Ag59, Ag65 or Ag461). In all mice the response was elicited by s.c. injection in the right hind footpad with 2x10^5 PFU UVI HSV-2 BRY 7 days post priming.](image)

Figure 5.8. Footpad DTH response to HSV-2 viral antigens in cyclophosphamide pretreated mice primed with various antigens. Mice pretreated with cyclophosphamide (150μg/g) were primed by i.d. injection at two sites on the flank with 20μg HI density gradient purified HSV-2 BRY or 20μg immunopurified gB-2 or 20μg immunopurified recombinant antigen (Ag59, Ag65 or Ag461). In all mice the response was elicited by s.c. injection in the right hind footpad with 2x10^5 PFU UVI HSV-2 BRY 7 days post priming.
As shown in Figure 5.8 the DTH response induced and elicited by HSV-2 produced a maximum swelling of around 1.2mm at 24 hours post antigen challenge. The DTH response to HSV-2 in mice primed with gB-2 was good with a maximum value at 24 hours around 0.9mm. Priming with either Ag59 or Ag65 also induced a DTH response to HSV-2 of approximately 0.4mm which was significantly higher than 0.15mm produced in mice primed with the control Ag461 (p<0.001).

5.3.2.3.) Effect of cyclophosphamide on the induction of DTH response to HSV-2 by the recombinant antigens.

The recombinant antigens were tested to see whether the DTH response induced by each of them was influenced by suppression. To do this parallel groups of mice that had or had not been pretreated with cyclophosphamide were set up for each antigen. Two additional groups, involving copriming with Ag59 and Ag65, were also set up to see if the low level of DTH induced by each contributed in an additive manner to the higher DTH response induced by gB-2.

The results (in Figure 5.9) showed once again the significant effect of cyclophosphamide treatment on the DTH response to the whole virus, with a 44% higher response (0.9mm with against 0.5mm without). On this occasion the response to priming with gB-2 was not as strong as it had been in the previous experiment (only 0.27mm here against 0.90mm previously), and in the absence of cyclophosphamide pretreatment there was only a very weak response (0.10mm). There was a small response induced by the recombinant antigens Ag59 and Ag65 in mice pretreated with cyclophosphamide (0.20mm and 0.17mm respectively). In the absence of such pretreatment there was little change in the response induced by Ag59 (0.22mm) and virtually no DTH response was induced by Ag65 (0.02mm). The effect of
copriming with Ag59 and Ag65 in the presence of cyclophosphamide did not affect the response significantly (0.25mm compared with 0.20mm for Ag59, p>0.1, and 0.17mm for Ag65, p>0.05). In the coprimed group without cyclophosphamide pretreatment, the response was noticeably reduced (0.07mm). There was no detectable response to Ag461 in either group (0.02mm and -0.04mm).

Figure 5.9. 24 hrs footpad DTH response to HSV-2 antigens in mice primed with various antigens: the effect of cyclophosphamide pretreatment. 48 hrs before priming one group of mice was treated with cyclophosphamide (150μg/g), the other remained untreated. Both groups were primed as described in Fig.5.8 with an additional pair of groups primed with 20μg of each of Ag59 and Ag65. Response in all groups was elicited with 2x10^5 PFU UVI HSV-2 BRY on day 7 and measured at 24hrs.
5.3.2.4.) Ability of the recombinant antigens to induce or elicit a virus specific DTH response in cyclophosphamide pretreated mice.

In this experiment all mice were pretreated with cyclophosphamide to increase any detectable responses. Thirteen groups of mice were prepared, the first primed with HI HSV-2 infected cell sonicate and elicited with the same antigen, the remainder were two parallel sets of six groups, one set primed with antigen (HI purified HSV-2, gB-2, Ag59, Ag65, Ag71 and Ag461) and elicited with HSV-2, the other set primed with HSV-2 and elicited antigen.

It was found that there was little difference in the DTH response to HSV-2 whether induced or elicited by the respective antigens (Figure 5.10). In all cases the response was slightly higher when elicited with antigen, with the exception of gB-2 where a slightly higher response was induced by priming with the antigen. Ag71 was included in this assay and was found to give a response as good as Ag65.
Figure 5.10. Effect of priming or eliciting with various antigens on the DTH response as measured by 24 hrs footpad swelling, in cyclophosphamide pretreated mice. Mice pretreated with cyclophosphamide (150µg/g) were primed with 20µg HI pHSV-2 BRY or 4x10^5 PFU HI HSV-2 BRY or 20µg immunopurified gB-2 or 20µg immunopurified recombinant antigen. DTH response was elicited seven days later by injection of 2x10^5 PFU UVI HSV-2 BRY or 10µg UVI pHSV-2 BRY or 10µg immunopurified gB-2 or 10µg immunopurified recombinant antigen into the right hind footpad, and swelling measured at 24 hrs post eliciting.
5.3.2.5.) Effect of various combinations of copriming on the DTH response to HSV-2.

The observation that Ag65 lost detectable DTH response in the absence of cyclophosphamide, whereas Ag59 did not, and that when coprimed Ag59 and Ag65 also lost detectable DTH response in the absence of cyclophosphamide (Figure 5.9), lead to the consideration of whether a similar response would be observed when Ag65 was coprimed with HSV-2. To answer this question a number of groups of mice were set up, without cyclophosphamide pretreatment treatment, as follows:

<table>
<thead>
<tr>
<th>Priming</th>
<th>Eliciting</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. pHSV-2</td>
<td>HSV-2</td>
</tr>
<tr>
<td>2. Ag</td>
<td>Ag</td>
</tr>
<tr>
<td>3. Ag</td>
<td>HSV-2</td>
</tr>
<tr>
<td>4. Ag/pHSV-2</td>
<td>HSV-2</td>
</tr>
<tr>
<td>5. PBS</td>
<td>Ag</td>
</tr>
<tr>
<td>6. PBS</td>
<td>HSV-2</td>
</tr>
</tbody>
</table>

Groups two to six were repeated for Ag59, Ag65, Ag71 and Ag461. In the case of copriming, 20μg of each antigen were used per mouse (i.e. 40μg mixed antigen per mouse).

The results (in Figure 5.11) showed that copriming of any of the immunopurified antigens with pHSV-2 caused a drastic reduction of the DTH response to HSV-2 compared to that primed by pHSV-2 alone (values on average only 30% of the maximum) comparing bar one with bar four on each chart. The bar two on each chart showed that only Ag59 could prime and elicit a clear response to itself (0.27mm), while antigens Ag65, Ag71 and Ag461 induced insignificant responses, in the range 0.04-0.10mm. Bar three showed again
that Ag59 could prime a clear, though small response to HSV-2 (0.25mm), while antigens Ag65, Ag71, and Ag461 induced responses of only 0.01-0.06mm. Bar five and bar six provided negative controls to show that these antigens or HSV-2 did not elicit a response in unprimed mice, values having been in the range 0.04-0.05mm, giving the baseline for the experiment.

Figure 5.11. Effect of various combinations of priming and eliciting antigens on the 24 hrs footpad DTH response in mice not treated with cyclophosphamide. For each of the four recombinant antigens a)Ag59, b)Ag65, c)Ag71 and d)Ag461, groups of mice were prepared which were primed with 20μg HI density gradient purified HSV-2 BRY or 20μg immunopurified recombinant antigen or a combination of 20μg of each or PBS. After 7 days a swelling response was elicited by injection of 2×10^5 PFU UVI HSV-2 BRY or 10μg of recombinant antigen or a combination of both.

P.T.O.
Effect of various combinations of priming and eliciting antigens on 24 hour footpad swelling response

<table>
<thead>
<tr>
<th>Priming</th>
<th>Antigen</th>
<th>Eliciting</th>
<th>Footpad swelling (mm x 10^{-2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. pHSV-2</td>
<td>HSV-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Ag 59</td>
<td>Ag 59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Ag 59</td>
<td>HSV-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Ag 59/pHSV-2</td>
<td>HSV-2</td>
<td></td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
<td>2. Ag 65</td>
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<tr>
<td>3. Ag 65</td>
<td>HSV-2</td>
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</tr>
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<td>Ag 71</td>
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</tr>
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<td>HSV-2</td>
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</tr>
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</tr>
<tr>
<td>6. PBS</td>
<td>HSV-2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.3.3.) Protection of mice against lethal challenge with HSV-2 strain BRY.

5.3.3.1.) Determination of LD\textsubscript{50}.

The most important factors affecting the value of the dose of virus lethal to the mice challenged are strain, age, sex and route of infection. The LD\textsubscript{50} for 10 week old BALB/c female mice of HSV-2 strain BRY, was determined following subcutaneous challenge in the hind footpad.

Infected animals showed two forms of disease progression. In the first case all animals appeared healthy until day 6-7 post infection (p.i.) when some began to show signs of restriction of hind limb movement. This progressed within the next 4-5 days (days 10-12 p.i.) to total paralysis in one or both hind limbs, open lesions visible on the skin of the hind limbs, a general malaise and finally death. No animals that displayed these symptoms recovered. In the other case some animals would appear healthy until day 6-7 p.i. when they would show signs of general malaise such as a lack of grooming, unsteadiness and slowness of movement, a hunched gait, but no sign of hind limb paralysis. Animals showing these symptoms also never recovered, dying within 3-4 days of the first signs.

The results are shown in Table 5.1 and Figure 5.12. From the graph the LD\textsubscript{50} for HSV-2 BRY in 10 week old BALB/c female mice was given the value $1.25 \times 10^4$ PFU/mouse, by subcutaneous injection.
### Table 5.1

<table>
<thead>
<tr>
<th>Virus dose (PFU/mouse)</th>
<th>In each group</th>
<th>Cumulative total for this dose</th>
<th>Mortality Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dead</td>
<td>Survivors</td>
<td>Dead</td>
</tr>
<tr>
<td>2x10^6</td>
<td>5</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>4x10^5</td>
<td>5</td>
<td>0</td>
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</tr>
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</tr>
<tr>
<td>3.2x10^3</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

**Figure 5.12.** Estimation of the LD$_{50}$ for HSV-2 BRY in 10 weeks old BALB/c female mice. Five groups each of five mice were infected by s.c. injection in both hind footpads with either 2x10$^6$, 4x10$^5$, 8x10$^4$, 1.6x10$^4$ or 3.2x10$^3$ PFU/mouse. Semi-logarithmic plot of the results summarized in Table 5.1 in order to determine a value for the dose of HSV-2 lethal to 50% of mice (of that age and sex). LD$_{50}$ = 1.25x10$^4$ PFU/mouse.
For use as a lethal challenge 10x LD50 i.e. 1.25x10^5 PFU/mouse was used. This should assure 100% mortality in unprotected mice of this strain, age and sex.

5.3.3.2.) Protection of mice against lethal challenge with HSV-2 by immunization with HSV-2, gB-2 or the recombinant antigens.

The results of T cell proliferation and DTH response had shown that the recombinant antigens were immunogenic and/or antigenic. It was therefore of interest to see whether or not the immune response that they induced was protective. Six groups of eleven mice were prepared. Three mice from each group were bled from the tail vein before the first infection. These mice were also tail bled 24 hours before challenge. They were kept separately in case the stress of bleeding caused a difference in response.

The tail bled mice seemed to behave in accordance with unbled mice receiving the same priming. Their results were therefore included with the others, shown in Figure 5.13. Immunization with purified HI HSV-2 antigen conferred complete protection against the lethal dose of infectious virus (no deaths or malaise). Immunization with gB-2 also conferred a high degree of protection, only one individual succumbing to infection at day 18. Animals primed with Ag461, Ag71 and Ag65 died between days 9 and 18, except for two individuals (one from the Ag461 group and one from the Ag71 group) which showed no signs of infection and survived the term of the experiment. Of the mice primed with Ag59, four individuals survived the term of the experiment equivalent to 36% protection; however statistical analysis of this result (observed vs expected) using X^2 for the significance of difference from the the Ag461 primed group gave a value X^2=2.33, which is not significant (P>0.1). Of
the individuals in this group that died a number survived longer than the fatalities in the other groups. For this reason a life table was constructed for the Ag59 primed group and for the Ag461 primed control group to allow longitudinal statistical analysis of the significance of these extended survival times.

![Graph showing survival rates over time](image)

**Figure 5.13.** Active protection of mice by immunization with various antigens. For each antigen a group of 11 mice (BALB/c females) were injected at 5 weeks of age with 20μg emulsified in FCA/mouse (of HI density gradient purified HSV-2 BRY or immunopurified gB-2 or immunopurified recombinant antigen). Each group was boosted with an additional injection with 10μg of the appropriate antigen (without adjuvant) at 7 weeks of age and again at 9 weeks of age. At 10 weeks of age all mice were challenged with 10xLD$_{50}$ (1.25x10$^5$ PFU/mouse) of HSV-2 BRY. Mice were monitored daily for mortality and signs of infection until 40 days post challenge.
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Number Survivors/Total</th>
<th>Survival %</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-2</td>
<td>11/11</td>
<td>100</td>
</tr>
<tr>
<td>gB-2</td>
<td>10/11</td>
<td>91</td>
</tr>
<tr>
<td>Ag59</td>
<td>4/11</td>
<td>36</td>
</tr>
<tr>
<td>Ag65</td>
<td>1/11</td>
<td>9</td>
</tr>
<tr>
<td>Ag71</td>
<td>0/11</td>
<td>0</td>
</tr>
<tr>
<td>Ag461</td>
<td>1/11</td>
<td>9</td>
</tr>
</tbody>
</table>
Table 5.3.
Life table for mice in the group primed with Ag461.

<table>
<thead>
<tr>
<th>x</th>
<th>O_x</th>
<th>d_x</th>
<th>q_x</th>
<th>p_x</th>
<th>p_x</th>
<th>SE(p_x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-9</td>
<td>11</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>9-10</td>
<td>11</td>
<td>2</td>
<td>0.182</td>
<td>0.818</td>
<td>0.818</td>
<td>0.116</td>
</tr>
<tr>
<td>10-11</td>
<td>9</td>
<td>2</td>
<td>0.222</td>
<td>0.778</td>
<td>0.636</td>
<td>0.145</td>
</tr>
<tr>
<td>11-12</td>
<td>7</td>
<td>2</td>
<td>0.285</td>
<td>0.715</td>
<td>0.455</td>
<td>0.150</td>
</tr>
<tr>
<td>12-13</td>
<td>5</td>
<td>2</td>
<td>0.400</td>
<td>0.600</td>
<td>0.273</td>
<td>0.134</td>
</tr>
<tr>
<td>13-14</td>
<td>3</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.273</td>
<td>0.134</td>
</tr>
<tr>
<td>14-15</td>
<td>3</td>
<td>1</td>
<td>0.333</td>
<td>0.667</td>
<td>0.182</td>
<td>0.116</td>
</tr>
<tr>
<td>15-16</td>
<td>2</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.182</td>
<td>0.116</td>
</tr>
<tr>
<td>16-17</td>
<td>2</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.182</td>
<td>0.116</td>
</tr>
<tr>
<td>17-18</td>
<td>2</td>
<td>1</td>
<td>0.500</td>
<td>0.500</td>
<td>0.091</td>
<td>0.086</td>
</tr>
<tr>
<td>18-19</td>
<td>1</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.091</td>
<td>0.086</td>
</tr>
<tr>
<td>19-20</td>
<td>1</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.091</td>
<td>0.086</td>
</tr>
<tr>
<td>20-21</td>
<td>1</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.091</td>
<td>0.086</td>
</tr>
<tr>
<td>21-22</td>
<td>1</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.091</td>
<td>0.086</td>
</tr>
<tr>
<td>22-29</td>
<td>1</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.091</td>
<td>0.086</td>
</tr>
<tr>
<td>29-30</td>
<td>1</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.091</td>
<td>0.086</td>
</tr>
<tr>
<td>30-40</td>
<td>1</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.091</td>
<td>0.086</td>
</tr>
</tbody>
</table>

x - time interval (days)
O_x - animals observed during interval
d_x - animals dying during interval
q_x - chance of dying during interval (d_x/O_x)
p_x - chance of surviving interval (1-q_x)
P_x - cumulative chance of surviving interval (P_xP_x-1)
SE(P_x) - Standard error of P_x (P_x/Σ[q_x/O_x-d_x])
Table 5.4.

Life table for mice in the group primed with Ag59.

<table>
<thead>
<tr>
<th>x</th>
<th>O_x</th>
<th>d_x</th>
<th>q_x</th>
<th>p_x</th>
<th>P_x</th>
<th>SE(P_x)</th>
<th>* z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-9</td>
<td>11</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.50</td>
</tr>
<tr>
<td>9-10</td>
<td>11</td>
<td>0</td>
<td>0.0</td>
<td>0.990</td>
<td>0.990</td>
<td>0.094</td>
<td>1.16</td>
<td>0.123</td>
</tr>
<tr>
<td>10-11</td>
<td>10</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.990</td>
<td>0.094</td>
<td>2.06</td>
<td>0.020</td>
</tr>
<tr>
<td>11-12</td>
<td>10</td>
<td>1</td>
<td>0.100</td>
<td>0.900</td>
<td>0.891</td>
<td>0.126</td>
<td>2.31</td>
<td>0.011</td>
</tr>
<tr>
<td>12-13</td>
<td>9</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.891</td>
<td>0.126</td>
<td>3.36</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>13-14</td>
<td>9</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.891</td>
<td>0.126</td>
<td>3.36</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>14-15</td>
<td>9</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.891</td>
<td>0.126</td>
<td>4.18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>15-16</td>
<td>9</td>
<td>1</td>
<td>0.111</td>
<td>0.889</td>
<td>0.793</td>
<td>0.146</td>
<td>3.27</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>16-17</td>
<td>8</td>
<td>1</td>
<td>0.125</td>
<td>0.875</td>
<td>0.694</td>
<td>0.158</td>
<td>2.61</td>
<td>0.005</td>
</tr>
<tr>
<td>17-18</td>
<td>8</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.694</td>
<td>0.158</td>
<td>3.34</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>18-19</td>
<td>8</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.694</td>
<td>0.158</td>
<td>3.34</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>19-20</td>
<td>8</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.694</td>
<td>0.158</td>
<td>3.34</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>20-21</td>
<td>8</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.694</td>
<td>0.158</td>
<td>3.34</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>21-22</td>
<td>7</td>
<td>1</td>
<td>0.143</td>
<td>0.875</td>
<td>0.595</td>
<td>0.164</td>
<td>2.72</td>
<td>0.003</td>
</tr>
<tr>
<td>22-29</td>
<td>6</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.595</td>
<td>0.164</td>
<td>2.72</td>
<td>0.003</td>
</tr>
<tr>
<td>29-30</td>
<td>6</td>
<td>2</td>
<td>0.333</td>
<td>0.667</td>
<td>0.396</td>
<td>0.158</td>
<td>1.69</td>
<td>0.046</td>
</tr>
<tr>
<td>30-40</td>
<td>4</td>
<td>0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.396</td>
<td>0.158</td>
<td>1.69</td>
<td>0.046</td>
</tr>
</tbody>
</table>

* Comparison of significance of the difference between life table 5.3. and life table 5.4.

\[ z = \frac{P_x - P'_x}{\sqrt{SE(P_x)^2 + SE(P'_x)^2}} \]

P - significance (probability).
5.3.3.3.) Determination of anti-HSV antibody in the serum of mice in the challenge experiment.

a) Solid phase radioimmunoassay (RIA) for anti-HSV antibody.

A solid phase RIA was developed to detect anti-HSV serum antibody from small samples of blood taken from a number of mice in the challenge experiment. The results in Figure 5.14 showed the dilution series of HSV-2 antigen against a 1:100 dilution of anti-HSV-2 antiserum to optimize the concentration of plate bound antigen.

![Graph](image)

**Figure 5.14.** Optimization of RIA of HSV-2 antigen on solid phase (adsorbed live virus) to detect anti-HSV-2 antibodies. Results of a titration of the amount of HSV-2 antigen bound to a solid phase followed by the specific immuno-binding of a 1:100 dilution of a known polyclonal anti-HSV-2 antiserum and detection by binding of $^{125}$I-rabbit anti-mouse antibody. Control is 1:100 dilution of normal mouse serum.
b) Assay of serum from mice in challenge experiment.

As expected, the pre-immune sera from mice in all groups showed no detectable anti-HSV-2 antibody titre, all giving titres <20 (results summarized in Table 5.5). The pre-challenge sera (following three immunizations with antigen) from HSV-2 immunized mice showed a very high titre (>2560) and from gB-2 immunized mice also showed a very good titre (320). The pre-challenge sera from mice immunized with either Ag461 or Ag71 showed no detectable anti-HSV-2 antibody titre (both giving <20), however the sera from mice immunized with Ag65 showed a rather low but detectable titre of 40. The titre of sera from mice immunized with Ag59 was only 20 and cannot be considered significant. These results were detecting antibody capable of binding to the native structure of the HSV-2 virion.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Anti-HSV-2 antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-immune</td>
</tr>
<tr>
<td>HSV-2</td>
<td>&lt;20</td>
</tr>
<tr>
<td>gB-2</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Ag59</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Ag65</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Ag71</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Ag461</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>
5.4.) DISCUSSION.

5.4.1.) Lymphocyte proliferation assay.

5.4.1.1.) Timecourse.

There was a difference in the timecourse of response of HSV-2 primed draining lymph node cells to HSV-2 and gB-2, following s.c. injection (Figure 5.1), the maximum response to gB-2 being at day 12 and to HSV-2 being day 6 or earlier. The peak of response specifically to gB-2 took longer to develop than the cumulative response to all virion components. The lymphoproliferative response to HSV is mediated by T lymphocytes (Horohov et al., 1985). These results, which represent a T cell response to gB-2, have demonstrated in HSV-2 a parallel to the lymphoproliferative response to gB-1 induced by priming mice with HSV-1 (Chan et al., 1985), which was observed at between 10 to 15 days post immunization.

5.4.1.2.) Response to recombinant antigens in vitro (antigenicity).

HSV-2 primed T lymphocytes (4x10^5 cells/well) were found to respond to doses of 10μg/well of Ag59 and Ag65, and to a lesser extent to Ag71 when immunized with higher doses (1x10^6 PFU/mouse) of HI HSV-2 strain 333 (Figure 5.2). However Ag71 failed to stimulate T cells primed with HSV-2 strain BRY (Figure 5.3). This may be due to antigenic changes arising from amino acid substitutions between the HSV-2 strains 333, BRY and HG52 within the putative T cell epitope covered by the 22 amino acids of gB-2 sequence in Ag71. Such a change may result in the loss of the Ag71 specific T cell epitopes in BRY. Alternatively the
discrepancy may result from the method of purification of the protein. The former results were obtained with substrate affinity purified protein. In the latter experiment, and all subsequent ones, the antigens were prepared by immuno-affinity chromatography.

Like purified HI virion, gB-2, Ag59 and Ag65 all stimulated HSV-2 primed T cells across a range of concentrations. The response curves and concentration range (0.2-50μg/ml, approx. 0.015-3.5μM) were similar to those published for lymphoproliferative response to purified influenza hemagglutinin (0.01-10μg/ml) (Mills et al., 1986) and synthetic peptides of bacteriophage lambda cI protein and hen egg lysozyme (0.001-0.1μM and 0.1-100μM) (Guillet et al., 1986; Babbit et al., 1985). These results indicated that the regions of gB-2 represented by Ag59 and Ag65 were antigenic for HSV-2 primed T cells, in vitro. The response curves for Ag71 and Ag461 both showed an increase at 50μg/well. It would be unlikely to be a specific response as Ag461 is not related to HSV-2. It may be an artifact of the high concentration of protein present in the wells, perhaps nearing the critical concentration of a minor impurity in the samples, which could then act as a non-specific mitogen.

5.4.1.3.) Response to recombinant antigens in vivo (immunogenicity).

Having established that Ag59 and Ag65 could stimulate HSV-2 primed T cells in vitro, the antigens were used to prime T cells in vivo. The results (Figure 5.4) showed that Ag59 could prime lymphocytes in vivo to proliferate in response to stimulation with either HSV-2 or gB-2, but Ag65 could not. Ag59, Ag65 and Ag461 all primed for a response to stimulation with Ag461 (GZ) as expected since they all contain GZ sequences, though the response primed by Ag65 was
low. The lack of an HSV-2 specific response to Ag65 priming was surprising since it suggested that whilst acting as a T cell antigen in vitro, Ag65 was not doing the same in vivo. This indicated a difference between the cells primed by the whole virus and those primed by Ag65 alone. Three possible explanations for this include:

1) the HSV-2 sequences in Ag65, in the form of a fusion protein, were unable to prime T lymphocytes because of the physico-chemical environment of the HSV-2 specific sequences and conformational constraints at the junction between the two components of the hybrid molecule.

2) in the response to the whole virus there was a heterogeneous population of T cells which perhaps provide help to produce a subset of cells capable of responding to Ag65 (synergy); whereas in isolation the cells induced by Ag65 alone were unable to respond to the same epitope on the virus (no synergy).

3) there may have been an epitope recognised by suppressor cells on Ag65, in addition to the epitope recognised by proliferative T cells. This may explain why the response to Ag461 was low in the cells primed with Ag65 (Fig.R.3.4).

The route of immunization with HSV-1 is known to affect the subsets of T cells that become primed for response, such that s.c. injection primes for DTH and proliferative response and i.v. injection primes for suppressive response (Nash et al., 1981a). The manner of presentation of the antigen also played a role such that viral antigen present on the surface of infected spleen cells, rather than as free virion, primes for a suppressive response (Schrier et al., 1983b). It was possible that the antigenic gB-2 peptide fragment, presented as part of a fusion protein, induced suppressive T cell subsets or T cell subsets unable to respond in this assay. This was not the case with Ag59, which suggests that this was a property of the Ag65-HSV-2
polypeptide and not the GZ portion of the molecule. The fusion of the epitope with GZ may have affected the structure or conformation in such a way that it could be recognised by primed T cells but could not itself serve to prime those T cells. Conformational constraint on recognition of altered antigen by T cell clones has been demonstrated with influenza hemagglutinin (Mills et al, 1986a), in which recognition was diminished or abolished by proteolytic cleavage, or alteration of epitope structure by treatment with low pH.

5.4.2.) Delayed-type hypersensitivity.

5.4.2.1.) Timecourse.

The timecourse of the induction of a DTH response by U.V. inactivated virus showed a maximum response between days 7 and 9 post priming (Figure 5.7). This corresponds with reports in the literature of day 6-7, though Schrier et al (1982) found the peak response to HSV-1 to be day 6. These results also show that the use of cyclophosphamide pretreatment on the peak swelling was to increase the value by approximately 34%. It has been suggested that this phenomenon is due to the sensitivity of a subset of Ts cells (which suppress T-DH cells) to concentrations of cyclophosphamide which do not affect the other T cell subsets (Liew, 1985). The timecourse of the footpad swelling response showed a maximum value at 24hrs post eliciting with a decrease to zero by 72 or 96hrs post eliciting (Figure 5.6). This is characteristic of the footpad swelling in response to a non-infectious antigen as described by Brand and Liew (1983) and in line with the presentation of 24hr swelling in published literature. Nash et al (1980ab) showed the swelling response in the ear pinna to be more prolonged. This was due to the use of infectious virus to elicit the
response, which would persist at the site of infection much longer than a passive antigen. All subsequent DTH measurements presented were for 7 days post priming and at 24hrs post eliciting.

5.4.2.2.) Response to gB-2 and the recombinant antigens.

The observation of a DTH response to gB-2 confirmed in HSV-2 similar observations of DTH response to gB-1 (Chan et al., 1985; Lukic et al., 1985; Blacklaws et al., 1987). There was also a small but significant response to Ag59 and Ag65 (Figure 5.8). Ag59 had already been shown to prime for proliferative T cells. Ag65 however had failed to prime for proliferative T cells but appeared to prime for T-DH cells. It was possible that this was due to the removal of suppressor cells by the pre-treatment of the mice with cyclophosphamide in DTH experiments. This tended to support the idea that one factor in the difference between the in vitro and in vivo response to Ag65 was the possible involvement of suppressor cells. It is known that the T cell response to HSV can be very sensitive to the effect of suppressor cells (Nash et al., 1981b; Schrier et al., 1982). The recombinant antigens elicited a DTH response in HSV-2 primed mice equivalent to the response elicited with HSV-2 in recombinant antigen primed mice (Figure 3.10), when all mice were pre-treated with cyclophosphamide. This indicated that the form of the gB-2 epitope presented as Ag65 was recognised by HSV-2 primed T cells. This again supported the suggestion that the failure of Ag65 to prime for proliferative T cells responsive to HSV-2 was due to the induction of cyclophosphamide sensitive suppressor cells, rather than a structural alteration to the epitope caused by the fusion to GZ. Ag71 was also included in this experiment and was able to induce and elicit a significant response. This indicated that although not antigenic for HSV-2 BRV
primed proliferative T cells (Figure 5.3), Ag71 was immunogenic.

5.4.2.3.) Effect of cyclophosphamide on the response to the recombinant antigens.

The next step was to compare the DTH response to these antigens in mice with or without cyclophosphamide pretreatment. The results (Figure 5.9) show a reduction in the swelling response to priming with HSV-2, gB-2 and Ag65, but not Ag59, antigen in mice not pretreated with cyclophosphamide. In addition, the response to priming with a mixture of Ag59 and Ag65 was also reduced in mice not pretreated with cyclophosphamide (Figure 5.9). It appeared that the DTH response to the portion of gB-2 represented in Ag65 was regulated by suppression, possibly mediated by suppressor cells induced by the antigen itself. The DTH response to HSV-2 and gB-2 also appeared to be regulated in a similar suppressor cell mediated (cyclophosphamide sensitive) manner.

A new experiment was designed to examine the possible effects of combination priming, in the absence of cyclophosphamide, such that an animal received both HSV-2 and the recombinant antigens at the same site. It was clear that copriming with any of the recombinant antigens caused a strong inhibition of DTH response to HSV-2 (Figure 5.11). This inferred that the effect may be a property of GZ, since it was common to the recombinant antigens, and the effect was seen in the control group using Ag461 (GZ). As previously described (Section 4.1.), this molecule was chosen as a carrier for a number of reasons amongst which was its immunogenicity. Krzych et al (1982) described a broad repertoire of proliferative T cells responding to GZ, of which the Th cell repertoire was a more limited component.
These authors found no indication of suppressive effects; however the earlier work of Sercarz et al. (1978) reported a transient period of suppression during the primary response, though still emphasizing the immunogenicity of GZ. This transient suppression was found to appear between days 3-11 predominating around day 7. Thus this GZ suppressive effect may have been interfering with the development of HSV-2 specific DTH, in an antigen non-specific fashion. If this was the case it would suggest that Ag59 was a particularly strong T cell immunogen since a detectable proliferative and DTH response were developed even in mice not treated with cyclophosphamide. In further characterising this apparent suppressive effect it would be important to examine the effect of Ag65 on the production of antibody forming cells or the proliferative T cell response to HSV-2.

An alternative approach to obtain a clearer picture of the immune response induced by the gB-2 specific portion of these recombinant antigens would be to separate them from the carrier (GZ). This is discussed in more detail in Section 6.3.

5.4.3.) Lethal challenge.

The lethal challenge of $1.25 \times 10^5$ PFU/mouse was based on 10x LD$_{50}$ such that it was greater than the LD$_{100}$ (at $9 \times 10^4$ PFU/mouse). It was administered s.c. to mimic a natural route of infection with replication of virus in the skin, so that it would be presented to the immune system in the same manner and to the same subsets of cells as the doses of immunizing antigen.
5.4.3.1.) Protection by immunization with HSV-2 and derivative antigens.

Immunization of mice with HI HSV-2 conferred complete protection against the challenge dose. This dose of whole viral antigen acted as a positive control. Immunization with gB-2 immuno-purified antigen also gave a very high level of protection at 91% (Table 5.2). This result with gB-2 established a parallel to the observation that the homologous protein in HSV-1, gB-1, can protect against a lethal challenge with HSV-1 (Chan, 1983; Dix and Mills, 1985). The work of Dix and Mills (1985), however, found that gB-2 did not provide protection against HSV-2 infection though it did against HSV-1 infection. These authors employed the i.p. route for immunization with a subsequent challenge by the s.c. route. They reported that mice immunized with gB-2 had significantly longer mean survival times, suggesting some degree of immune enhancement. It is possible that this route of immunisation did not induce an appropriate immune response or recruit the subsets of T cells necessary to provide a protective response. The s.c. route of immunization reported in this thesis, however, did stimulate an immune response sufficient to protect against a lethal challenge.

The baseline of time, rate and percentage of deaths in unprotected mice was provided by immunization with Ag461 (GZ). In this group 91% of the animals died by day 18 (Figure 5.13) with a mean survival time of 11.4 days. This was paralleled by Ag65 and Ag71 primed animals which had total mortality of 91% and 100% and mean survival times 11.3 days and 11 days respectively. These two HSV-2/gB-2 related antigens provided no protection against infection. However, 36% of mice immunized with Ag59 survived the lethal challenge and those that died had a mean survival time of
18.5 days. Though this final number of survivors, 4/11, was not significantly different from unprotected mice, 1/11, (P>0.1) longitudinal analysis using life tables for these two groups showed a significant difference. This indicated that the immune response elicited by Ag59 was providing a degree of protection against lethal challenge with HSV-2, and providing a significant increase in the life expectancy of these mice (P<0.005) between day 12 and day 29, the critical period of this experiment.

Ag59 contains only approximately 6% of the gB-2 polypeptide. Thus it probably represents only a fraction of the total immune repertoire of the gB-2 antigen. This may explain why the protection against lethal infection provided by this antigen was only partial. This effect had been noticed in the case of an antigenic synthetic peptide from gD-1/2. Watari et al (1987) used an acylated-peptide-liposome complex to immunize mice prior to lethal challenge and found that it provided 80% protection against a 7xLD50 infection. These authors demonstrated 100% protection with this antigen against a lower dose (4xLD50) challenge following the same type of immunization. The response was shown to be mediated by T cells. Such partial protection may have resulted from the recruitment of a limited subset of immune cells which were capable of responding to infection. If their response did not quickly clear infectious virus a focus of infection could be established, the subsequent spread of which would result in the death of the individual.

5.4.3.2.) Antibody response.

The serum anti-HSV-2 antibody level of three individuals from each of the groups was measured both prior to the series of immunizations and, as expected, no specific
antibodies were detected in any pre-immunised groups. Both HI HSV-2 and gB-2 immunized mice showed HSV-2 specific antibody post immunisation even before challenge, with titres of >2560 and 320 respectively. These results confirmed in the HSV-2 system the antibody response induced by gB-1 observed by Lukic et al (1985). gB-2 has also been shown to induce a sustained antibody response from an early time post immunization in previously seronegative human patients (Ashley et al, 1985). The importance of this antibody response to gB, as previously discussed (Section 1.3.), may be relevant to control of the spread of the virus to the CNS, but does not mediate protective immunity (Lukic et al, 1985). This was further substantiated by the higher antibody response produced in Ag65 primed mice compared with Ag59 primed mice and yet the former group demonstrated no protection against lethal challenge.

The post immunization pre-challenge anti-HSV-2 antibody levels induced by immunization with the recombinant antigens and GZ were very low (titres from 40 to <20). It was possible that an antibody response was elicited but that the avidity of the gB specific antibodies produced was too low for them to be clearly detected in this RIA. It was also possible that the GZ linked suppression detected in the DTH experiments was affecting development of the antibody response. This seemed unlikely, however, since the report of Sercarz et al (1978) described the generation of a good anti-hapten antibody response from repeated hapten-GZ priming despite the transient appearance of suppressor cells during the response. The antibody response raised may be improved by adjusting the immunization regime by changing the adjuvants used, altering timings of boosts, and changing the route of immunization.
5.4.4.) Summary.

The immuno-purified glycoprotein gB-2 was shown to be a potent immunogen. It elicited strong responses in the subsets of T cells mediating both in vitro proliferation and DTH. It also induced a good antibody titre. The overall immune response induced by gB-2 was able to confer a very high degree of protection against a lethal challenge with HSV-2, almost as effective as immunization with whole inactivated virus. The recombinant antigens showed a mixture of responses. Both Ag59 and Ag65 stimulated a proliferative T cell response in HSV-2 primed cells but only Ag59 primed for such a proliferative response to whole virus. When mice were pretreated with cyclophosphamide both Ag59 and Ag65 primed for DTH responses to the virus. In the absence of cyclophosphamide pretreatment only Ag59 primed for such a response. This implied an involvement of suppressor cells in the response to Ag65, though this was not conclusive. Ag71 stimulated a proliferative response to HSV-2 333 primed cells, and appeared to be a good immunogen for the induction of a DTH response to HSV-2. Of the recombinant antigens produced during the course of this work, Ag59 (representing gB-2 codons 339-394) appeared the most potent HSV-2 specific antigen and immunogen, an observation supported by its conferring a significant degree of protection against a high dose lethal challenge.
6.) GENERAL DISCUSSION.

6.1.) Identification of the gB-2 gene.

The results described in Section 3. showed homology between the 4.7kbp BamHI to KpnI DNA, fragment representing 0.345-0.376 MU in the HSV-2 genome, and a 3.2kb mRNA present in HSV-2 infected cells at 14 hours p.i.. This transcript, isolated from total HSV-2 infected cell mRNA by hybridization to the 4.7kbp DNA fragment, translated in vitro to a 92kDa polypeptide. This product co-migrated, in SDS PAGE analysis, with a polypeptide which was immuno-precipitated by a gB specific monoclonal antibody from an in vitro translation of total HSV-2 infected cell mRNA. This complemented observations by Rafield & Knipe (1984) of a 3.4kb mRNA encoding HSV-1 gB mapping to 0.343-0.386 MU and data which co-mapped gB-1 and gB-2 to 0.34-0.38 MU by polypeptide analysis HSV intertypic recombinant viruses (Ruyechan et al.,1979; Honess et al.,1980; Pereira et al.,1982a). A restriction map of this region of the HSV-2 genome (Section 3.3.6. and 3.4.4.) showed strong homology to a map derived from the sequence of the gB-2 gene subsequently published by Bzik et al. (1986). Furthermore sequence data from clones expressing HSV antigenicity (Section 4.3.2.) proved to be homologous to the both the gB-1 and gB-2 sequences.

It was interesting to note that the predominant products of in vitro translation of hybrid-selected mRNA representing gB-2 were of a lower molecular weight than the full length gB-2 gene product, which was a relatively minor band. As discussed in Section 3.4.3 proteolytic cleavage of the full length product would be unlikely and mRNA secondary structure causing early termination of translation was not detected by computer analysis. An alternative explanation
could be internal initiation of translation. Analysis of the gB-2 sequence revealed 24 ATG codons within the reading frame, some of which would yield product approximating to the sizes observed (see Table 6.1.). Since there were clearly less than 24 observed products, computer analysis for eukaryotic ribosome binding sites (RBS) was applied (Stadan and McLachlan, 1984) to suggest products that may occur preferentially. Neither of the predicted products (48 kDa or 42 kDa) nearest the 45 kDa product which is most prevalent, had an adjacent RBS.

Internal initiation of translation has been observed with the mRNA encoding HSV-1 thymidine kinase. Hybrid arrested in vitro translation coupled with two dimensional gel electrophoresis of products indicated that, in addition to the expected 45 kDa product of this gene, 39 kDa and 38 kDa products were also translated from its mRNA (Marsden et al, 1983). Furthermore the difference in the pi values for these products was compatible with predicted losses of basic and acidic residues if they were internally initiated at two AUG codons corresponding to predicted products of 35 and 34 kDa. This form of analysis might clarify the origin of the products observed in Section 3.3.5.

It was interesting to note that the gB-2 AUG known to be used in vivo lacks a computer predicted RBS. This may reflect a failure of the predictive algorithm or it may suggest that initiation of translation is subtly altered during viral infection to favour specific viral genes. In eukaryotes nine or more factors are required for the initiation of translation (Lewin, 1983). It is possible that one or more of the as yet uncharacterised gene products of HSV (McGeoch et al, 1988) could influence the initiation of translation. This may explain why the 92 kDa product was so poorly translated from the hybrid selected mRNA. Some form
of as yet uncharacterized regulation of transcription has been noted for gD-1 expression. Synthesis of gD-1 declines after 6 hours p.i. despite continued synthesis of its mRNA which was still functional in in vitro translation (Johnson and Spear, 1984). However in considering this possibility it was recognised that the mRNAs encoding gB-1, gC-1, gD-1 and gE-1 have been successfully translated in vitro to the expected polypeptide products (Rafield and Knipe, 1984; Lee et al, 1982; Frink et al, 1983).

Table 6.1. Potential sites for internal initiation of translation (AUG codons) in the qB-2 gene.

<table>
<thead>
<tr>
<th>Position (kDa)</th>
<th>Product size (kDa)</th>
<th>Adjacent RBS</th>
<th>Nearest observed product (kDa)</th>
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<tr>
<td>548</td>
<td>100.060</td>
<td>-</td>
<td>93</td>
</tr>
<tr>
<td>1019</td>
<td>84.001</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1079</td>
<td>81.477</td>
<td>-</td>
<td></td>
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<tr>
<td>1184</td>
<td>77.526</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1226</td>
<td>75.840</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1421</td>
<td>68.355</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1628</td>
<td>60.457</td>
<td>+</td>
<td>60 63, 62</td>
</tr>
<tr>
<td>1655</td>
<td>59.130</td>
<td>-</td>
<td></td>
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<td>1802</td>
<td>53.862</td>
<td>-</td>
<td>54</td>
</tr>
<tr>
<td>1937</td>
<td>48.651</td>
<td>-</td>
<td>50, 47 50</td>
</tr>
<tr>
<td>2096</td>
<td>42.519</td>
<td>-</td>
<td>43 45</td>
</tr>
<tr>
<td>2225</td>
<td>37.719</td>
<td>-</td>
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<td>2246</td>
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<td>-</td>
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<td>-</td>
<td>34, 32</td>
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<td>25.106</td>
<td>+</td>
<td>30 28</td>
</tr>
<tr>
<td>2729</td>
<td>17.609</td>
<td>-</td>
<td></td>
</tr>
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<td>2849</td>
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<td>-</td>
<td></td>
</tr>
<tr>
<td>2954</td>
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<td>-</td>
<td></td>
</tr>
<tr>
<td>3083</td>
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<td>-</td>
<td></td>
</tr>
<tr>
<td>3095</td>
<td>6.778</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3113</td>
<td>6.214</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3182</td>
<td>5.641</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

1 Nucleotide position of the AUG codon.
2 Predicted molecular weight of product of internal initiation at specified AUG.
3 Computer predicted (Stadan and McLachlan, 1984).
6.2.) Expression of fragments of the gB-2 polypeptide.

Despite a number of problems, the techniques employed in Section 4.2. led to the production of fusion proteins expressing gB-2 specific sequences that were both recognised by HSV-2 specific sera and active in an immunological assay. The data obtained also confirmed the location of the gB-2 gene.

The problems encountered were revealed by nucleotide sequencing of clones. Firstly, it was apparent that a number of multiple ligation events had occurred, presumably reflecting an excess of small fragments. A method of size fractionation of the DNA prior to ligation would alleviate this problem. Second, exonuclease activity during preparation of the vector caused reading frame shifts in recircularised vector and led to frequent (1 in 3) isolation of blue colonies (GZ expression positive) which contained no insert DNA. Finally, the result of exonuclease activity was also observed at the ligation junctions between vector and HSV DNA inserts. These junctions should represent, respectively, one half of the Smal cloning site and half of either an AluI, Rsal or Thal site. The Smal sites were always found. This was only true, however, for the HSV junctions in pXH71. In all other clones the junctions ranged from 4-45 nucleotides from these specific sites indicating clearly that a certain degree of exonuclease activity had occurred at some point prior to ligation. Fortunately, because the vector was designed to accept random fragments, this did not prevent expression of gB-2 specific sequences.

A more effective means of addressing this task in future, whilst still employing a fusion protein approach, would be to create progressive deletions from the C or N terminus of the coding sequence. This strategy was
successfully performed on the 65kDa mycobacterial antigen, by Lamb et al (1987) and Thole et al (1988) using the lambda phage vector gt11, to elucidate its antigenic structure both in terms of antibody and T cell response. Problems may be encountered in expressing certain domains of gB-2 in the prokaryotic system as discussed in Section 4.4.1, but it is quite possible that the majority of the extracellular domain could be stably expressed. Examination of the antigenicity of the transmembrane and cytoplasmic anchor regions could be carried out in eukaryotic expression systems. Indeed Cai et al (1987) and Highlander et al (1988) have recently described the construction of linker insertion mutants of gB-1, which have progressive deletions of the C terminus, to identify monoclonal antibody binding domains.

6.3.) New approaches to recombinant gene expression.

A recently developed technique known as the polymerase chain reaction, or PCR (Saiki et al, 1988), can be applied to the preparation of specific DNA fragments for expression (explained below). This may prove to be a considerable improvement over methods, such as those used in Section 4.2., which relied on random cloning or manipulation of any available restriction sites in a gene sequence. Potentially any defined region of a protein can be expressed, subject to tolerance of expression by host cells. Regions of a protein which differ between clinical isolates, strains or mutant forms can be analysed by sequencing of amplification products (Green et al, 1989) and/or by specific assay following expression (S. Comerford, personal communication).

The PCR technique was originally developed to amplify, in vitro, low abundance, specific sequences for the detection of sickle cell disease (Saiki et al, 1985). Amplification of a region of DNA is achieved by thermal
cycling which promotes denaturation of target DNA, hybridisation of two paired primers then polymerisation by the thermostable Tag DNA polymerase. The result of >30 thermo cycles is a population of molecules whose ends are dictated by the primers. The technique can be applied to expression by modification of the primers. They can be selected to encode specific restriction enzyme sites compatible, in a chosen reading frame, with a particular expression vector (see below). This is achieved by allowing certain base pair mismatches with the target sequence which create the required restriction site whilst retaining specific hybridisation.

In light of the results of the DTH experiments, discussed in Section 5.4.2.3, it would be useful to separate the gB-2 specific sequences expressed in the pXH clones from the carrier, GZ, or to couple them to a different carrier molecule. This could be achieved by use of an oligonucleotide inserted in the BamHI site, between the gB-2 fragment and GZ, encoding the tetra peptide sequence Ile Glu Gly Arg for Factor X specific cleavage (Nagai and Thogersen, 1984), or Leu Val Pro Arg Gly Ser for thrombin specific cleavage (Chang, 1985). Alternatively the sequences could be transferred to another compatible vector which expresses the insert as a fusion to another gene for, instance trpE in the vector pXY533 (Wellcome Biotec Ltd). Two other vectors are currently available which use compatible EcoRI and BamHI restriction sites, with respect to pXY460, one which produces C terminal fusion to Staphylococcus aureus protein A, pRIT2T (Nilsson et al, 1985), and the other which produces C terminal fusion to Schistosoma japonicum glutathione S transferase, pGEX-3X (Smith and Johnson, 1986). The later also contains the Factor X cleavage site between the carrier and its partner.
6.4.) T cell immunogenicity/antigenicity of the gB-2 recombinant antigens.

The results in Section 5 show that the recombinant gB-2 fusion proteins Ag59, Ag65 and Ag71 are recognised by T cells. Recognition of protein antigens by T cells only occurs in the context of specific major histocompatibility (MHC) antigens (Zinkernagel and Doherty, 1979). This usually requires processing of the antigens (proteolytic cleavage) inside specific antigen presenting cells, often macrophages (Unanue, 1980). Presentation of the recombinant gB-2 antigens was demonstrated, both in vivo by DTH response (Section 5.3.2.) and in vitro by lymphoproliferative response (Section 5.3.1.). It is now known that one or more of the resultant peptide fragments of a processed protein bind to either a class I or class II MHC molecule (Babbit et al., 1985; Buus et al., 1986) prior to recognition by specific T cell receptor (reviewed by Kronenberg et al., 1986). Both of the responses observed in Section 5.3. are generally characterised by recognition in the context of class II MHC. Particular MHC alleles will only bind certain peptides (Buus et al., 1987) and present them as antigens. This provides, at the molecular and genetic level, an explanation for variations in cell-mediated immune responses between species, strains and individuals to protein antigens. In this work immune responses to the recombinant gB-2 antigens were studied in an inbred mouse strain (BALB/c which is H-2\d). Therefore it is possible that the strength of antigenicity and type of response elicited will differ in other mice strains or in humans. However there is some evidence to suggest that Ag65, and to a lesser extent Ag59, are antigenic to human T cells as measured by the in vitro proliferative response (Chan et al., 1989 in press).
The fact that Ag59 was identified by HSV-2 immunostaining suggests that it contained at least one B cell epitope. This was supported by the assignment of a type-common antigenic site (site III at residues 283-380) overlapping Ag59 (at residues 339-394) by Highlander et al (1988). This may imply some proximity between the B cell epitope(s) and the T cell epitope(s) in this region of gB-2. Such proximity of B cell and T cell epitopes has been noted in influenza hemagglutinin (Thomas et al, 1987), though its significance is not clear.

Much interest has focused on what basic parameters govern binding of an antigenic peptide to an MHC allele and thus influence the potential T cell antigenicity of a peptide. Models of the association between antigenic peptides and MHC molecules have been proposed by Berzofsky et al (1987) and Rothbard et al (1987). Since there were observed T cell responses to the gB-2 recombinant antigens (Section 5.3.) it was of interest to examine their relationship to these models. Berzofsky et al (1987) have used computer analysis on thirty empirical peptide defined T cell epitopes derived from sequence data of a number of proteins, with examples of both class I and class II MHC restricted responses. A correlation was made between recognition of these epitopes and the ability of the peptides to form an amphipathic alpha helical structure. It was suggested that the hydrophobic face of the amphipathic alpha helix would associate with the hydrophobic pocket in the surface of the MHC protein (Bjorkman et al, 1988) leaving the hydrophilic face of the peptide exposed for interaction with the T cell receptor. Rothbard et al (1987), using essentially the same database of epitopes, identified a motif of four amino acids with conserved physical characteristics within the epitopes. In position one the
amino acid was either charged or glycine, in position two and three hydrophobic and in position four polar (charged or uncharged). In nine cases position four was hydrophobic, in all of these position five was then polar (charged or uncharged). It was suggested that the central portion of the motif would associate with the MHC molecule and that remaining amino acids would be recognised by the T cell receptor.

Figure 6.1. An illustration of the distribution of regions of amino acid sequence within gB-2 which have characteristics found in known T cell epitopes as described by Berzofsky et al. (1987) [AMPHI computer analysis performed by Dr. C. Beddell, Wellcome, Beckenham] and by Rothbard et al. (1987) [analysis performed manually]. Also shown are the locations of Ag59, Ag65 and Ag71. Dots above indicate sequences with characteristics of both types of analyses. S - signal sequence, TM - transmembrane domain.
Neither of these models claim to define T cell epitopes. They do, however, give some indication of characteristics of known T cell epitopes which may be applied prospectively to locate potential epitopes. Similar analyses to those used by these groups were applied to gB-2, looking particularly at the regions represented by the recombinant antigens. The results of these analyses (Figure 6.1.) show that each of the recombinant gB-2 antigens contain amino acid sequences conforming to either one or both models. These characteristics appear to be quite uniformly distributed throughout the gB-2 sequences, the regions represented as recombinant antigens not being particularly notable. In view of this, it would be interesting to examine further isolated regions of the gB-2 polypeptide and determine the relative distribution of antigenic domains. An approach using recombinant antigens and chemically synthesised short peptides was used by Lamb et al (1987) to determine T cell epitopes to within seven to nine amino acid sequences in Mycobacterial 65kDa antigens. Chemical synthesis of specific peptides could be applied to the fine determination of epitopes within Ag59 and Ag65. First it will be important to complete the antigenic map of the gB-2 polypeptide in terms of both the mouse experimental model and human lymphoproliferation.
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APPENDIX

DNA SIZE MARKERS

The following DNA size markers were used in electrophoresis to estimate the size of experimentally generated fragments. These were obtained from BRL.

1KB Ladder DNA

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<th>6</th>
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<td>1.636</td>
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<td>3.054</td>
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<td>Size(kbp)</td>
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123bp Ladder DNA

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lambda HindIII DNA

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SUPPLIERS OF CHEMICALS AND REAGENTS.

Chemicals and Solvents

Sigma Chemical Company Limited,
Fancy Road,
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256
Nucleic acids, restriction endonucleases, DNA modifying enzymes and fine reagents.

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**BRL (Gibco BRL)**
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Uxbridge,
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**New England Biolabs.**
C/O CP Laboratories,
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Hertford CM23 3DH.

**Pharmacia LKB Biotechnology Limited,**
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Central Milton Keynes,
Buckingham MK9 3HP.