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Expression of Pseudorabies Virus and Rous Associated Virus Antigenic Determinants in *Escherichia coli*

By
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Thesis submitted to the School of Graduate Studies and Research in partial fulfillment of the requirements for the M.Sc. degree in Biology.

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Stephen Bertrand

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Abstract

Both pseudorabies virus and the various strains of avian sarcoma and leukosis viruses are economically important pathogens. In Canada, pseudorabies is an exotic virus which primarily infects swine. The disease is rapidly fatal in most species although adult pigs may become carriers. Because pseudorabies virus (PrV) is exotic to Canada, imported animals are rigorously tested for the presence of antibody titers to the virus. The leukosis caused by Rous associated virus (RAV) has a major impact on the health of poultry flocks. Different strains of the avian leukosis viruses cause damage of differing severity but the general characteristics of infection include decreases in both egg and meat production. There would be significant benefits if non-infectious, antigenic fractions from both of these viruses could be produced in bacterial cells. These proteins could be used in serum antibody testing to make the detection of both viruses considerably simpler than the currently used ELISA and virus neutralization tests. As well, the production of large quantities of RAV *env* protein could simplify studies of the mechanisms of host specificity and provide additional information about chicken cell receptors.

I have attempted to produce an antigenically recognizable protein from both pseudorabies virus and Rous associated virus. Attempts were made to express such a protein from pseudorabies virus by cloning either cDNA or genomic DNA fragments into plasmid vectors followed by colony immunodetection using a polyclonal antibody against PrV prepared in pig. Although several recombinants produced positive immunological results, further examination showed that none contained PrV DNA sequences. Standard screening methods were modified and improved during the course of this work. Subsequently, other laboratories identified significant difficul-

ties in using unpurified polyclonal antibodies prepared in swine which provided an explanation for the results I observed.

A cDNA library constructed from RNA prepared from RAV-1 was screened using a probe prepared from *env* sequences in pSR-XD2, a plasmid containing much of the RSV sequence. Transformants containing RSV homologous DNA were identified. During purification of these clones and subsequent screenings many of the plasmids identified as containing RSV homologous sequence lost their inserts. Additional studies demonstrated that plasmids which exhibited the strongest homology with the RSV probe and which contained the longest inserts, lost these inserts at a high rate.

A second cDNA cloning using a different bacterial host and a different viral strain (RAV-2) produced several positive clones, with insert lengths ranging from 250 bp to 950 bp. These inserts were sequenced and compared to known RSV and RAV sequences. A high degree of homology was observed. The longest RAV-2 cDNA fragments included most of the *env* gp37 polypeptide coding region. In order to test for expression of an antigen, inserts from two independently isolated recombinants were subcloned into λgt11 in each of the three reading frames. A polyclonal antibody prepared against Rous sarcoma virus was used to test for the expression of any recognizable antigenic determinants. No antigen production was detected even though nucleic acid plaque hybridizations demonstrated that the subcloning had been successful in introducing RAV-2 sequences into λgt11. Possible explanations for this, as well as a discussion of the composition of the RAV-2 sequences and their placement on the RSV genetic map are considered.

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Introduction

There is a large body of work which shows that it is possible to produce foreign proteins in bacterial cells (see reviews and methodologies by Remaut *et al.*, 1987 and Snyder *et al.*, 1987). The presence of the foreign protein is typically verified through immunological methods. I have endeavoured to produce antigenically recognizable proteins in *E. coli* through the use of both plasmid and phage expression vectors; attempts were made to produce and detect envelope proteins from both pseudorabies virus (PrV) and two strains of Rous associated virus. Preparation of the proteins was accomplished through recombinant cloning directed at introducing nucleic acid sequences coding for fragments of these proteins into a variety of *E. coli* expression vectors. The results of these clonings were then tested using polyclonal antisera prepared against the proteins in the native hosts. The reaction of this antiserum with polypeptides en-coded by the cloned DNA indicated the presence of an insert DNA fragment capable of producing the appropriate antigenic epitopes. The recombinant protein-producing bacteria could then be used as a source of purified viral antigen for use in infection-screening activities, vaccine production and in research into the composition of the viral envelope and its importance in the actual infection process. The potential uses of such an engineered expression system are well illustrated by work done by Motz *et al.* (1986) who were successful in expressing an antigenically active epitope of one of the immediate early proteins from Epstein-Barr virus and using it for subsequent diagnostic work.

Foreign Protein Expression

Through the use of recombinant DNA techniques it is possible to clone all or part of a protein-coding sequence of a gene into a specialized vector which can then be intro-

duced into either a eucaryotic or bacterial cell line. The new host cell may then be able to express the foreign protein dependent upon a series of conditions (Silhavy *et al.*, 1984).

Fusion Proteins

The simplest method of doing this is to clone the gene, or portion of the gene, into a vector sequence which already codes for a protein. If the cloning can be done such that the open reading frame of the cloned DNA is in frame with the open reading frame under transcriptional control of the vector sequence's promoter then a fusion

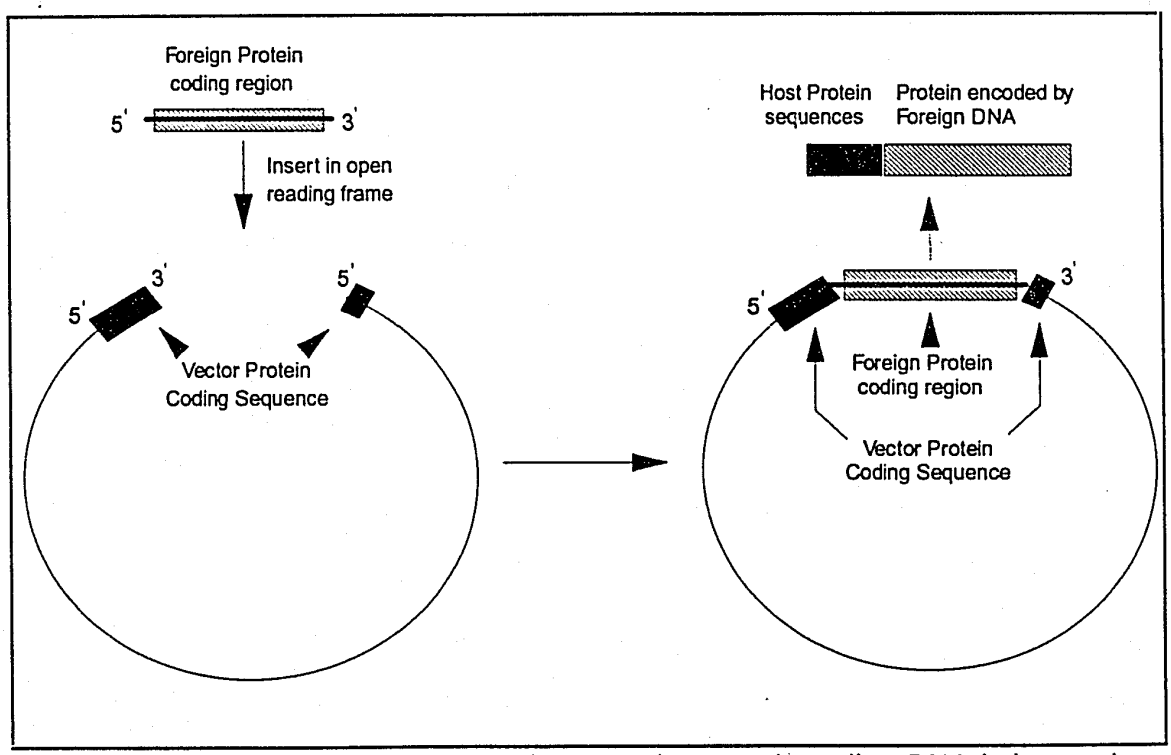

Figure 1 Fusion Protein Construction: Foreign protein-coding DNA is inserted into the vector in such a way that when the vector protein is expressed, translation "runs" into the foreign protein coding region.

protein will result. The result is a polypeptide consisting of sequences coded by both the gene in the vector and the cloned DNA segment.

Actual expression of a fusion protein and production of the protein in measurable quantities is considerably more complicated than this. The new protein may be toxic to the host cell, creating a cycle in which the growth of all cells encoding the protein is severely retarded, thereby greatly diminishing actual production of the protein (Remaut *et al.*, 1983). Typically this problem is solved by regulating expression through the inclusion of a repressor of the native promoter within the host vector. Cell replication is permitted to proceed with the production of the potential fusion protein being repressed. Repression is experimentally released after significant cell replication has occurred, allowing for a short burst of protein production as the colony or culture reaches maturity. β-galactosidase fusion proteins can comprise up to 4% of total cellular protein when produced in this way (Young and Davis, 1984). Other fusion products can reach even higher percentages of total cellular protein when released from repression (eg. Lautenberger *et al.*, 1983).

The fusion protein produced in this manner is frequently unstable in its new host cell, particularly in the case of recombinant eucaryotic proteins in prokaryotic cells (Young and Davis, 1983). Fusion of the eucaryotic moiety with all but a small portion of β-galactosidase has been shown to enhance the stability of the eucaryotic residues (Itakura *et al.*, 1977). This fusion can be achieved by choosing an insertion site in *lac*Z that will result in N-terminal (host-cell native) β-galactosidase translation combined with translation of the foreign polypeptide sequence.

Cloning the Foreign Gene

Several methods exist to clone the gene, or portion of the gene, of interest into the expression vector. These methods are defined by the technique with which the DNA of interest is prepared for insertion. In the case of DNA viruses such as the herpesviruses, the genomic viral DNA can be isolated and cleaved into manageable size fragments through the use of either the exonuclease activity of DNA polymerase or digestion by restriction enzymes. The latter approach results in a non-random distribution of restriction fragments but, where the sequence of the viral DNA is known,

may result in a better chance for ligating the polypeptide encoding fragment into an open reading frame. Alternatively, cDNA may be prepared from RNA isolated from infected cells and ligated into the host vector. This approach alters the chances of finding an antigenically active polypeptide in a ratio corresponding to the relative population of mRNA's. The genomes of the retroviruses are much less complex than those of any of the DNA viruses. Because of this, the importance of differences in the relative ratios of various transcripts in the mRNA population is essentially eliminated as a concern for cDNA construction. The DNA resulting from the reverse transcription of retroviral RNA (a stage in the cellular integration process) can also be used as a source of genetic material.

Expression Vectors

Types of Expression Vectors

Two general types of expression vectors exist for *E.coli* — bacteriophage and plasmids. Neither vector system is universally preferred; rather the selection of a vector system is dependant upon the specifics of the current study. In general, plasmid vectors can be tailored more closely to the requirements of the expression system because of the ease with which promoter and insertion sites can be altered. This allows for studies on protein secretion and modification to be conducted within the context of the cloning vehicle (Duffuad *et al.*, 1987). A large number of plasmid expression vectors currently exist. As well, plasmid vectors are generally more suitable for use when large quantities of the expressed protein are to be isolated. However, since most plasmid vectors exist in high copy numbers within *E. coli* cells, the lethality of the foreign protein becomes a major concern. Many laboratories have found it very difficult to completely repress expression of the foreign protein as a result of this gene dosage effect (eg. Koenen *et al.*, 1983); where the expressed protein is harmful to the host cell, viable growth is not possible. Practical detection of protein expressed from plasmids is also slightly more difficult in practice since release of the protein from the bacterial colony and removal of bacterial cell matter

prior to detection are troublesome. If all cell matter is not removed prior to application of the antisera, a high background level results (Muller-Hill, 1984).

From a practical perspective, bacteriophage are easier to screen in large numbers for the presence of a given clone because of the high number of plaques that will fit on a petri plate. Phage vectors are also more suitable for studying highly toxic gene products since their propagation in the host cell as a single copy genomic insert (ie. in a lysogenic state) enhances the viability of the host by facilitating repression of the foreign genetic information. This eliminates many of the problems which exist when working with high copy number plasmids. However, it is considerably more difficult to adjust the expression controls within a phage vector and there are many fewer phage vectors to choose from. This was especially true at the time that this work was carried out (Windle, 1986). The experiments described in this thesis used both plasmid and phage expression vectors.

The Plasmid Vector — pUC 8

Many modifications that emphasize the highly efficient production of protein have been made to the pBR322 system in recent years; frequently these modifications are specific to the expressed protein (eg. Lautenberger *et al.*, 1983; Bröker, 1986; Duffaud *et al.*, 1987). One set of early modifications to pBR322 resulted in the pUC series of plasmids. pUC8 (Vieira and Messing, 1982) was used as a plasmid vector for much of the work presented in this thesis. pUC8 is a 2.7 kb plasmid (see Figure 22 in Appendix A[1]) containing coding regions for ampicillin resistance (*bla*) and β-galactosidase (*lac*Z).

Restriction sites in a multiple cloning site, such as the *EcoRI* site in the *lac*Z gene, provide target sites for the insertion of foreign DNA. pUC8 typically exists in twenty to thirty copies per cell so that expression levels gained from a recombinant clone

[1] All maps of vectors and clones can be found in Appendix A.

should be very high. In *E. coli* the *lac*Z gene is normally repressed; IPTG acts as an inducer. Under this control, potentially harmful foreign proteins will theoretically not be produced from the recombinant plasmid until the inducing agent is present. In fact, during the construction of cloning vectors such as pUC8 the repression mechanism has been lost and must be provided by the host. Because of this and the multi-copy nature of pUC8, some transcription may occur, even in the absence of an inducer, and it is possible that some cells which contain recombinant plasmids coding for a foreign protein may be adversely affected.

These potential problems are present because pUC8 was not specifically designed for foreign protein expression; despite this pUC8 has been successfully used as an expression vector by several laboratories (eg. Okayama and Berg, 1982; Nakamura *et al.*, 1986). Helfman *et al.* (1983) used pUC8 as the sole vector in studies which identified clones encoding chicken tropomyosin by immunologically screening a cDNA expression library. Although more sophisticated expression plasmids were developed during the course of my work on this project, other laboratories also continued to use pUC8 because of their familiarity with it (eg. Guise *et al.*, 1985). It was chosen as an expression vector because of the documented successes and its accessibility.

The Phage Vector — λgt11

λgt11 is a lambda phage derivative, constructed by Young and Davis (1983) especially to allow the isolation of unknown proteins encoded by cloned DNA. The insertion site in λgt11 is a unique *Eco*RI cleavage site located 53 bp upstream from the β-galactosidase translation termination codon within the *lac*Z gene. The vector will permit a maximum insert size of 7.2 kb. The fusion protein that results from a λgt11 cloning should be extremely stable in *E. coli* because of the positioning of the insert site at the 3′ end of the lacZ gene. The size of the preceding portion of native β-galactosidase protein is sufficient to shield the foreign regions from breakdown by cellular proteases (Young and Davis, 1983).

The phage vector produces a temperature-sensitive repressor allowing control of growth; the repressor is inactive at 42°C. Combined with an amber mutation (*S100*) that renders the phage lysis defective, lysogens can be systematically induced by a temperature shift, resulting in the accumulation of large quantities of phage particles. This reduces the potentially toxic effects of foreign protein production during amplification of the phage. It also places a precise control over the timing of foreign protein production thereby minimizing the effects of eucaryotic protein degradation in bacteria. Alternatively, in a *sup*F host, plaques can be screened. When the number of infected cells (where the host cell is chosen for its production of large amounts of the *lac* operon repressor) surrounding the plaque is sufficiently large, *lac*Z directed expression is induced by inactivating the repressor with IPTG.

The choice of host cell is extremely important for λgt11. Y1090 is the preferred strain of *E. coli* for plaque screening of λgt11 recombinants (Y1088 is a similar strain that allows screening of lysogens). The most important characteristic of Y1090 is that it is a *lon*- strain, ie. it is deficient in *lon* protease. This permits the β-galactosidase fusion proteins to accumulate to much higher levels than in wild-type hosts (Young and Davis, 1984). Y1090 also harbours pMC9, a pBR322 plasmid including the *lac*I gene encoding for the *lac* repressor. This provides a high level of repression of the foreign protein until an experimentally controlled time. Finally, the *sup*F gene encodes a suppressor tRNA which suppresses the *S100* mutation of λgt11 and permits plaque formation.

Antigen Production and Reactions

General Theory

The basic theory of antibody-antigen interaction is well understood. It is known that antibody recognition of an antigen occurs at specific points on the antigen structure and that only these areas, known as the antigenic determinants (or epitopes), need to

be present for recognition to occur. Most antigens are proteins or protein attached moieties, although nucleic acids and even some non-organic compounds may generate immune reactions. Because of the exclusive dependence upon the antigenic determinant it is possible for an antibody to recognize a segment or part of a polypeptide from the native protein even though the entire protein is not produced. This may occur when the polypeptide corresponds to an antigenic determinant. This is the basis for the immunological detection of expression from recombinants (Skalka and Shapiro, 1976; Sanzey *et al.*, 1976).

Factors Determining Antigenicity

Several factors determine whether such a polypeptide will be recognizable by an antibody. The most important criterion is the primary structure of the polypeptide. The residue composition must match that of the parent protein very closely for any possibility of detection to exist. The second factor is the physical conformation of the polypeptide. In the native protein, regions surrounding the actual antigenic determinant will impart a tertiary structure to it that is essential to the antibody recognition process (Crumpton, 1974). In order for a given polypeptide sequence to function as an antigenic determinant it must be capable of assuming the tertiary structure of the recognition region in the whole protein. This is a major factor in determining the minimum number of amino acids that will result in antibody recognition.

In many cases, when mammalian proteins are made in *E. coli* they are produced as insoluble aggregates of denatured proteins without proper formation of disulphide bonds (Wetzel and Goeddel, 1983). Post-translational modifications to the protein, such as glycosylation, phosphorylation and methylation, also significantly affect the recognition of an antigenic determinant in eucaryotic cells. The most significant of these modifications for determining antigenicity is glycosylation. Glycosylated residues often form an integral part of an antigenic determinant; in the absence of the carbohydrate group the region may no longer be recognized by a given antibody (Katz *et al.*, 1986). All of these factors have an impact on the creation of an antigenically

recognizable polypeptide outside of its normal environment. For a foreign protein expressed in *E.coli* we would not expect any significant post-translational modifications to occur; this has an important impact on the choice of immunological detection strategies.

Immunological Detection of Expressed Proteins

In order to ascertain that successful production of an antigenically active protein or polypeptide has been attained, a method for its detection is necessary. Two very different methods are available to resolve this question dependent upon the choice of antibodies that is made.

Monoclonal Antibodies

The first approach is to use a monoclonal antibody. The primary advantage of using a monoclonal is that the antibody will be specifically targeted towards the single expressed epitope of interest. As a result, background proteins, due to the expression of host or vector genes, will not generate false positive results unless they contain very similar epitopes. However, there are several difficulties with this approach. In order to produce the monoclonal some of the protein of interest must have been previously partially purified to be used as an antibody inoculum. This is difficult for proteins which have been poorly characterized or are present in only small quantities within their native organism. Secondly, the **exact** target epitope for the monoclonal must be produced in the bacterial cell in order for successful detection to occur. Effectively, this means that full-length expression of the foreign protein must be achieved in order to be certain that the monoclonal will be capable of recognizing it. Even this may not be sufficient in the case of recombinant eucaryotic proteins produced in *E. coli* because of the lack of post-translational processing, resulting in the potential absence of native secondary and tertiary structure in procaryotic hosts. As noted earlier, the inability to create disulphide linkages among the component pep-

tides and the resultant formation of denatured protein aggregates may also make the production of a reactive monoclonal difficult. Finally, time and financial constraints make monoclonal antibodies impractical under many circumstances.

Polyclonal Antibodies

Polyclonal antibodies can provide a good, albeit less elegant, alternative and solve many of the problems associated with the monoclonals. However, their use does introduce new difficulties. The major advantage of a polyclonal antibody preparation is that it will recognize multiple epitopes on a given protein. It will also be capable of recognizing not only full-length antigens, but also many partial proteins that contain various regions of antigenicity. This is important since genomic and cDNA inserts will often be less than full-length and thus will code for only a portion of the complete protein of interest. As well as allowing for the detection of partial proteins with respect to primary structure, components of the polyclonal preparation may also recognize polypeptides with deviations in secondary and tertiary structure. For example, a polyclonal antiserum is much more likely than a monoclonal to permit the detection of a protein that is glycolsylated or acytlated in its natural state but lacks these modifications in the cloning host.

By their nature, polyclonal antisera are much simpler and quicker to develop than monoclonal antibodies. They are especially useful when the protein of interest is not well characterized and in general, it is not necessary to affinity purify the polyclonal antiserum (Snyder *et al.*, 1987). This advantage also explains the major disadvantage of the polyclonal detection system — polyclonals will typically lead to the detection of proteins other than the one of interest, particularly if the serum is developed against an impure protein. Many antisera, including those developed in rabbits and pigs (used in these experiments) also contain high levels of anti-*E. coli* antibodies. This can also lead to high levels of background signals during the detection process and special care must be taken to reduce these background levels.

Examples and Comparisons

Both monoclonal and polyclonal antibody preparations have been used successfully in the detection of foreign proteins produced in *E. coli*. Allen and Yeargan (1987) used specific monoclonal antibodies to detect each of six glycoproteins in equine herpesvirus (a close relative of the pseudorabies virus studied in my experiments). Helfman *et al.* (1983) provides one of the earliest examples of using a polyclonal antisera to detect fusion proteins (β-galactosidase-chicken tropomyosin) produced in pUC8; Bröker (1986) used a polyclonal antiserum preparation to detect products of a β-galactosidase-herpes simplex virus 1 fusion protein. There was little data on the relative success of using monoclonal versus polyclonal antisera in immunoscreening studies at the time that my work was initiated. Since that time the literature has favoured the polyclonal approach (or at least the use of multiple monoclonals recognizing different determinants) in most experiments where partial proteins are likely to be produced or where the lack of post-translational modifications is likely to affect the number of epitopes on the fusion protein (Snyder *et al.*, 1987).

The Viruses

Economic Importance

Both pseudorabies virus (PrV) and the various strains of Rous associated virus (RAV) are economically important agricultural pathogens. PrV infects swine, causing Aujesky's disease, a degenerative neurological condition which causes high losses within infected herds in many countries around the world. Pseudorabies is classified as an exotic virus in Canada and government agencies monitoring strict importation regulations have effectively limited its penetration into this country. In most countries where the virus is present, attenuated strains are used to vaccinate swine herds. Because of the attendant risk of infection Canadian herds are not vaccinated; rather should a herd become infected then all pigs in the herd are slaughtered. At the time of my experiments, methods for the detection of pseudorabies virus infection in

swine herds were considered to be time-consuming and not perfectly reliable; an enzyme linked immunosorbent assay (ELISA) was the most prevalent method although some serum neutralization testing was also in use (Dr. Gilles Dulac, Animal Diseases Research Institute, personal communication).

The exotic status of the pseudorabies virus confers an extra degree of complexity upon the research as supplies of viral material are limited and specific restrictions are placed upon working locations and conditions. Conversely, because of its special status in Canada very stringent detection methods for the virus are required which do not exist and are unlikely to be developed by any group other than Canadians. The best way of obtaining the requisite sensitivities of detection is to produce a non-infective subset of the viral envelope proteins which include the antigenic determinants. This could then be used in serum antibody testing of animals suspected of being infected with the virus. If this subset could be produced using a readily available expression vector then test materials could be produced easily and inexpensively.

Rous associated viruses are members of the avian sarcoma and leukosis virus group of Retroviridae. Different strains infect fowl both endogenously and exogenously and both vertical (mother to offspring) and horizontal (chick to chick) transmission is possible (Coffin, *et al.*, 1978). Although less pathogenic than the oncogenic sarcoma viruses, infection with RAV causes a measurable decline in egg and meat production even in the absence of other externally obvious symptoms (Dr. Parvis Sabour, ADRI, personal communication). A variety of host range specific subgroups can be identified (see below for immunological criteria) and although there are adequate detection procedures for RAV they are not capable of distinguishing among the variety of subgroups that are recognized as causing damage of different degrees. As well, little is known about the manner in which host-range specificity is established nor about type-specific immunological differences among particular strains. Since this could have a major impact upon any immunization program, additional information in this area would be very helpful.

Pseudorabies Virus

Pseudorabies virus is a herpesvirus containing a class 2 DNA molecule (Honess and Watson, 1977), consisting of two unique sequence segments with the shorter of the two (U_s) bracketed by inverted repeats. Inversion of the U_s sequence allows two different isomeric forms (I_L and I_S) of the genome to exist in a population. The total size of the double-stranded DNA molecule is approximately 9.2×10^7 daltons (approx. 140 kb) in size (Ben-Porat, 1982). The architecture of the virus is similar to that of other herpesviruses. It is constructed of an isocahedral capsid containing the genomic DNA surrounded by a protein and carbohydrate envelope. The envelope contains approximately 50% of the total viral protein, including all of the glycoproteins (Kaplan and Ben-Porat, 1970). There are four major glycoproteins (gIIa, gIIb, gIIc and gIII) and three minor ones (gI, gIV and gV) that exist in a variety of complexes with one another and with at least one non-glycosylated protein (Hampl *et al.*, 1984).

At the time that the experiments outlined in this thesis were conducted the exact arrangement and functions of the various proteins was not known. However, the glycoproteins from the closely related herpes simplex virus (HSV) had been more completely characterized. In that case it has been shown that at least some of the glycoproteins are responsible for the binding of the virus to the cell-surface (Fuller and Spear, 1985) and fusing to infected cells (Noble *et al.*, 1983). Furthermore, individual HSV glycoproteins have been shown to interact with specific immune system components (Baucke and Spear, 1979; Friedman *et al.*, 1984; Spear, 1985). Because of the strong evolutionary linkages between all of the herpesviruses most literature assumes a high degree of similarity between the herpes simplex and pseudorabies viruses. A number of the PrV genes have been shown to have similarity with HSV genes but there appears to be some differences in the post-transcriptional modifications of the gene products (Robbins *et al.*, 1984; Robbins *et al.*, 1986; Petrovskis *et al.*, 1986). As well, it has been shown that while a majority of the PrV genome is

colinear with the I_L arrangement of the HSV I genome, at least two genes are inverted relative to one another in the two species of virus (Ben-Porat *et al.*, 1983). At the time that the work outlined in this thesis was conducted this was another incentive to more fully document the PrV proteins, their antigenic properties and their gene identities without reference to corresponding results in herpes simplex virus I.

Rous Associated Virus

The group of Rous associated viruses are closely related to Rous sarcoma virus (RSV) with the primary difference being their lack of the *src* gene, thereby rendering them transformation defective. Their structure is typical of avian retroviruses. A glycoprotein-containing viral envelope derived from the plasma membrane of the host cell surrounds a single stranded RNA molecule which is 9.3 kb long in RSV. The two viral glycoproteins extend from the contour of the envelope to form a spike and ball arrangement joined by disulphide bonds (Bolognesi *et al.*, 1972; Bolognesi, 1974, Leamnson and Halpern, 1976). Although these protrusions are often a major locus for subgroup specific antigenicity and are responsible for the host range of the virus (Weiss, 1976) most, if not all, viral proteins contain both type and subgroup specific antigenic determinants (Strand and August, 1974).

The various strains of RAV and RSV are classified into five subgroups (in chickens), A through E, as defined by a series of criteria. The first classification criterion is based on the fact that different isolates of virus display different characteristics of infectivity when used to infect genetically defined chicken lines (Vogt and Ishizaki, 1966; Vogt, 1977). Interference patterns between viral isolates also define subgroups (Weiss, 1969; McMahon *et al.*, 1986). For example, infection of chick embryo fibroblast cells with RAV-0 (a type E avian sarcoma virus) appears to confer immunity to infection by other subgroup E virus as well as subgroup B virus. However, subgroups A and C are still able to infect the cells (Crittenden, 1968; Vogt,1977). Interference appears to be a result of blockage of the cellular receptors required for entry (Robinson *et al.*, 1967; Hanafusa, *et al.*, 1970). Finally, neutraliz-

ing antibody cross-reactions provide phenotypic differentiation between the sub-groups; immune chicken sera show reaction specificity to those viruses in the originally infecting sub-group with occasional lesser reactions to some other sub-groups. Within each subgroup, specific strains may belong to one of several immunologically defined types (Ishizaki and Vogt, 1966).

The genome of the avian leukosis virus contains three genes — *gag*, *pol* and *env*. These genes are mapped in the order 5'- *gag* - *pol* - *env* - polyA - 3'. In RSV an additional gene, *src*, is found immediately downstream of the *env* gene. The *gag* gene codes for the proteins of the viral core, *pol* encodes the reverse transcriptase enzyme and *src*, when present, codes for the protein responsible for neoplastic transformation of the host cell. The *env* gene encodes the viral envelope proteins. Each of these genes may encode a large precursor polypeptide which is post-translationally processed by the host to create the active proteins (see review by Bishop, 1978). In the avian leukosis and sarcoma virsuses a single 95 K precursor protein is coded by *env*. It is cleaved and processed to form two glycoproteins — gp85 and gp37 (England *et al.*, 1977; Moelling and Hayami, 1977; Hayman, 1978; Klemenz and Diggleman, 1978). The amino termini of both of the mature glycoproteins are located within the *env* gene (Hunter *et al.*, 1983; Schwartz *et al.*, 1983). The two glycoproteins are linked together in a 1:1 ratio by disulphide bonds (Leamnson and Halpern, 1976) in the viral envelope. Together, they form the "spike and ball" arrangement protruding from the envelope with gp85 as the "ball" and gp37 serving as the anchoring "spike" (Bolognesi *et al.*, 1972; Bolognesi, 1974).

It is now clear that the *env* gene is responsible for encoding the proteins responsible for host-range specificity in chickens; that is, it encodes the differences in chicken cell receptor recognition which mediate subgroup specificity. The original evidence for this came from T1 oligonucleotide mapping studies of a variety of viral isolates (Joho *et al.*, 1975; Coffin *et al.*, 1978), which showed that segments of the gp85 region of the *env* gene could be mapped using subgroup-specific oligonucleotides. Subsequently, heteroduplex analysis of viral isolates from different subgroups demon-

strated a region of reduced homology in the *env* region (Hu *et al.*, 1978). More recently, molecular recombinants have been used to show that a recombination event in the *env* region between viruses of two different subgroups can extend the host range of the virus to include both subgroups (Dorner *et al.*, 1985) and that type-specific neutralization is associated with the gp85 polypeptide (Bova *et al.*, 1988).

Finally, direct sequence data has clearly shown that while there is generally a very high degree of conservation throughout the genomes of various isolates of avian

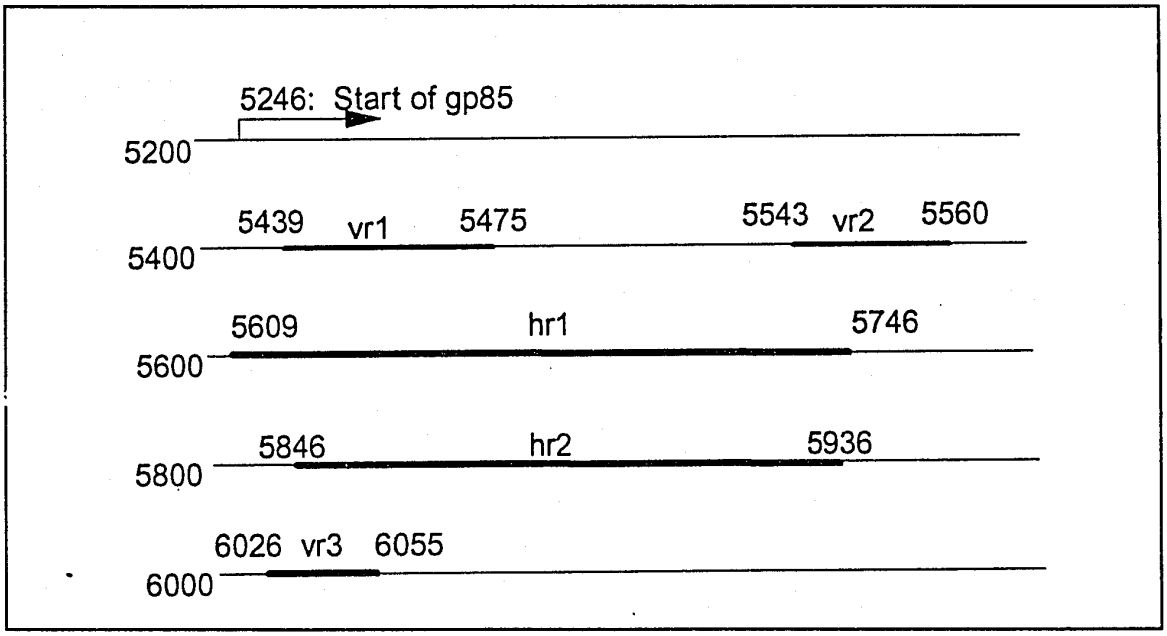

Figure 2: Hypervariable Regions in the Rous Sarcoma Virus: Determined by sequence comparison among strains of various subgroups (Bova *et al.*, 1988).

retroviruses (the exception being the presence of *src* in the avian sarcoma viruses) several well-defined, highly variable regions are located within the *env* regions of most subgroups. The locations of these "hypervariable" regions are outlined in the above diagram.

Summary of Intentions

My goal was to isolate clones expressing epitopes from pseudorabies virus or one of the Rous associated virus strains in *E.coli*. Since I was using immunological detection methods I expected to primarily isolate DNA segments encoding portions of surface glycoproteins from the viral envelopes along with some capsid polypeptides. The cloning strategies that were followed with the pseudorabies virus relied almost exclusively on immunological detection of recombinants of interest. This decision was made as much out of necessity as out of choice since when this project was started I could not gain access to any PrV DNA in Canada. The initial PrV library was constructed in France. In contrast, when the work with Rous associated virus began, DNA for several strains of the highly homologous Rous sarcoma virus was available. I was fortunate to be able to use a previously constructed plasmid, pSR-XD2 (Cross and Hanafusa, 1986) as a source of probe DNA in nucleic acid hybridizations. pSR-XD2 was constructed by inserting most of the Rous sarcoma virus genome into pBR322. The restriction map and protein encoding regions of this plasmid are shown in Appendix A.

The enormous economic consequences of the pseudorabies and avian leukosis viruses in Canada make the study of both extremely important. The study of each of the two viruses also promises to hold extended benefits with respect to our knowledge of the herpesviruses and retroviruses. Finally, the ability to produce antigenically active, non-glycosylated viral envelope/capsule proteins from *E. coli* has not yet become the simple process that it undoubtedly will (although during the course of research into this thesis, acceptance of this idea has become common and other demonstrations of its validity have been achieved). Therefore, a demonstration of this extremely powerful technique using these viruses would be significant not only on the basis of the impact of its application but also as further confirmation that the methods involved are effective.

Materials and Methods

I. Bacterial and Virus Manipulations

Bacterial Manipulations

Culture Media

A variety of different culture media were used for different bacterial strains and for different end uses of the bacteria. Basic culture medium was LB medium[2]. This was used in most broth and plate media used to grow *E. coli* strains C600, Y1090, Y1089, and HB101. DH5α was grown on SOB media. Y1090 and Y1089 infected with lambda phage were plated on NZ media. Transformed cells to be used for protein preparation were grown in a rich minimal medium (Murialdo and Simonovitch, 1971).

Ampicillin (100 μg/ml) and tetracycline (15 μg/ml) were used for selection of appropriate plasmid-containing resistant colonies and in some broth media where it was essential to ensure that selection maintained the presence of the plasmid through generations.

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[2] All solution compositions may be found in the Table 1 at the end of this section.

Viral Manipulation

Collection of Virus

Due to its exotic nature and government restrictions upon its use and storage, pseudorabies virus was never directly manipulated in this study. Previously purified PrV DNA was provided by the Animal Diseases Research Institute (ADRI).

RAV-2 viral RNA was prepared by modifications to the method of Murphy (1987). Roller bottles of chick embryo fibroblasts after a three week infection with the virus were provided by Dr. Lloyd Spencer (ADRI). The viral supernatant was separated from any remaining cell debris by centrifugation at 12,000 xg for 10 minutes at 4°C and then decanted. Viral particles were precipitated through the successive additions of NaCl to 2% (w/v) followed by PEG-8000 to 8% (w/v). Precipitation was carried out at 4°C for 4 hours. Virus was collected by centrifugation at 12,000 xg for 10 minutes at 4°C. The pellet was resuspended by rotating in 100 mM NaCl, 10 mM Tris, 1 mM EDTA pH 7.5 for 20 minutes at 4°C. Following pronase addition to 2 mg/ml, the solution was incubated at 37°C for 30 minutes and then spun through a sucrose step gradient (20%/70% in TES) at 98,000 xg for 1 hour. The band appearing at the lower gradient interface was collected by fractionation and diluted 5 times in TE. Virus particles were collected by centrifugation at 90,000 xg for 30 minutes, the pellet resuspended in TES and virus concentration determined by spectrophotometry at 260 nm (1 A_{260} = 50 μg/ml).

Lysis buffer was added in a 2:1 ratio, Proteinase K was added to a final concentration of 500 μg/ml and the viral solution was incubated at 37°C for 30 minutes. Two phenol/$CHCl_3$ extractions were performed followed by a $CHCl_3$ extraction. NaAc was added to 300 mM and RNA was precipitated overnight in two volumes of 99% ethanol at -20°C. Viral RNA was recovered by centrifugation and, in preparation for cDNA synthesis, was resuspended in water.

II. Plasmid & Phage: Preparation and Manipulation

DNA Manipulation

Plasmid Preparation

Rapid

Plasmids were prepared for quick analysis and confirmation of insertions/excisions, etc. using the method of Birnboim and Doly (1979).

Large Scale

Large scale plasmid preparations (Kahn et al, 1979) were performed for any plasmids which were to be subjected to further experimentation, eg. ligation, sequencing, etc.. Briefly, transfected bacterial cells were grown overnight in L-broth at 37°C with agitation. Cultures were centrifuged at 4,000 xg for 15 minutes and the cell pellet was resuspended in 10 ml of 20% sucrose, 40 mM Tris pH 7.5. The resuspended cells were lysed by incubating with 30 mg/ml lysozyme on ice for 10 minutes, followed by a 10 minute incubation on ice with 20 ml 200 mM EDTA pH 8.0. Lysis was completed with the addition of 10 ml of 2% Triton-X. Cellular debris was removed by centrifuging the lysate at 35,000 rpm for 45 minutes in a Beckman 42.1 Ti rotor. 1.03 g/ml Cesium chloride and 1.3 mg/ml ethidium bromide were added to the supernatant and the plasmid DNA was then equilibrated on this gradient at 44,000 rpm for 16 hrs. in a Beckman 50 VTi rotor.

After equilibration the supercoiled DNA was removed from the gradient; ethidium bromide was removed by extraction with either CsCl saturated isopropanol or isobutanol equilibrated with TE. The DNA solution was dialysed for two hours against 1 l of TE to remove CsCl. Further purification was achieved by treatment of the DNA

solution with 1 μl of 10 mg/ml RNAase A for 1 hr. at 65°C followed by incubation with 100 μl/ml Proteinase K and 0.2% SDS for 2 hrs. at 37°C. The DNA was precipitated in 100 mM NaCl and 2 volumes 99% ethanol at -20°C. The precipitate was collected by centrifugation at 5,000 xg and then lyophilized. Resuspension was in TE and the final concentration of DNA was determined by spectrophotometry at 260 nm.

Lambda Phage

Lambda Phage Preparation

Initial preparations of the bacteriophage lambda were made by infecting suitable host cells from stationary phase cultures in TM buffer and plating on NZCYM-1.2% agar media. Incubation was typically for 6 hrs. at 37°C. Individual phage plaques were then picked and eluted into 1 ml of SM buffer with several drops of $CHCl_3$. The phage solution was allowed to stand at room temperature to permit the phage to disperse into solution.

To prepare the stock phage solutions 100 μl of phage-infused SM was added to 100 μl of a stationary phase culture of an appropriate strain of *E. coli* (Y1088, Y1090) along with 4 ml of NZCYM broth. This was then agitated with aeration at 37°C until lysis was apparent (typically 5 to 6 hours after infection). 100 μl of $CHCl_3$ was added and agitation was continued for 15 minutes to complete the lysis of all infected cells. Cellular debris was removed by centrifugation at 4,000 xg for 10 minutes at 4°C.

If large quantities of the bacteriophage were required then 500 ml cultures containing $1x10^{10}$ bacterial cells in NZCYM media were infected with the small lysate with a multiplicity of infection of 0.5. This culture was agitated with aeration until lysis was visible (5 to 7 hours). 5 ml of chloroform and a small amount of DNAase (approximately 1 μg/μl) were added and agitation was continued for 15 minutes. Cell debris

was removed by centrifugation at 8,000 xg for 10 minutes at 4°C; the resultant solution was filtered through cheesecloth.

The bacteriophage particles were precipitated from solution through the addition of NaCl to 500 mM followed by the addition of PEG 8000 to a final concentration of 10%. This was allowed to stand for 1 hr. at 4°C and the phage was then collected by centrifugation at 8,000 xg for 10 minutes at 4°C. The phage particles were then resuspended in 10 ml SM buffer and CsCl was added to 0.75 g/ml. The phage were centrifuged to equilibration on the CsCl gradient for 16 hours at 45,000 rpm in a 50 VTi rotor. The band of phage was removed from the gradient and dialysed against SM to remove CsCl.

Small-Scale Lambda Phage DNA Preparation

Preliminary analysis of insert DNA was performed on DNA preparations from small lysates of recombinant phage. Recombinant phage solutions were first purified through incubation with 10 μg/ml DNAase I at 37°C for 1 hr. The phage particles were then precipitated by the sequential addition of NaCl (to a final concentration of 500 mM) and PEG 8000 (to a final concentration of 10%) followed by 1 hour incubation at 4°C. The precipitated phage were collected by centrifugation and the pellet was resuspended in 0.2 ml SM buffer. This was further purified by two extractions with $CHCl_3$. Phage lysis was accomplished by the addition of EDTA to a concentration of 10 mM; sequential extractions with equal volumes of phenol, phenol/$CHCl_3$ and $CHCl_3$ were performed to purify the DNA. The phage DNA was precipitated in 300 mM NaCl and 2 volumes 99% ethanol at -20°C and then collected by centrifugation. After lyophilization the pellet was resuspended in TE.

Large-Scale Lambda Phage DNA Preparation

For preparation of vector phage and detailed analysis of inserts, higher quality DNA obtained from CsCl gradients was used. Lambda phage was prepared as detailed

above (pg 21). The phage protein coat was removed from purified phage in SM buffer by treatment with 10 mM EDTA pH 8.0, 0.3% SDS and 100 mg/ml Proteinase K for 1 hr. at 37°C. The DNA was extracted with multiple phenol/$CHCl_3$ extractions and the final aqueous phase was dialysed against TES overnight at 4°C. DNA concentration was estimated by spectrophotometry at 260 nm.

Packaging of Recombinant λgt11 DNA

Recombinant constructions consisting of λgt11 vector and either pseudorabies or Rous associated virus DNA inserts were packaged into bacteriophage particles to allow testing of the antigenicity of any expressed proteins. Packaging reactions typically contained 0.5 μg of DNA in a total volume of 10 μl. Packaging system extracts were purchased from Boehringer Mannheim and later from Promega Biotech. Packaging was performed as indicated in the manufacturers' protocols.

Subcloning

All protocols are from Maniatis (1982) unless otherwise indicated.

Restriction Digestion

All nucleic acid restriction digests were performed as per restriction enzyme manufacturers' specifications. Buffers used were either exactly as specified by the manufacturer or generic high, medium, or low salt buffers (see Table 2). Enzymes were purchased from New England Biolabs, Boehringer Mannheim, Promega Biotech and Pharmacia. DNA to be used as a vector for subsequent ligations was treated with calf intestinal alkaline phosphatase (Boehringer Mannheim). EDTA and SDS were added to 20 mM and 0.5% respectively and the reaction heated at 65°C for 10 minutes. During work with RAV-1 and RAV-2 the vector was purified with GeneClean; work with pseudorabies virus used vectors that were purified using successive ex-

tractions with phenol, phenol/chloroform and chloroform followed by ethanol precipitation at -20°C.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed as per standard protocols. Gels were composed of .7% to 1.2% agarose in TAE or TBE; higher percentage gels were used for the separation of DNA fragments smaller than 1 kb. Gels intended for fragment extraction through GeneClean procedures were always run in TAE buffer. Gels were stained for 10 minutes in ethidium bromide followed by destaining in gel buffer and visualization with a shortwave UV transilluminator.

Gel Fragment Purification

Restriction fragments were passed over TAE agarose gels ensuring sufficient separation from surrounding fragments. Fragments were removed from the agarose gel using the GeneClean glass bead protocols and reagents (BIO 101). After extraction fragments were stored in TE buffer.

Ligation

Typically, insert and vector DNA were mixed in a 3:1 molar ratio in a buffer of 10 mM Tris, 10 mM $MgCl_2$, 2 mM EDTA, 1 mM ATP, 100 μg/ml BSA and 1 mM DTT. 1 to 2 units of T4 DNA ligase (Boehringer Mannheim, Pharmacia) were used for each ligation. For blunt-ended ligations the reaction was left overnight at 15°C; cohesive end ligations were incubated at room temperature for 4 hours. In some cases the restriction enzymes used in preparing the insert and vector DNA were incompatible. Under these conditions the protruding ends left after restriction enzyme digestion were filled using the Klenow fragment of DNA polymerase I. Ligation products were either examined on agarose gels or directly transfected into competent *E. coli* cells.

Linker Addition

Testing of protein expression was accomplished by inserting viral fragments into λgt11 at the *Eco*RI site (Young and Davis, 1983). Since insert fragments rarely had ends that were compatible with the *Eco*RI cleaved λgt11 additional steps prior to ligation were required. The insert fragment was prepared to have blunt ends (either through restriction enzyme digestion or by repair). Methylation of internal fragment *Eco*RI sites was then performed using methylase from ProMega Biotech and reagents shipped with it. All manipulations were performed as per the manufacturer's instructions. Methylase reagents were removed either by phenol/chloroform extraction or by passing the viral DNA across an agarose gel and then purifying it using the GeneClean method (pg. 24).

Following this, *Eco*RI linkers (New England Biolabs, ProMega Biotech) with a sequence of d(pGGAATTCC) were ligated to the insert fragment. Ligation was performed as per standard conditions with the exception that linker sequences were in a 100:1 molar excess over viral DNA fragments. Ligation was terminated by addition of EDTA to 10 mM and heating at 65°C for 15 minutes. The linker sequences were then cleaved with *Eco*RI resulting in cohesive ends. Cleaved linker fragments were separated from viral DNA by passing the ligation mixture across a high percentage agarose gel and excising and purifying only the band corresponding to viral DNA. This fragment, with linkers attached, was used for ligation with λgt11 arms.

Preparation and Transformation of Competent Cells

A media broth was inoculated with a single colony and incubated until the beginning of logarithmic growth (as determined by light absorbance of the culture). Cell walls were then perforated through successive treatment of the culture with $CaCl_2$ and either $MgSO_4$ or $MnCl_2$. If maximum efficiency of transformation was desired the competent cells were used immediately; for routine work the cells were aliquoted and frozen at -70°C in 15% glycerol. Transformation was accomplished by adding 50 to

200 ng of DNA to 0.2 ml of competent cells. DNA binding to the cell surface was allowed to occur by standing the mixture on ice for 40 minutes. The cells were then heat shocked at 37°C for 5 minutes; culture media was added and growth was initiated by incubating with aeration for 60 to 90 minutes. The cells were then plated on selective media appropriate to the subcloning (Hanahan, 1985). Using these methods transformation efficiencies of between 10^6 and 10^9 transformants/μg of DNA were achieved.

Glycerol Gradients

Glycerol gradients were composed of 10-40% glycerol in 10 mM Tris-HCl, 1mM EDTA, 300 mM sodium acetate. Total gradient volume was 2.4 ml. 25 μg DNA including approximately 1 μg of end-labelled DNA was loaded onto gradients and spun 21 hrs. at 38K in SW41 Beckman rotors. Gradients were collected into 0.3 ml fractions. The fractions containing DNA were identified by liquid scintillation counting. The size of DNA fragments in these fractions was determined by passing aliquots of the fraction over an agarose gel and then Southern blotting the gel. Autoradiography allowed the identification of the fractions containing DNA of the appropriate size range.

cDNA Preparation

cDNA was prepared for RAV-2 RNA using the method of Rutledge *et al.* (1988). Prior to beginning the cDNA preparation a sample of the RNA was run over an agarose gel to verify its quality. Because complete RNA was collected from the RAV-2 virus particles no initial preparation of poly(A+) RNA was performed. All cDNA synthesis work was done in the laboratory of Dr. Martin Tenniswood at the Faculty of Health Sciences, University of Ottawa.

2 to 5 μg of isolated RNA was added to a mix of 8 μl reverse transcriptase buffer, 5 μl 1 mg/ml oligo dT, 3 μl 10 mM dCTP, 3μl 10 mM dATP, 3 μl 10 mM dGTP, 3 μl 10 mM TTP and 5 μl ^{32}P-dATP. This was heated at 60°C for 2 minutes and then cooled on ice for 5 minutes. 2 μl of reverse transcriptase (Life Sciences 27 Units/μl) was added and first strand synthesis was allowed to proceed for 45 minutes at 42°C. Enzyme activity was stopped by heating at 65°C for 15 minutes. Second strand synthesis was accomplished by the addition of 10 μl nick translation buffer, 1.25 μl RNAase H (Pharmacia, 800 Units/ml), 3 μl Pol1 (Life Sciences, 20 Units/μl) and water to a final volume of 100 μl. This was incubated at 15°C for 2 hours.

The reaction was stopped by extraction with phenol/$CHCl_3$/isoamyl alcohol (25:24:1) in equal volume followed by a second extraction with 24:1 $CHCl_3$/isoamyl alcohol. 1 μl of cDNA was counted in 100 μl of water and 5 μl of scintillation fluid and the yield of double stranded cDNA was estimated. Typically 6 μg of viral material resulted in the eventual production of 200 ng of double-stranded cDNA. We theorized that the relatively low yield compared to other work done in Dr. Tenniswood's laboratory was due to the poor condition of the starting RNA.

II. NUCLEIC ACID ANALYSIS

Nucleic Acid Probes

Radioactively labelled fragments of DNA were used as probes to identify homologous nucleic acids and as markers for various processes. Three types of radioactive labelling were performed.

Nick Translation

Nick translations were performed according to the methods of Rigby *et al.* (1977) and Kelly *et al.* (1970). Typically 100 ng of purified DNA was mixed with 100 μCi of α^{32}P-dCTP and 100 μCi of α^{32}P-dATP in a total reaction of 100 μl (25mM KH_2PO_4, pH

7.4. 5mM $MgCl_2$, 10 μM each dGTP and dTTP, 50 μg/ml BSA). The DNA was nicked through the addition of activated DNAase I (approx 1 pg) for 3 minutes at 15°C. 5 units of DNA polymerase I (Boehringer Mannheim) was then added and the reaction was incubated for 60 minutes at 15°C. After incubation the reaction was stopped by heating at 65°C for 10 minutes in the presence of 100 mg/ml denatured salmon sperm DNA in HENS buffer. Nick translated DNA was purified from unincorporated nucleotides by passing the reaction over a 10 ml G-50 Sephadex column equilibrated with HENS buffer and collecting the appropriate fraction. Prior to using the DNA for hybridization it was boiled for three minutes to ensure denaturation. Specific activities of 10^7 to 10^8 cpm/μg were typically achieved with this method.

Random Oligonucleotide Synthesis

For work done later in the course of study double stranded probes were made following the random priming method (Feinberg and Vogelstein, 1984) using a kit from Amersham Corp.. This procedure produced smaller labelled fragments than the nick translation method with a high specific activity — typically 10^9 cpm/μg. After labelling the DNA fragments with ^{32}P dCTP the reaction mixture was passed over a Sephadex G-50 spin column as described by Maniatis *et al* (1982).

End-Labelling

In several experiments the quantities of DNA being used were too small to allow for standard monitoring of the progress of DNA through various stages using agarose gels. In order to follow these reactions a small quantity of end-labelled DNA, of a similar size, would be added to the reagents. End-labelling was accomplished in the manner of Davies (1982). 1 μg of digested DNA was mixed with 1 μl $\alpha^{32}P$-dATP (800 Ci/mmol)(or other suitable nucleotide) in a buffer of 10 mM of each of the other three dideoxy nucleotides, 50 mM NaCl, 7 mM Tris-HCl pH 7.4, 7 mM $MgCl_2$, 1 mM DTT. 5 units of DNA polymerase I, (Klenow fragment) were added and the reaction was allowed to proceed at room temperature for 30 minutes. The reaction was

stopped by heating to 70°C for 10 minutes. If required, unincorporated nucleotides were then removed by passing the reaction mixture over a G-50 Sephadex column.

Nucleic Acid Analysis

Southern Transfer

100 to 200 ng of DNA to be hybridized for homology analysis was transferred to membranes after digestion and electrophoresis through agarose gels. Transfer was accomplished using the methods of Meinkoth and Wahl (1982) and Reed and Mann (1985). Both modified nitrocellulose (Zetabind, BioRad) and nitrocellulose (Schleicher & Schuell) were used as support mediums. Transfer to Zetabind was carried out in 25 mM $NaPO_4$ pH 6.5 while transfer to nitrocellulose was carried out in 20X SSC.

Northern Transfers

Five micrograms of RNA per sample were electrophoresed on 1.5% denaturing agarose gel containing formaldehyde as described in Maniatis *et al.* (1982). Lanes containing the *E. coli* rRNA markers were stained and the unstained portion of the gel containing the sample RNAs was used for transfer. Following a one hour wash of the gel in distilled water, an overnight transfer was performed onto a Biotrans membrane in 20X SSC. The RNA was crosslinked to the membrane by UV treatment following transfer, and the gel was stained as described in Maniatis *et al.* (1982) to verify complete transfer of the RNA.

DNA Hybridization

Hybridizations were carried out following the procedures of Thomas (1980) and Maniatis (1982) with modifications as suggested by the suppliers of the support media. Southern transfer filters were prehybridized in 5X Dernardt's solution, 5X SSPE, 0.1% SDS, 0.2 mg/ml denatured salmon sperm DNA or 0.5 mg/ml denatured

herring sperm DNA. In order to increase the stringency of hybridization 6% to 10% Dextran sulfate (Wahl, 1979) was added to the mixture in some cases. Prehybridization was performed at 42°C in the presence of 50% formamide or at 65°C in its absence for 4 hrs.

After the completion of prehybridization all fluid was removed from the filter and replaced with fresh solution; approximately 10^6 to 10^7 cpm of radiolabelled probe was then added. The filter was incubated overnight at either 42°C or 65°C. After the completion of hybridization, the filter was washed according to the desired stringency. In general, the filter was washed twice in 2X SSC, 0.1% SDS for 15 minutes at room temperature, followed by an additional two washes in 0.1 to 2 X SSC, 0.1% SDS at 65°C. In later experiments some homology was discovered between sequences in pGEM4 and RAV. In order to prevent undesirable hybridization very high stringency washes (room temperature washes were followed with two 10 minute washes in 10X SSC at 50°C) were used.

The filters were allowed to dry slightly before being wrapped in Saran wrap. Autoradiography was at -70°C for 2 hrs. to 4 days in the presence of an intensifying screen, depending upon signal strength.

Colony Hybridization with Nucleic Acid Probes

Colony hybridizations were performed as per the manufacturer's instructions for Hybond (Amersham). Competent bacterial cells were transfected with the DNA of interest and plated on selective media as described above (pg. 25). After overnight growth at 37°C the plates were overlaid with circles of Hybond nylon membrane. After 1 minute of binding on the agar the membrane was removed and placed on 3MM Whatman paper soaked in 1.5M NaCl, 500 mM NaOH for seven minutes to denature the DNA. The filters were then neutralized on 3MM Whatman soaked in 1.5M NaCl, Tris-HCl pH 7.2, 1 mM EDTA for 7 minutes. The filters were washed by

laying them on Whatman paper soaked in 2X SSC. Finally the DNA was fixed to the nylon membrane by exposure to UV light for five minutes.

Hybridization of a radioactive probe was performed by incubating the filters in a buffer of 1 M NaCl, 50 mM NaPhosphate pH 7.0, 5 mM EDTA, 0.2% SDS, 0.1 mg/ml herring sperm DNA and 5X Dernhardt's Solution. 10 ml of buffer was used for each filter and 10^6 counts of radioactively labelled denatured DNA probe were added. This was incubated at 65°C overnight. The filters were then sequentially washed as for normal DNA hybridizations. Additional, more stringent washes were performed if high levels of non-specific radioactivity could be detected using a handheld Geiger counter. Autoradiography was at -70°C for 4 to 48 hrs. in the presence of an intensifying screen.

III. Immunological Studies

Immunoblotting

Throughout the course of the work several different techniques were used for the immunological detection of the production of proteins from various types of constructions. All of these followed the same general process.

1/ DNA of interest was subcloned into plasmid or phage vectors such that its expression would be under the control of the vector DNA *lac* operon control sequences.

2/ Recombinant phage or plasmids were transfected/ transformed into various cell lines. After a period of growth, IPTG was added to the environment to induce the production of fusion proteins. Typically this induction was done by overlaying the media with an IPTG saturated nitrocellulose filter. This had the effect of allowing the

simultaneous production and transfer to the nitrocellulose filter of the expressed proteins.

3/ After adsorption, unused binding sites on the membrane were saturated (blocked) with an immunogenically non-active compound to prevent antibody probes from binding directly to the membrane.

4/ The membrane was probed with an antibody specific for the protein of interest. This antibody was previously incubated with concentrated *E. coli* proteins in order to reduce the binding of any contaminants in the serum to *E. coli* proteinatious matter.

5/ The membrane was incubated with a secondary antibody. This antibody was conjugated with an enzyme or with compounds providing for the binding of multiple detection reagent molecules.

6/ The colouring agent capable of being activated by the enzyme conjugated to the 2° antibody or a radioactively labelled detection reagent able to complex with the 2° antibody was added and positive plaques were identified.

Colony Immunoblotting

Colony immunoblotting was only performed during the pseudorabies virus experiments. It was performed essentially as outlined above. Either C600 or HB101 strains of *E. coli* were transformed with recombinant pUC8 plasmids created by ligating fragments of PrV homologous DNA (extracted from a λgt10 library) into the *Eco*RI site. After plating on LB media, colonies were grown to a small size. These were then replica plated onto similar selective agar plates. The original plates were overlaid with IPTG soaked nitrocellulose filters and incubated at 37°C for four hours. After that time the filters were removed and the colonies were lysed by a combination of the methods of Hsu *et al.* (1985) and Koenen *et al.* (1983).

Non-specific binding sites were blocked with TBS and 3% gelatin for 30 minutes with agitation. After removing the blocking solution by washing in TBS-Tween the primary antibody solution was added to the filters. Primary antibody solution in the pseudorabies virus experiments consisted of 0.1% crude serum antibody extracted from swine (provided by Dr. Gilles Dulac, Animal Diseases Research Institute) in TBS-Tween with 1% gelatin that had been incubated with 1% to 10% *E. coli* lysate to block non-specific antibodies.

After incubation with primary antibody for three to six hours the filters were again washed with TBS-Tween. Biotinylated anti-pig IgG (Cappel Laboratories) was added and incubated for two hours[3]. It was then removed with TBS-Tween; Tween residues were removed by a final fifteen minute wash with TBS. Either ^{125}I or ^{35}S conjugated with streptavidin (Amersham) was then added and the filters were incubated for thirty minutes. After final washing in TBS the filters were air dried and autoradiographed.

Plaque Immunoblotting

Plaque immunoblotting was performed in much the same manner as colony immunoblotting. The methods used for this procedure were derived from the BioRad Express-Blot kit and all components except antibodies were provided by BioRad. Initial work with the pseudorabies virus was performed using the pig serum antibodies (provided by Dr. Gilles Dulac) as the primary antibody with biotinylated goat anti-pig secondary antibodies (Cappel Laboratories). Detection was accomplished using streptavidin-^{35}S (Amersham).

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[3] Biotinylated antibodies were used in conjunction with streptavidin conjugated detection materials in order to amplify the signal. Up to seven streptavidin molecules may bind to each biotin molecule; in this way a single occurrence of primary-secondary antibody reaction results in the fixation of seven radioactive or chromogenic molecules (conjugated with the streptavidin molecule) to the reaction location.

Experiments with Rous sarcoma virus used chicken serum antibodies provided by Dr. Lloyd Spencer (ADRI). Rabbit anti-chicken conjugated with horseradish peroxidase (Cappel Laboratories) was used as the secondary antibody. Detection was based upon a colour development reaction. After removal of unbound secondary antibody, filters were incubated with 0.5% diaminobenzidine, 0.2% H_2O_2 in TBS until colour development was observed. The colour reaction was stopped by removing the colour development reagent and washing the filters in water.

Protein Isolation and Detection

Protein Isolation

JM109 cells were rendered competent as described above (pg. 25). 500 ml of rich minimal broth was inoculated with a single colony and incubated with aeration overnight. After the culture entered a stationary growth phase, cells were removed by centrifugation. The pellet was resuspended in 500 μl of 50 mM Tris pH 8.0/20% sucrose. Cells were lysed by adding 3 mg of lysozyme in 100 μl water and incubating on ice for 10 minutes. 250 μl of EDTA pH 8.0 was then added, followed by the addition of 50 μl of 2% Triton-X100. Cell debris was removed by centrifugation at 30,000 rpm for 45 minutes in a Beckman 42.1 Ti rotor. The supernatant was recovered, $CaCl_2$ and NaCl were added to concentrations of 5 mM each and the protein solution was stored at 4°C.

Western Blotting

SDS-PAGE gels were typically constructed using a 12.5% acrylamide separating gel under a 5% stacking gel (Hames, 1981 as modified by BioRad TransBlot protocols and Dr. Pierre Moreau). Protein samples were denatured by boiling before loading. Gels were run at 35 to 40 V overnight for separation of the protein bands of interest.

Proteins were transferred from acrylamide gels to Zetabind nylon membranes using the BioRad Transblot kit following BioRad procedures. Transfer was performed for four hours at 100 v using a 4°C circulating water bath to cool the apparatus. Nylon membranes were allowed to dry slightly before staining.

In all cases duplicates were prepared; one of the membrane halves was readied for standard Coomassie Blue staining while the other half was stained using antibodies. The Western Blot staining was performed as for the colony immunoblot staining (pg. 33) using non-radioactive staining techniques and antibodies conjugated with enzymatic colour development reagents (either HPTG or diaminobenzamine).

Table 1: Buffers and Other Solutions

Buffers and solutions indicated here are from Maniatis *et al.*, 1982, unless otherwise stated.

LB media: (per l)	10 g tryptone, 5 g yeast extract, 5 g NaCl, pH 7.2.
SOB media: (per l)	20 g tryptone, 5 g yeast extract, 0.57 g NaCl, 4.84 $MgSO_4$, pH 7.0.
NZ media: (per l)	10g NZ amine, 5g NaCl, 5g Yeast extract, 1g casamino acids, 2 g $MgSO_4.7H_2O$ per litre, pH 7.5.
Rich minimal: (per l)	1.75 g Na_2PO_4, 0.85 g KH_2PO_4, 10 ml 25X Salts (5 g NH'_4Cl, 1.23 g $MgSO_4.7H_2O$, 7.5 g KCl, 8.0 ml Glycerol, 1.5 ml 0.1 M $FeCL_3$), 3 ml 20% glucose, 10 ml 10% casamino acids, 0.5 ml 100 mM $CaCl_2$ (Murialdo & Simonivitch, 1971).
Restriction Digest Buffers	
	High salt: 500 mM NaCl, 250 mM Tris-HCl, 50 mM $MgCl_2$.
	Med'm salt: 250 mM NaCl, 50 mM Tris-HCl, 50 mM $MgCl_2$.
	Low salt: 50 mM Tris-HCl, 50 mM $MgCl_2$.
20X TAE: (per l)	96.8 g Tris base, 32.8 g sodium acetate, 7.4 g disodium EDTA pH 8.2 with the addition of glacial acetic acid.

10X TBE: (per l)	108 g Tris base, 55 g boric acid, 8.5 g disodium EDTA, pH 8.3.
TE buffer:	10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0.
TM buffer:	10mM Tris, 10 mM $MgSO_4$ pH 7.5.
SM buffer:	10 mM Tris, 10 mM $MgSO_4.7H_2O$, 50 mM NaCl pH 7.5.
10X ligase buffer:	300 mM Tris pH 8.0, 70 mM $MgCl_2$, 12 mM EDTA, 100 mM DTT, 100 mM ATP, 500 μg/ml BSA.
Lysis buffer:	100 mM NaAc pH 5.0, 200 mM NaCl, 20 mM EDTA and 1% SDS.
TES buffer:	10mM Tris pH 7.5-7.9, 1mM EDTA, 100 mM NaCl.
HENS buffer:	100 mM HEPES pH 7.2, 1 mM EDTA, 10 mM NaCl, 0.1% SDS.
20X SSC:	3 M NaCl, 0.3 M sodium citrate, pH 7.0.
20X SSPE:	3.6 M NaCl, 0.2 M sodium phosphate pH 8.3, 0.02M EDTA.
50X Denhardt's sol'n:	1% (w/v) Ficoll (400 000 M. Wt., 1% (w/v) polyvinyl-pyrolidone (360 000 M. Wt.), 1% (w/v) bovine serum albumin (Denhardt, 1966).
TBS:	20 mM Tris, 500 mM NaCl pH 7.5.

TBS Tween:	20 mM Tris, 500 mM NaCl, 0.05% Tween, pH 7.5.
Reverse Transcriptase buffer:	480 mM Tris pH 8.3 at 42°C, 560 mM KCl, 40 mM $MgCl_2$, 80 mM β-mercaptoethanol (Rutledge *et al.*, 1988).
Nick Translation buffer:	30 mM ß-mercaptoethanol, 30 mM $MgCl_2$, 250 mM Tris pH 7.9, and 250 μg/ml BSA.
7% acrylamide gel for sequencing:	33.6 g urea, 37 ml water, 14 ml 40% bis/acrylamide (20:1), 4 ml 10X TBE, 56 μl TEMED, 560 μl ammonium persulfate (100 mg/ml).

Table 2: Bacterial Strains

C600	supE44, thi-1, leuB6, lacY1, tonA21, λ-...Restriction: (rk+,mk+), mcrA(-), mcrB(+)
HB101	F-, hsd S20 (rB^-,mB^-), supE44, ara14, λ-, galK2, lacY1, proA2, rpsL20, xyl-5, mtl-1, recA13...Restriction: (rk+,mk+), mcrA(-), mcrB(+)
JM109	recA-, $endA^1$, gyrA96, thi, hsdR17, supE44, relA1λ-, Δ(lac proAB)F, traD36, proAB, $lacI^qZ$, ΔM15
Y1089	Δ(lacU169), proA+, Δ(lon), araD139, strA, hflA150 [chr::Tn10],(pMC9)
Y1090	Δ(lacU169), proA+, Δ(lon), araD139, strA, supF, [trpC22::Tn10],(pMC9). Restriction: (r+,m+), EcoP1(-), mcrA(-), mcrB(+)
DH5α	F^1, ϕ80dlacZ, ΔM15, Δ(lacZYA,-argF), U169, endA1, $recA^1$, hsd R17, (r_k-,m_k+), thi-1, supE44,λ^-gyrA96, relA1

Results

I. Cloning and Expression of Pseudorabies Virus Glycoprotein Genes

Introduction

In the first phase of my work I attempted to obtain expression of an antigenically active fragment of a pseudorabies virus (PrV) protein. Two approaches were used in trying to clone portions of PrV DNA capable of encoding a recognizable epitope. These were the identification of the DNA region from a previously prepared PrV cDNA library and the shotgun cloning of the pseudorabies virus genomic DNA. In general, the approach that was followed consisted of isolating putative protein-encoding DNA fragments, cloning these into an expression vector, and attempting to detect expression of antigen using polyclonal antibodies prepared from pseudorabies-infected swine along with indirectly radioactively-labelled or enzyme-conjugated secondary antibodies.

Much of the work was constrained by the exotic nature of the pseudorabies virus and the restrictions placed upon experimentation with it. This was especially true in the early part of my studies when Agriculture Canada regulations did not permit any experimentation with PrV genomic DNA. Later, limited work in this area was permitted; pseudorabies viral DNA was treated as a biohazard.

Cloning Pseudorabies Virus cDNA into pUC8

Isolation of the Pseudorabies Virus DNA Inserts

A λgt10 cDNA library prepared from RNA isolated from pseudorabies virus infected cells was provided by Dr. Pierre Moreau. Earlier work done by Dr. Moreau had characterized the library as containing a total of $5x10^8$ plaque forming units (pfu's); the proportion of recombinants in the library was unknown. This library was amplified in *E. coli* Y1090 and DNA isolated from phage was collected. 200 μg of the purified DNA was digested with *Eco*RI to release the cDNA inserts. A small amount of this digest was end-labelled and combined with the remainder of the digest to provide a means for monitoring fragment separation on a gradient. After centrifugation through a glycerol gradient, fractions were collected and counted. The chart in Figure 3 shows the seven peak fractions which were further analyzed. An aliquot of each of these gradient peaks was subjected to electrophoresis, transferred to a nitrocellulose membrane and autoradiographed (see Figure 4).

Figure 4 shows that fraction 49 was the peak fraction which contained the excised DNA inserts; no other fraction appeared to contain DNA in the expected size range (note that the maximum insert size in λgt10 is 7.6 kb (Huynh *et al.*, 1985). This fraction corresponded to cDNA inserts with a size range of 500 to 1000 bp as determined by gel electrophoresis. In addition to the putative insert-containing fraction, the figure shows peaks corresponding to the restricted lambda arms (fractions 5,13 and 16) and a fraction which was hypothesized to correspond to the unincorporated nucleotides, marking the end of the fractionation profile (fraction 56). In order to ensure that all insert DNA was collected, fractions 47 to 50 were combined and the insert DNA was recovered by precipitation. 20 ng of this cDNA was ligated into 100 ng of pUC8 restricted with *Eco*RI . The results of the ligation were used to transform *E. coli* C600 cells which were then plated on L-agar with ampicillin.

Figure 3: Fractionation Profile — λgt10 cDNA Library Restricted with *Eco*RI

The PrV cDNA library (supplied by Dr. Pierre Moreau) was digested with *Eco*RI. A portion of the library was end-labelled prior to loading the digest on a glycerol gradient. After centrifugation, 0.2 ml fractions were collected and counted by liquid scintillation counting. Peak fractions, marked on the graph, were diluted with water and electrophoresed through agarose gels for further characterization.

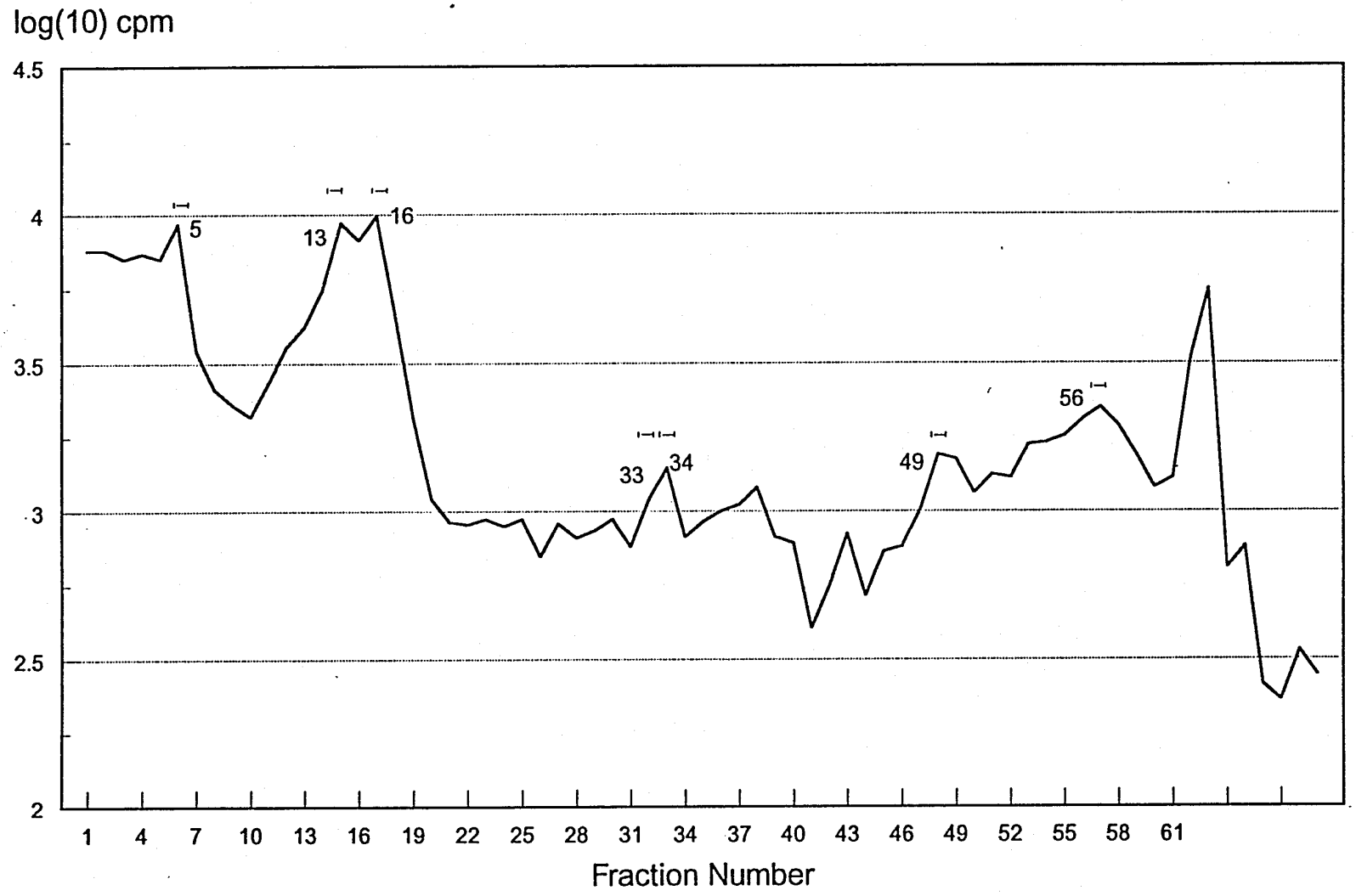

log(10) cpm
4.5
4
3.5
3
2.5
2
5
13
16
33
34
49
56
1 4 7 10 13 16 19 22 25 28 31 34 37 40 43 46 49 52 55 58 61
Fraction Number

Figure 4: Autoradiography of Glycerol Gradient Fractions Analyzed by Gel Electrophoresis

Selected glycerol gradient fractions (see Figure 3) were electrophoresed through an agarose gel which was then transferred to nitrocellulose membrane and autoradiographed.

Fraction 5:	33.1 kb arm from λgt10;
Fractions 13 & 16:	10.6 kb arm of λgt10;
Fractions 33, 36 & 56:	No definitive identification;
Fraction 49:	500 to 1000 bp excised insert DNA.

SELECTED PEAK FRACTIONS

5 13 16 33 34 49 56

At this point it would have been appropriate, especially in hindsight, to have attempted to hybridize this end-labelled insert DNA with PrV genomic DNA. Unfortunately, the restrictions imposed by Agriculture Canada prevented this experiment; confirmation that the insert fragments were homologous with PrV DNA could not be obtained at the nucleic acid level. For similar reasons, the original λgt10 cDNA library from which the fragments were isolated could not be fully characterized until much later in the project.

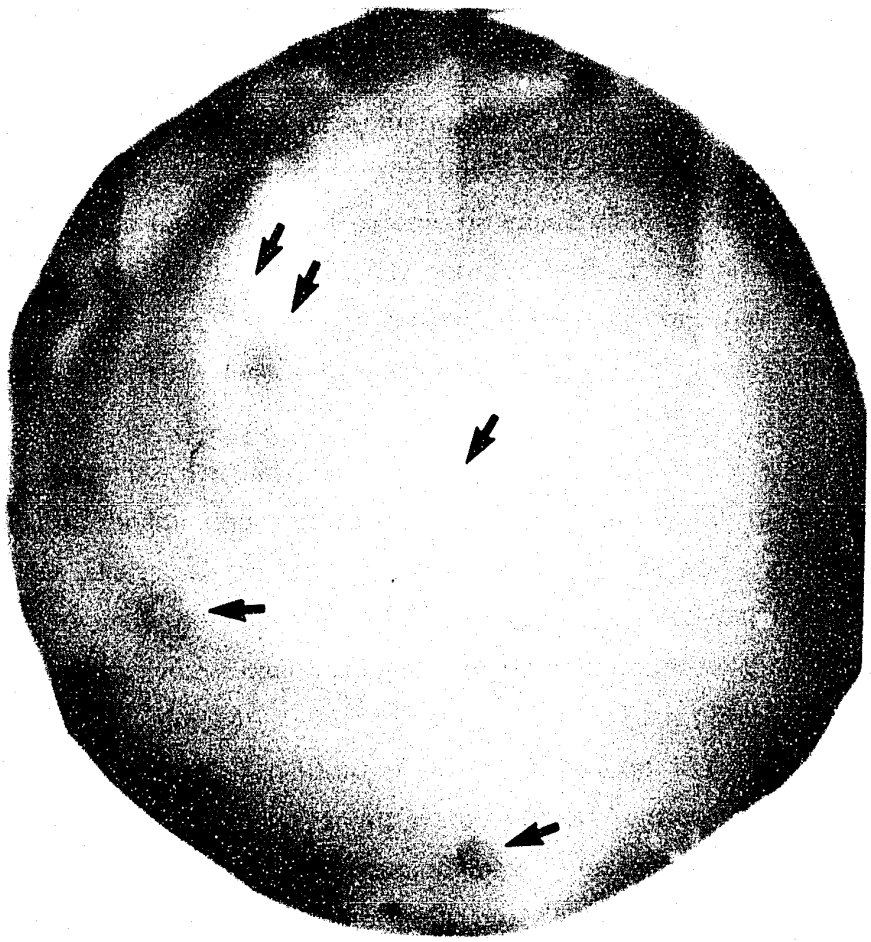
Colony Immunoblotting

Since characterization of the inserts could not be accomplished by traditional DNA hybridization experiments, the work immediately proceeded to an immunological description of the insert characteristics. Approximately 10,000 colonies were screened by plating the pUC8-PrV cDNA transformants on L-agar-ampicillin to a density of 300 to 400 colonies per plate. After growth, the colonies were lifted onto nitrocellulose and treated according to normal immunoblotting methods using a secondary antibody conjugated with radioactive ^{125}I as the probe.

High background levels were seen on all filters. In addition, because of disruption to the plate surface during the first screening, caused by the removal of the Hybond filters, it was difficult to identify signal-generating colonies. This resulted in a low proportion of first screening isolates generating a signal during the second screening. Despite this, several colonies gave clear positive results which persisted through several colony purifications and screenings (Figure 5). In addition, those colonies which produced positive results on the immunoscreening assays had a morphology clearly different from the negative controls. These colonies were considerably smaller than non-reacting colonies and their edges were not as smooth as those of normal C600 colonies. As the C600 strain had not been engineered specifically for cloning and expression of foreign polypeptides we proposed that the different morphology might result from the inclusion and expression of foreign DNA within the transfected C600 cells. Persons *et al.* (1985) noted a similar change in morphology when isolat-

Figure 5: Second Screening of PrV Transformed Colonies

Positive signal-generating *E. coli* C600 cells transformed with pUC8-cDNA recombinants were replated at a density of approximately 25 to 50 colonies/plate. This autoradiogram shows five positive colonies. Detection was with pig anti-pseudorabies virus primary antibody (ADRI) cleared with *E.coli* lysate (1:100), biotinylated goat anti-pig (1:2000) secondary antibody (Cappel Laboratories), visualized with ^{125}I-Streptavidin.



ing a plasmid with an insert encoding the 500 amino acids of the glycoprotein B (gB) of herpes simplex virus type 2 (HSV-2). They noted that "Upon induction of *lac*Z-promoted transcription, some of the bacteria became filamentous and produced inclusion bodies containing a large amount of a 65 kilodalton peptide that was shown to be precipitated by broad-spectrum antibodies to HSV-2 and HSV-1".

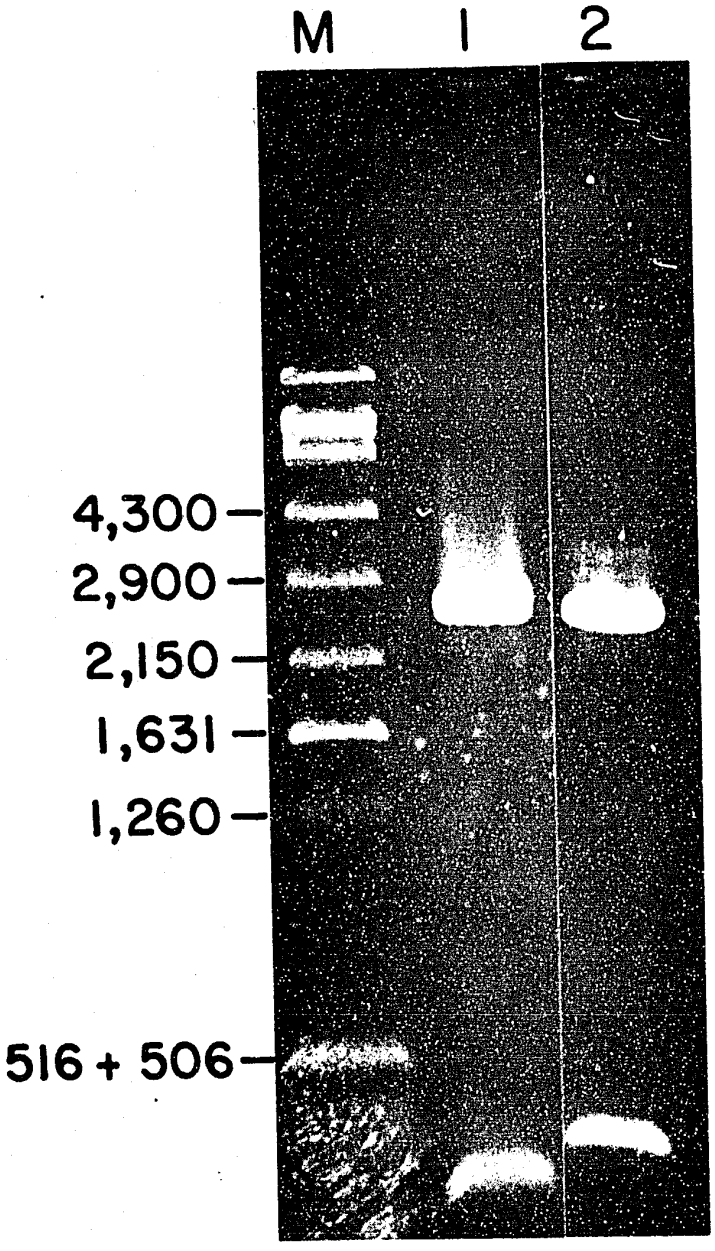
Initial experiments were conducted using C600 as a host cell solely because of its convenience; at the time these early experiments were conducted it was our standard lab strain of *E. coli*. Later, as my experimental studies became more complex and additional information on foreign protein expression appeared in the literature, different strains of *E. coli*, eg. JM109, more highly optimized for expression, were used. It should be noted that the morphological differences which were observed with the C600 transformants were not seen with these other strains in later experiments.

In order to characterize the plasmids, DNA was isolated from colonies which appeared positive on the immunoscreenings and digested with *Eco*RI to release the putative PrV cDNA inserts. Restriction digests of plasmids isolated from two such positive colonies are shown in Figure 6. Immunoscreening studies resulted in the identification of six putative positive colonies; plasmids isolated from these colonies had the following sizes: pPR255-7, 750 bp; pPR282-3, 320 bp; pPR282-5, 320 bp; pPR2124-4, 630 bp; pPR2125-1, 380 bp; pPR2125-3, 380 bp. In addition, in order to maintain negative controls, two plasmids were selected from colonies that did not provide positive results during the immunoscreenings. These were pPRN2-2 and pPRN2-3; both had inserts of approximately 500 bp. These insert sizes were in the expected range given that the original fractions which were isolated from the glycerol gradients showed a DNA smear in this size range.

Figure 6: Inserts from pPR282-3 and pPR2125-3

*Eco*RI and *Bam*HI double digests of recombinant pUC8-cDNA isolated from positive second-screening colonies were analyzed by gel electrophoresis.

Lane M: DNA marker;
Lane 1: 320 bp insert in pPR282-3;
Lane 2: 380 bp insert in pPR2125-3.


M
1
2
4,300
2,900
2,150
1,631
1,260
516 + 506

Plasmid Nomenclature

At this point it is appropriate to discuss the nomenclature used to describe the plasmids. After the initial identification of putative positive colonies, second immunoscreenings were typically performed on multiple daughter colonies. From these screenings, plasmids were given a two part name. The first part corresponds to the colony which provided a positive signal on the first screening. A hyphenated second part corresponds to a purified daughter colony which provided a positive signal on the second immunoscreening. For example, pPR2125-1 and pPR2125-3 are isolated from two purified daughter colonies derived from a single first screening, signal-generating colony. As such, they should be expected to contain identical inserts.

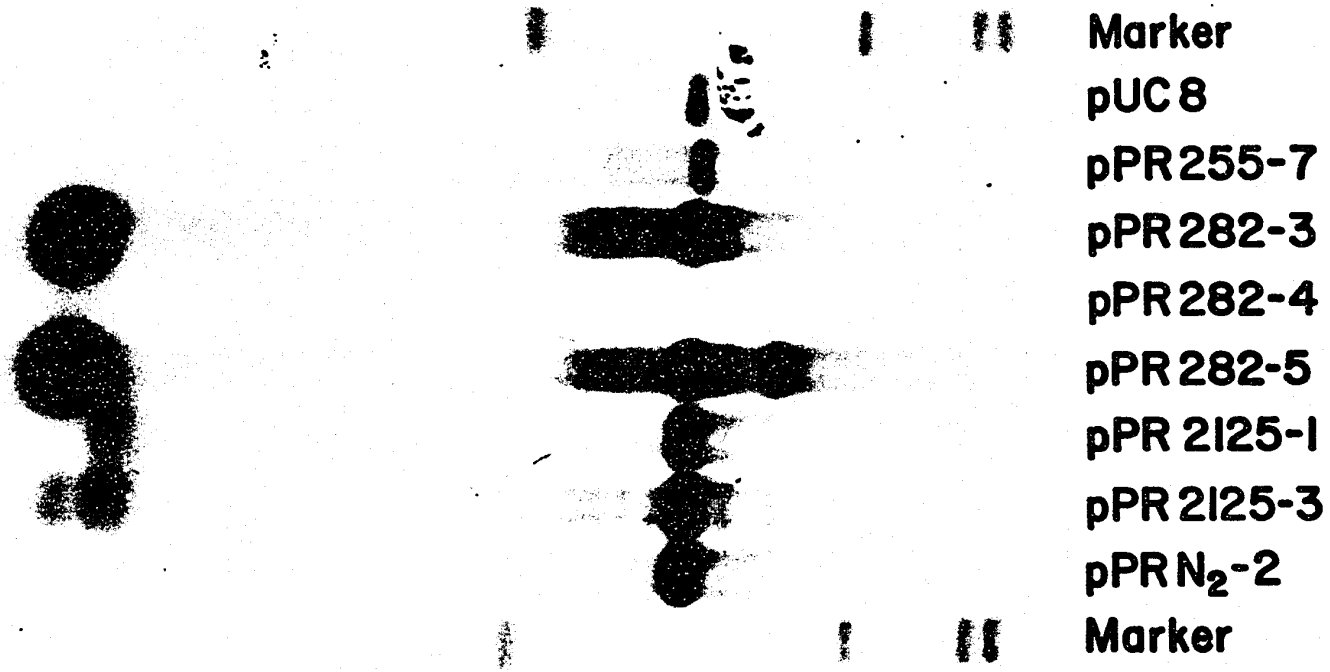
Characterization of the Inserts

In order to further define the inserts that had been obtained, large amounts of DNA were prepared from plasmids pPR255-7, pPR282-3, pPR282-5, pPR2125-3 and the negative control pPRN2-2. Several attempts at culturing pPR2124-4 were unsuccessful, even when ampicillin was included in the nutrient broth. We theorized that the production of a polypeptide expressed by the insert DNA was interfering with bacterial growth.

At this point in the study Agriculture Canada still did not permit the use of PrV genomic DNA outside of its biohazard laboratories; this made direct hybridization of the inserts with PrV DNA impossible. However, it was clear that further examination of the isolated DNA was necessary. The potential antigenic regions were expected to be fairly small in PrV; it was possible that if different but overlapping portions of one region had been isolated, there would be DNA sequence homology among the inserts. If this was true then cross-hybridization between some selected inserts should occur. Additionally, to reinforce the validity of the experimental controls, we wished to ensure that the plasmids obtained from daughter cells derived from a single positive colony (during the first screening) were homologous.

Figure 7: Cross-Hybridization Study

250 ng of DNA from each immunologically positive recombinant plasmid was restricted with *Eco*RI to release the insert and then electrophoresed through a 1% agarose gel in TAE buffer. The Southern blot was probed with nick translated pPR282-3 and autoradiographed for 48 hrs. at -70°C.


Marker
pUC 8
pPR 255-7
pPR 282-3
pPR 282-4
pPR 282-5
pPR 2125-1
pPR 2125-3
pPR N_2-2
Marker

A series of cross-hybridization experiments were performed in an attempt to determine if there was any homology among the inserts that had been isolated. Inserts from three of the clones, pPR282-3, pPR2125-3 and pPR255-7, were excised by *Eco*RI and *Bam*HI double digestion, purified by gel electrophoresis, radiolabelled by nick translation and used as probes against Southern blots of plasmid DNA digested with *Eco*RI plus *Bam*HI. These studies showed cross-hybridization between the two clones isolated from colony 282 and colony 2125. Figure 7 is a Southern Blot of the putative positive plasmids which has been probed with nick-translated pPR282-3 — note the hybridization of the probe to the two lanes containing pPR2125-1 and pPR2125-3. As expected, there was clear homology between daughter colony plasmids that had been isolated from the same putative positive parent; Figure 7 shows the strong hybridization of the pPR282-3 probe against the insert in pPR282-5. No cross-hybridization between any two sequences was detected for probes derived from pPR255-7 or pPRN2-2 (a negative control from colony immunoblotting that contained an insert sequence).

Probe↓ ←Plasmids digested with *Eco*RI and electrophoresed→

	255-7	282-3	282-5	2125-1	2125-3	N2-2
255-7		None	None	None	None	None
282-3	None		Strong	Weak	Weak	None
2125-3	None	Weak		Strong	Strong	None

Table 3 Cross-Hybridization Matrix: All plasmids are from the pPR plasmid series. The matrix contents describe the intensity of hybridization observed on autoradiograms of Southern transfers.

The matrix above summarizes the cross-hybridization observed; note that in the matrix the hybridization of a plasmid to itself is not shown. This hybridization was not performed at the same time as the others because the relative strength of the self-hybridization reaction reduced the resolution of the remainder of the auto-radiogram.

The fact that there was weak cross-hybridization between only two of the three 'parental' plasmids, *ie*. between plasmids isolated from daughter cells of the pPR282 and pPR2125 plasmid series, suggests that different epitopes were detected during immunological screening. This result is not unexpected given the polyclonal nature of the antibody; it was initially hoped that the presence of antibodies against different epitopes would increase the likelihood of immunodetection. Thus, although some clones were expected not to show homology between one another due to the fact that the polyclonal antibody should select for separate epitopes, we expected to obtain some clones that were producing similar antigens.

The cross-homology pattern seen in these experiments seemed to confirm this hypothesis. pPR255-7 appeared to contain cDNA insert sequences which were unique amongst the immunoselected clones, while the pPR282 and pPR2125 series showed some evidence of containing sequences homologous to one another. We assumed that this homology resulted from the fact that the two cDNA inserts encoded the same antigenic determinant. It is important to note that these cross-homology experiments are only meaningful when a positive result is produced; negative results provide no information about a given clone or its origin. The absence of any cross-hybridization for pPR255-7 did not suggest that it was any less (or more) likely to contain PrV homologous insert DNA than the other isolated clones; it was possible to hypothesize that this clone contained non-overlapping DNA, coding for a completely different epitope.

Confirmation of Putative Positive Insert Homology with Pseudorabies Virus DNA

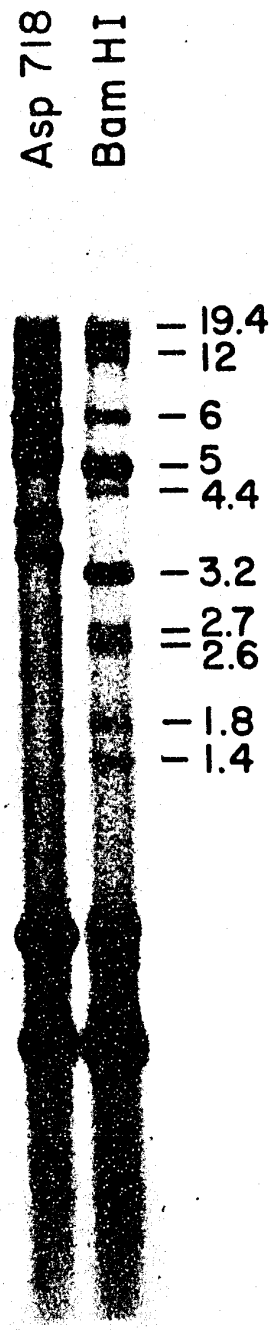
Six months after the isolation of the first putative positive colonies by immunoblotting, permission was received to test the isolated DNA for homology against actual pseudorabies virus DNA. The viral DNA was provided by Dr. Gilles Dulac of the Animal Diseases Research Institute (ADRI) and all work was carried out at the ADRI facility in Hull. Two aliquots of PrV DNA were restricted with *Bam*HI and *Asp*718 respectively. Multiple pairs of these digests were then run on an agarose gel and Southern blotted. This experimental methodology ensured that the digest, electrophoresis and transfer conditions were identical for each of the hybridization probes. Nick translated insert DNA, excised with *Eco*RI from each of the clones — pPR255-7, pPR282-3, pPR2125-1 and pPR2125-3 — was hybridized with strips from the blot. Two additional strips were hybridized with nick translated pUC8 DNA and PrV DNA to establish negative and positive controls respectively. No hybridization was seen between any DNA from the putative positive clones and the immobilized PrV DNA (data not shown). Under identical conditions, the self-hybridization tests showed a PrV restriction pattern corresponding to that previously published for the Ka strain of PrV (Lomniczi *et al.*, 1984) (see Figure 8). The results from these hybridization experiments conclusively demonstrated that there was no homology between the DNA isolated from colonies identified as positive through immunoblotting and the pseudorabies viral DNA.

The nature of the reaction between the swine PrV antibody and the clones identified as putative positives remains unclear. It is certain that the plasmids isolated from these colonies did contain inserts with considerable homology amongst themselves; however, once I had determined the lack of homology with PrV, the DNA inserts were not further characterized. It is unlikely that the insert DNA was vector DNA since the inserts did not hybridize with the negative controls (pUC8 DNA) during Southern blotting experiments. The most likely interpretation of the source of these inserts is that they originated in contaminant DNA introduced during the original creation of the cDNA library in λgt10.

Figure 8: Hybridization of Putative Positive Recombinant DNA with Pseudorabies Virus DNA

Southern blot of the restricted PrV DNA (positive control) self-hybridized with nick translated PrV DNA. Restricted DNA isolated from immunologically positive colonies and transferred to the same nitrocellulose membrane showed no hybridization with the labelled PrV DNA probe.

Lane 1: PrV digested with *Asp*718;
Lane 2: PrV digested with *Bam*HI.


Asp 718
Bam HI
19.4
12
6
5
4.4
3.2
2.7
2.6
1.8
1.4

Antigen Detection Methodology

Signal to Noise Background

During the course of the first attempts at colony immunoblotting it became clear that the generally high background level caused by the immunoscreening methodology was making identification of positively hybridizing colonies difficult. To eliminate this factor from the experimental design, several tests were conducted in order to improve the signal to noise ratio seen during the immunoscreenings. Because of the polyclonal nature of the antibody and its isolation from pig it was expected that there would always be a certain amount of background due to the presence of *E.coli* antigenic determinants in the antiserum; antiserum prepared from pig is known to contain significant amounts of anti-*E. coli* antibodies (Smith and Wengeling, 1977). Current literature suggested that ^{125}I, previously the most common method of detection, generated particularly high background levels (Hsu *et al.*, 1985), although the exact cause of this high background has not been explained. In order to improve the detection system, tests were conducted on new immunoblots using a variety of positive control marker reagents, primary and secondary antibodies. As well, during this period permission was received from Agriculture Canada to use PrV antigen in our laboratories. This enabled testing of the efficiency of the primary antibody under the conditions of the immunoblot methodology for the first time. It also allowed tests in which the effectiveness of varying antibody concentration and detection methodology could be evaluated to determine the optimum combination of reagents for maximizing the signal to noise ratio.

Adjustments to the immunoanalysis were tested in four major areas: 1/ the visualization agent was changed from ^{125}I to one of ^{35}S, diaminobenzidine (DAB) or horseradish peroxidase; 2/ the concentration of antibody in solution was varied between 1/100 and 1/5000; 3/ rabbit anti-swine and goat anti-pig sera were both tested as

secondary antibodies and 4/ the concentration and incubation time of the *E. coli* lysate was varied between 1/10 and 1/1000 and between 30 minutes and 16 hours. A summary of the results obtained by changing these components in the detection process is presented in the table in Table 4. A specific example of the results obtained by varying antibody concentration, lysate incubation time and chromogenic reagent is shown in Figure 9.

Detection Methodology Results

The extensive experimentation performed with various immunological detection strategies resulted in a number of changes to the protocols used in subsequent experiments, both for pseudorabies virus studies and later for work with Rous associated virus. The greatly improved signal to noise ratio associated with ^{35}S conjugated secondary antibody (as compared to ^{125}I conjugates) led to an immediate switch to the use of ^{35}S as the primary visualization tool (not shown). Although the chromogenic reactions provided by detection of the biotinylated secondary antibodies with streptavidin peroxidase were superior even to the detection reactions generated by ^{35}S conjugates it was some time after these test studies before a consistent method for reproducible results in colour reactions could be established. Once chromogenic reaction methodologies were stabilized, most work was done using diaminobenzadine as the detection reagent because of its darker colour (compare Row 5, Column 3 (HRP) with Row 5, Column 4 (DAB)).

The length of incubation of the primary antibody with *E. coli* lysate and the concentration of the lysate had a marked effect upon the hybridization of the primary antibody. By optimizing these two factors I was able to minimize background detection of colony material. Note the background levels of both the control antigen and the C600 lysate preparations in Row 2 and 3 of Figure 9 — where there was a high

Table 4: Effectiveness of Immunoscreening Methodologies

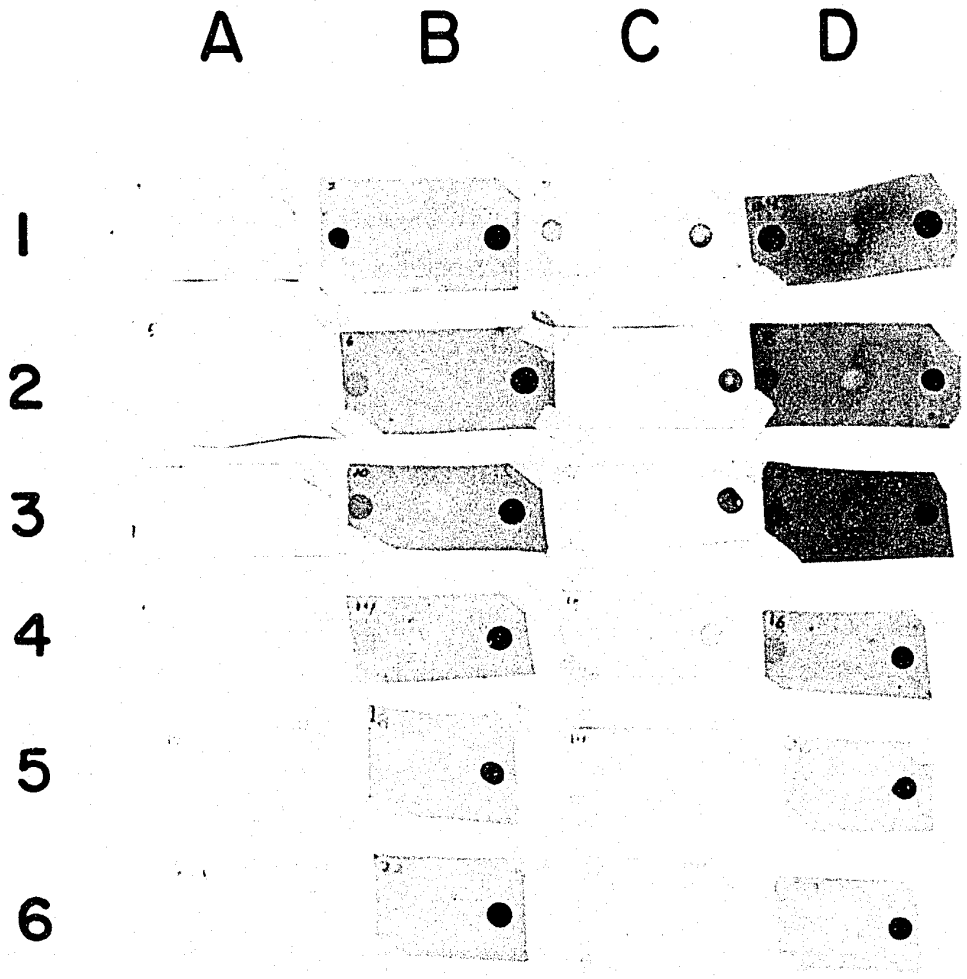
All tests were performed according to the basic techniques described in the Methods section. Control antigens and primary antibody prepared against pseudorabies virus in swine were provided by ADRI. Additional tests were conducted against *E. coli* C600 cells, some of which were transformed with recombinant pUC8 plasmids.

Change made	Result
■ ^{35}S marker reagent	Reduced filter background. No effect on colony background.
■ *E. coli* lysate preparations changed	Slight reduction in overall colony hybridizations as lysate concentration increased. Clear effect on colony background as concentration varied.
■ Streptavidin-peroxidase instead of radioactively labelled streptavidin	Marked reduction in overall filter background and in colony background. Pure antigen detected at extremely low concentrations.
■ Change secondary antibody from goat anti-pig (GAP) to rabbit anti-swine (RAS)	Significantly better signal to noise ratio with RAS antibodies than with GAP.

Figure 9: Effectiveness of Varying Antibody and Lysate Concentrations

A test series comparing the effectiveness of varying antibody and *E. coli* lysate concentrations and incubation times with non-radioactively conjugated secondary antibodies. Each test square had three aliquots of protein dotted — from left to right: *E.coli* C600 lysate, negative control antigen (ADRI), positive PrV antigen (ADRI). All test squares were blocked with gelatin prior to incubation with the antibody solution, as per protocols described in Materials and Methods.

Column A:	Secondary antibody: Colour detection:	Goat anti-pig; Horseradish peroxidase.
Column B:	Secondary antibody: Colour detection:	Goat anti-pig; Di-aminobenzadine.
Column C:	Secondary antibody: Colour detection:	Rabbit anti-swine; Horseradish peroxidase.
Column D:	Secondary antibody: Colour detection:	Rabbit anti-swine; Di-aminobenzadine.
Row 1:	Primary antibody conc.: Lysate concentration:	1/100 0
Row 2:	Primary antibody conc.: Lysate concentration:	1/100 1/50
Row 3:	Primary antibody conc.: Lysate concentration:	1/100 1/100
Row 4:	Primary antibody conc.: Lysate concentration:	1/500 1/50
Row 5:	Primary antibody conc.: Lysate concentration:	1/1000 1/50
Row 6:	Primary antibody conc.: Lysate concentration:	1/1000 1/100


A
B
C
D
1
2
3
4
5
6

concentration of primary antibody. Even high lysate concentrations were not sufficient to eliminate the background (compare Row 2 to Row 3 where test strips in Row 2 had double the lysate concentration). When the antibody concentration is reduced and the lysate concentration is kept relatively high (Row 5) the background signal is virtually eliminated while the positive antigen generated signal remained clearly evident for all four combinations of secondary antibody and colour detection method. Finally, although the rabbit anti-swine secondary antibody was generally superior to the goat anti-pig antibody (note the strong signal in Column 3, Rows 2 to 4 compared to the signals in the same rows of Column 1) it was not possible to secure a consistent supply of a correctly conjugated antibody; thus goat anti-pig was used as the secondary antibody in all subsequent PrV studies.

Cloning the Pseudorabies Virus Genomic DNA into pUC18

The improvements in the detection techniques that had come from altering antibody concentration, developing more stringent antibody clearing methods and changing the actual detection reagents, as described above, provided significantly clearer results in test situations. With these improvements we felt that it would be possible to eliminate many of the problems that had been associated with the background levels of detection during the first sets of immunoscreenings.

Abandoning the cDNA Library

A decision was made to discard the original λgt10 library from which the first expression library had been made primarily because of concerns surrounding the source of the inserts that had generated positive results in the first set of screenings. Additionally, although we had originally accepted the relatively low average size of the cDNA insert fragments (as shown by autoradiography of samples taken from the glycerol fractionation in Figure 4) we were hopeful that a different approach would yield larger PrV DNA inserts. Also, we were not certain that the small average size of

the inserts isolated from the glycerol gradient offered the best chances of locating sequences coding for antigenically recognizable polypeptides. The limited coding capacity of a small fragment places constraints upon the tertiary structure formation of expressed proteins; this tertiary structure is often a critical element in antibody recognition.

In order to obtain an antigen-producing clone, a new expression library was constructed from fragments of pseudorabies virus DNA resulting from cleavage with a number of restriction enzymes. This decision was made possible by the fact that Agriculture Canada had modified its policies and now permitted the release of the pseudorabies virus DNA from their laboratories. For the first time, it became possible to work directly with full length viral DNA to construct the library and to directly test the inclusion of viral DNA fragments in the library.

Construction of a Genomic Library

Purified pseudorabies DNA was supplied by Dr. Gilles Dulac of ADRI. Four separate aliquots of the DNA were each restricted with one of four different restriction enzymes: *Alu*I (AG|CT), *Rsa* (GT|AC), *Sau*3A (|GATC) and *Hae*III (GG|CC). Multiple restriction enzymes were chosen so that when the resulting restriction fragments were combined the likelihood of all three reading frames being represented would be increased. Restriction endonucleases that resulted in blunt ends were chosen to ease the ligation of the resulting fragments with linker fragments.

After restriction, the *Alu*I, *Rsa* and *Hae*III fragments were combined together and methylated with *Eco*RI to protect internal *Eco*RI sites from restriction digestion. The combined fragments were then ligated with *Eco*RI linkers, restricted with *Eco*RI to digest excess linkers, and passed over Biogel columns to separate the pseudorabies virus DNA fragments from the restricted linker preparation. Progress of the DNA over the column was monitored using end-labelled *Sau*3A fragments. A peak fraction,

corresponding to the fragment DNA ligated with the linkers, was successfully identified and collected.

Screening the Library

The collected, linked PrV fragments were ligated into pUC18 (Norrander *et al.*, 1983) previously restricted with *Eco*RI and treated with calf intestinal alkaline phosphatase prior to ligation. The vector was changed from pUC8 as a result of current literature which identified pUC18 as a potentially superior expression vector (Yamashita *et al.*, 1987). After transformation of the pUC18 recombinants into JM109, colony hybridization, using nick translated PrV DNA as a probe, was conducted against the library. Approximately 68% of the library was shown to contain PrV homologous DNA (data not shown).

The pUC18 recombinants were then tested using the immunoscreening methods developed during work with the first library. From total platings of 8,000 recombinants, four highly positive colonies were identified. These colonies continued to show significantly greater binding to the antibody reagent than negative controls through three rounds of colony screening (see Figure 10). These four colonies were named 301, 305, 306 and 309; the plasmids isolated from them were similarly designated pPR301, pPR305, pPR306 and pPR309.

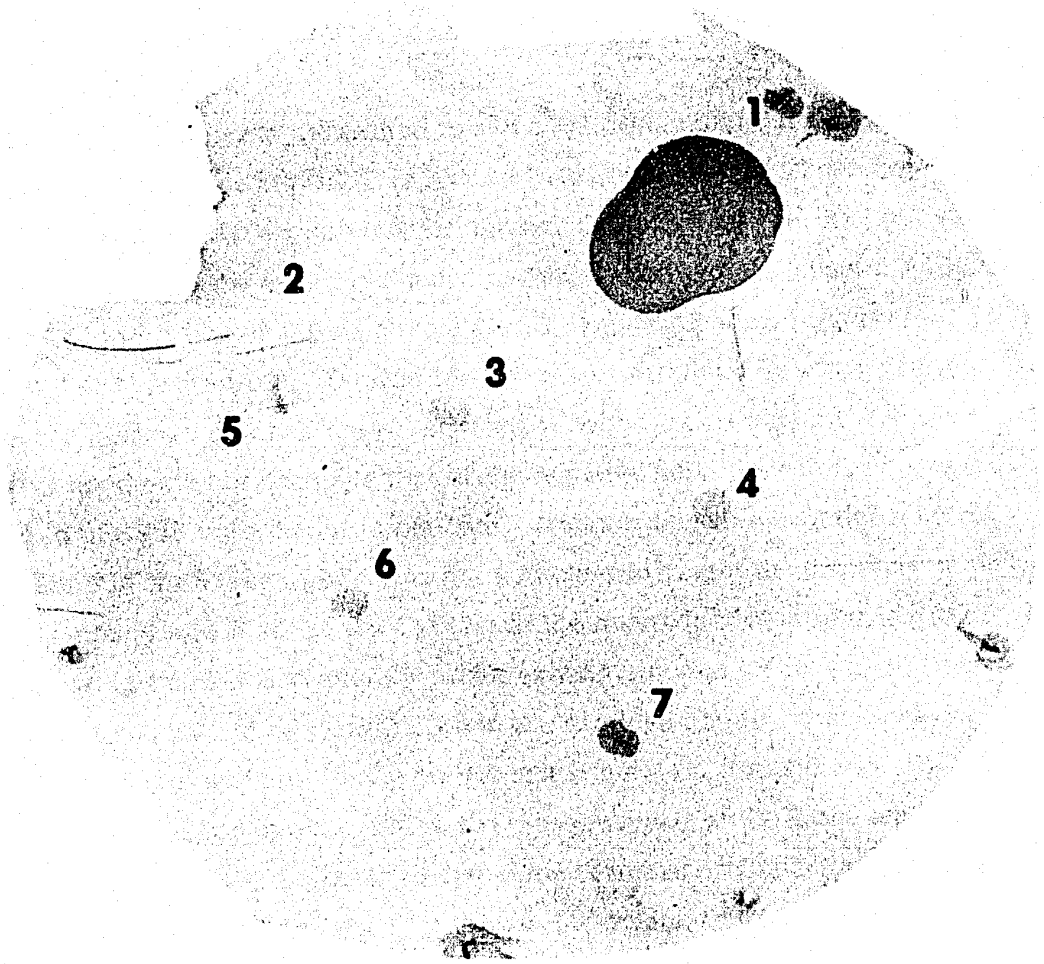
Characterization of the Clones

Once these colonies were isolated, additional DNA and protein analyses were conducted. Digestion of the pUC18 recombinants showed inserts in the range of approx-

Figure 10: Colony Immunoscreening of Shotgun Cloning.

Second screening of four putative positive colonies; JM109 was transformed with recombinant pUC18 created from the shotgun cloning of PrV digest fragments. Labels identify colonies from which the plasmids listed below were isolated. These plasmids were later investigated for protein expresssion (see Figure 11).

1/ Dot of PrV positive control antigen;
2/ Negative control — JM109 transformed with recombinant plasmid — no signal observed after first screening;
3/ pPR309;
4/ pPR306;
5/ JM 109 transformed with non-recombinant pUC18;
6/ pPR305;
7/ pPR301.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imately 850 bp to 1 kb[4]. After confirmation that the plasmids did contain inserts, DNA and protein analysis were carried out in parallel. Proteins were prepared from cultures of each of the four transformed JM109 preparations. The identified clones consistently generated signals on protein preparation dot blots. Figure 11 shows the strong signals that were generated from protein dot blots for the putative positives; negative controls in Figure 11 show the low background levels that were observed. Based upon these results the protein extracts were purified for Western blot analysis.

In the Western blot analysis, I expected to see a unique band in the putative positive lanes. The *lacZ* sequence in pUC18 is 231 bp long and codes for 77 amino acids; the resulting protein would be expected to be approximately 8.5 kD. If the DNA insert in pUC18 was 1 kb then the resulting β-galactosidase fusion protein (approx. 410 amino acids) would have a size of approximately 45 kD. Figure 12 shows the presence of a unique band at approximately 30 kD in the protein preparations isolated from two of the three the colonies that had shown significant hybridization during the dot blot tests. This band was not seen in either of the negative control lanes (compare the lane marked pPR306 with the left-most lane, corresponding to a negative control). The resolution of the Western blot was not sufficient to detect the presence or absence of a band at 8.5 kd (at the position of the non-recombinant β-galactosidase). The exception to these observations was the protein preparation developed from colony 305; it showed no bands not seen in the negative control, despite having generated a strong signal on the protein dot blot (Figure 11, Spots 3 and 10). All lanes showed a high background due to the cross-reaction of the *E.coli* antibodies with JM109 antigens; I was unable to completely eliminate this background regardless of the blocking conditions used. Although the source of this unique band is unknown we believed it possible that it reflected the fusion protein from a smaller PrV DNA insert. The additional band was suggestive of an insert of approximately 750 bp. Since this band was unique to the putative positives it seems likely that it

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[4] Initial digests were performed with *Pvu*II which resulted in two bands being observed by gel electrophoresis — the vector band at 2350 bp and the insert band bounded by 331 bp of vector DNA.

Figure 11: Screening of Proteins Produced from Putative PrV Recombinants

Proteins were prepared from each of the four putative recombinant colonies and 5 μl samples were dotted, in duplicate, on nylon membrane. Detection is with ^{35}S-Streptavidin using biotinylated goat anti-pig secondary antibody.

1/ Positive Antiserum (ADRI);

2, 12/ Proteins from pPR301/JM109;

3, 10/ Proteins from pPR305/JM109;

4, 9/ Proteins from pPR306/JM109;

5, 6/ Proteins from pPR309/JM109;

7, 11/ Proteins from pUC18/JM109;

8,13/ Proteins from transformed JM109 that did not produce a signal in earlier screenings;

14/ Negative Control: Pre-immune Serum (ADRI).

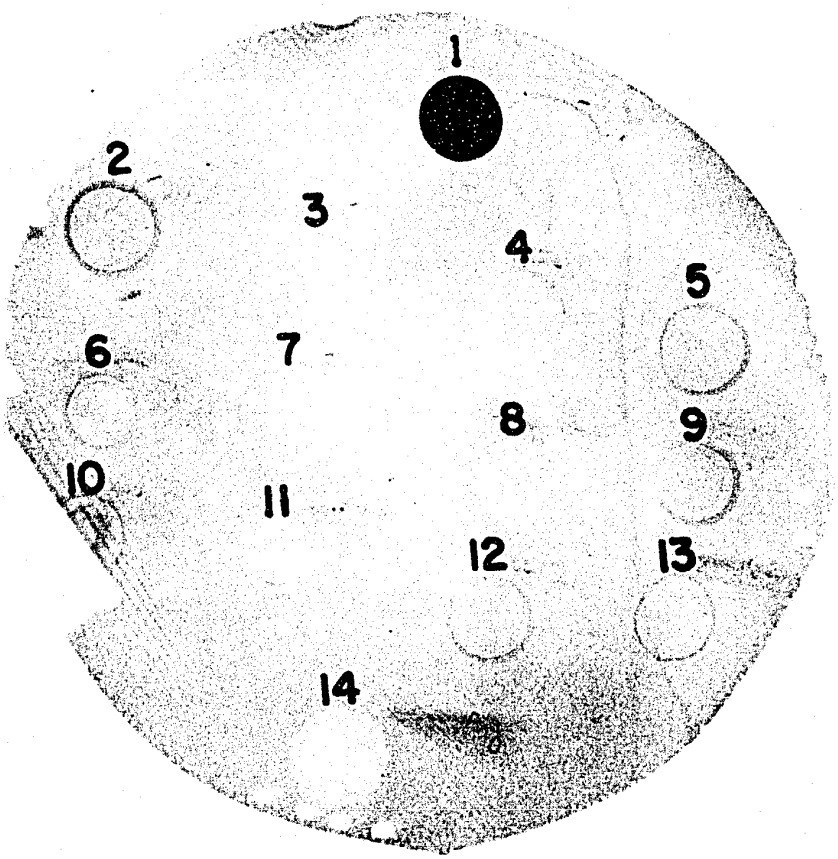
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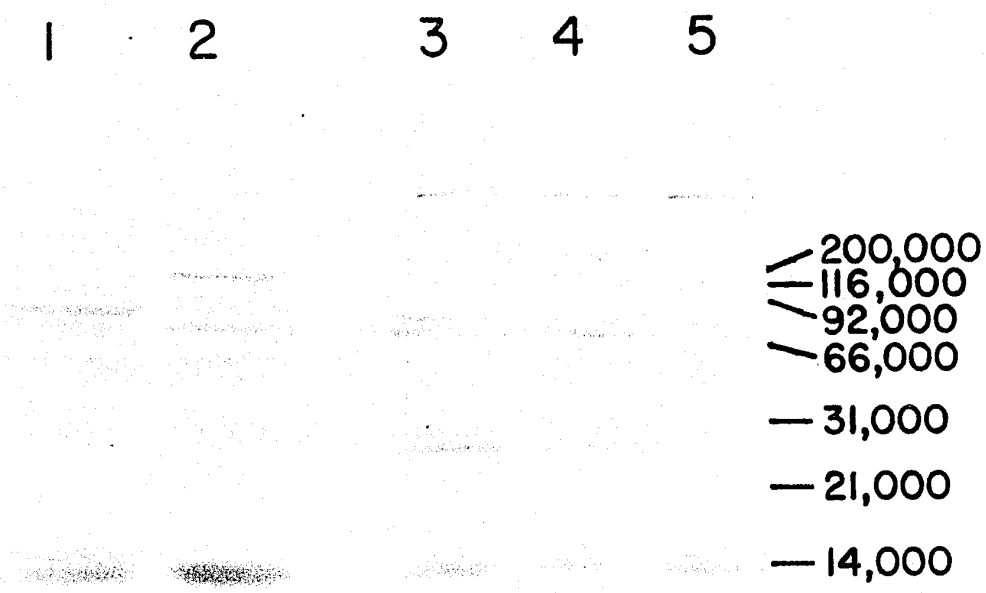
Figure 12: Western Blot Analysis of the Putative Positives

Protein preparations were derived from cultures of each of the putative positives grown in rich minimal media. Protein samples were run through a 12.5% SDS-PAGE gel overnight @ 40v. After electroblotting, protein samples were immunoscreened with 1/500 primary antibody cleared with a 1/50 *E.coli* lysate for 2 hours. The secondary antibody was peroxidase conjugated goat anti-pig and detection was with DAB.

Lanes 1 & 2: JM109 Negative controls;
Lane 3: pPR309/JM109;
Lane 4: pPR306/JM109;
Lane 5: pPR305/JM109.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200,000
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31,000
21,000
14,000

was responsible for the antigenic signals observed during the colony immunoscreening. However, no explanation for the absence of this band in protein preparations derived from clone 305 was possible.

Concurrently with the protein studies, DNA from the putative positives was used in the preparation of nick-translated DNA probes and was hybridized to Southern blotted PrV DNA. No homology was seen with any of the recombinants. Separately, rapid plasmid preparations were performed on the putative positives and inserts in the 850 bp to 1 kb size range were observed for all four of the selected clones. Since only a limited supply of PrV DNA was available, the DNA hybridization was only tested twice; both trials produced negative results. Because of the negative DNA results it was concluded that the recombinant pUC18 clones that had shown positive results on the immunoassays had incorporated non-PrV DNA that expressed an antigen capable of being recognized by the antibody used in screening. Because of the polyclonal nature of the primary antibody this was not unreasonable. The antibody had previously been shown to be highly reactive with *E. coli* proteins; the high degree of impurity typically found in swine proteins is well documented in the literature (Snyder *et al.*, 1987). Further exploration of this issue can be found in the Discussion.

Summary

At this point it was apparent that the limiting factor in the isolation of a PrV DNA fragment capable of producing antigenically recognizable protein was the quality of the antiserum. The only source for this antiserum in Canada was the Animal Diseases Research Institute. I had demonstrated that the antiserum was recognizing a protein not found in negative controls but that the DNA inserts from clones that were producing protein in this band contained no homology to PrV DNA. Subsequently, other researchers confirmed significant difficulties when working with antiserum raised against PrV infected swine. Petrovskis *et al.* (1986) indicated that they had been unsuccessful in immunoscreening using sera from pigs which had

recovered from PrV infection. Timmins *et al.* (1986) found that antisera that efficiently neutralized PrV, immunoprecipitated PrV glycoproteins, and recognized infected cells in enzyme linked immunosorbent assays, did not detect λgt11 recombinants known to synthesize PrV proteins in host *E. coli*. They recommended that antisera be prepared against PrV proteins eluted from SDS-PAGE gels in order to enhance recognition of PrV fusion proteins expressed by recombinant phage. After discussions with ADRI it was decided that it was not feasible to develop an antiserum prepared in this manner. Furthermore, Canadian agricultural policy prevented the import of antisera from other countries. Without a suitable antiserum the work with pseudorabies virus was discontinued.

II. Cloning and Expression of the Rous Associated Virus *Env* Gene

Introduction

The goal of the second phase of my work was to obtain expression of an antigenically active fragment of the *env* protein of Rous associated virus. In contrast to the work with pseudorabies virus, the approach in this phase was to first isolate an identifiable region of the *env* gene by virtue of its homology to Rous sarcoma virus sequences. After protein-encoding portions of the gene had been isolated they would be subcloned into expression vectors in an attempt to produce antigens recognized by antibodies prepared against Rous sarcoma virus-infected chicken.

Two strains of Rous associated virus were used during these studies. They were RAV-1, a subgroup A strain and RAV-2, a subgroup B strain. At the time that these experiments were conducted, limited details about the sequences of these two strains were known. However, the location of hypervariable regions within similar subgroup A and B strain viruses had been identified (Dorner *et al.*, 1985). It was generally believed that a high level of sequence conservation existed between various strains of RAV and RSV throughout most of the genome, with specific regions of high variability (the "hypervariable regions") responsible for determining host range. It was my goal to isolate RAV DNA with the intent of being able to demonstrate a strain-specific antigenic reaction.

Preparation of the Nucleic Acid Probes

The plasmid, pSR-XD2, (Cross and Hanafusa, 1986; provided by Dr. John Bell, University of Ottawa) was used as the source of all nucleic acid probes developed during work with Rous associated virus (see Appendix A for the structure of pSR-XD2). Two DNA segments were isolated from the plasmid. These were the 700 bp *Nco*I

fragment and the 1.7 kb *Sma*I fragment. The *Sma*I probe contains the entire contiguous section of *env* as prepared in pSR-XD2 while the *Nco*I probe contains only the 3' coding region of the gene. Both probes were used in hybridization experiments but it was found experimentally that the *Nco*I probe generally exhibited lower background levels during colony hybridization. Radioactive probes were routinely capable of detecting picogram quantities of homologous DNA under the hybridization and washing stringencies used.

RAV-1 cDNA preparation

Partially purified RAV-1 virus was supplied by Dr. Parvis Sabour of Agriculture Canada. After treatment with proteinase K, phenol/chloroform extraction and ethanol precipitation (as per Materials and Methods), spectrophotometric analysis revealed a yield of 12.9 μg of RNA from 1.5 mg of viral material.

The viral RNA was divided into two aliquots and one was used for cDNA synthesis. A double-stranded cDNA yield of 260 ng was achieved from 6.45 μg of RNA. To verify that the cDNA preparation did contain RAV-1 sequences, dilutions of the cDNA, along with a number of negative controls, were prepared for dot blotting and hybridized to the 700 bp *Nco*I fragment of pSR-XD2. Hybridization with the cDNA was detectable at the 100 pg level. There was no hybridization with any of the negative control DNA's, including EMBL3, pBR322 and pGEM 4B, and strong hybridization with all expected positive DNA's, pSR-XD2, pGEM 4B with the *Nco*I fragment of pSR-XD2 inserted into it and an unligated aliquot of the cDNA library (data not shown).

Colony Hybridization

The cDNA was ligated into pGEM 4B previously restricted with *Sma*I and treated with calf intestinal alkaline phosphatase. This approach permitted excision of the insert with an *Eco*RI/*Bam*HI double digest. The ligated DNA was transformed into competent JM109 cells and was plated on L-agar-ampicillin plates with X-gal and IPTG. After overnight growth at 37°C, colony lifts and hybridizations with the *Nco*I or *Sma*I probes were performed. Numerous colonies showed strong signals (see Figure 13A); generally, the *Nco*I probe produced much lower background than the *Sma*I probe. Hybridizing colonies were purified and screened again with the *Nco*I probe (Figure 13B). Rapid plasmid preparations were performed on putative positive colonies in order to determine insert sizes.

The results shown in Figure 14A were particularly confusing. Each of the restriction digests was performed on plasmids isolated from positively hybridizing colonies following a second colony screening. Yet fewer than 50% of the plasmids show any insert. Additional sets of rapid plasmid preparations were performed with the same results. Insert-containing plasmids from these additional preparations were identified and collected into one experimental group. After restriction with *Eco*RI and *Bam*HI, these insert-containing plasmid preparations were electrophoresed through an agarose gel. The Southern blot of this gel after hybridization with the *Sma*I probe is shown in Figure 14B. The autoradiogram shows that only five of the nineteen insert-containing plasmids have any homology with the RSV probe. Four of these were large inserts, approximately 1000 bp (Lanes 3, 4, 10 and 17), while the remaining one was approximately 300 bp (Lane 7). In an attempt to further characterize those plasmids which had inserts showing homology to the 1.7 kb *Sma*I probe, large scale plasmid preparations were performed for all five. We were unable to grow cultures of three of the plasmid-carrying colonies in the presence of ampicillin. After large scale preparation of the remaining two plasmids, restriction analysis revealed that neither plasmid contained an insert (data not shown).

Figure 13: Cloning of RAV-1 cDNA into pGEM 4B

JM109 was used as the host strain for colony hybridizations of the RAV-1 cDNA library cloned into pGEM 4B.

Figure 13A: First Screening
(opposing page)

Recombinant colonies were plated at a density of approximately 800 colonies/plate and grown overnight at 37°C. The plates were prepared for colony hybridization with the 700 bp *Nco*I fragment of pSR-XD2 as per Materials and Methods. Five colonies that are typical of the positive signal-generating colonies which would be selected for further screening are identified with arrows.

Figure 13B: Second Screening
(next page)

Colonies which generated a positive signal in the first screening were picked, transferred to a second plate, and processed as in Figure 13A. Marked colonies indicate those from which plasmid DNA was isolated and further analyzed by gel electrophoresis and Southern transfer (see Figure 14A).


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Figure 14: RAV-1 Recombinant Plasmids from Hybridizing Colonies

Colonies which provided positive immunological results through two screenings were selected for further characterization.

Figure 14A: Restriction Analysis:
(opposing page)

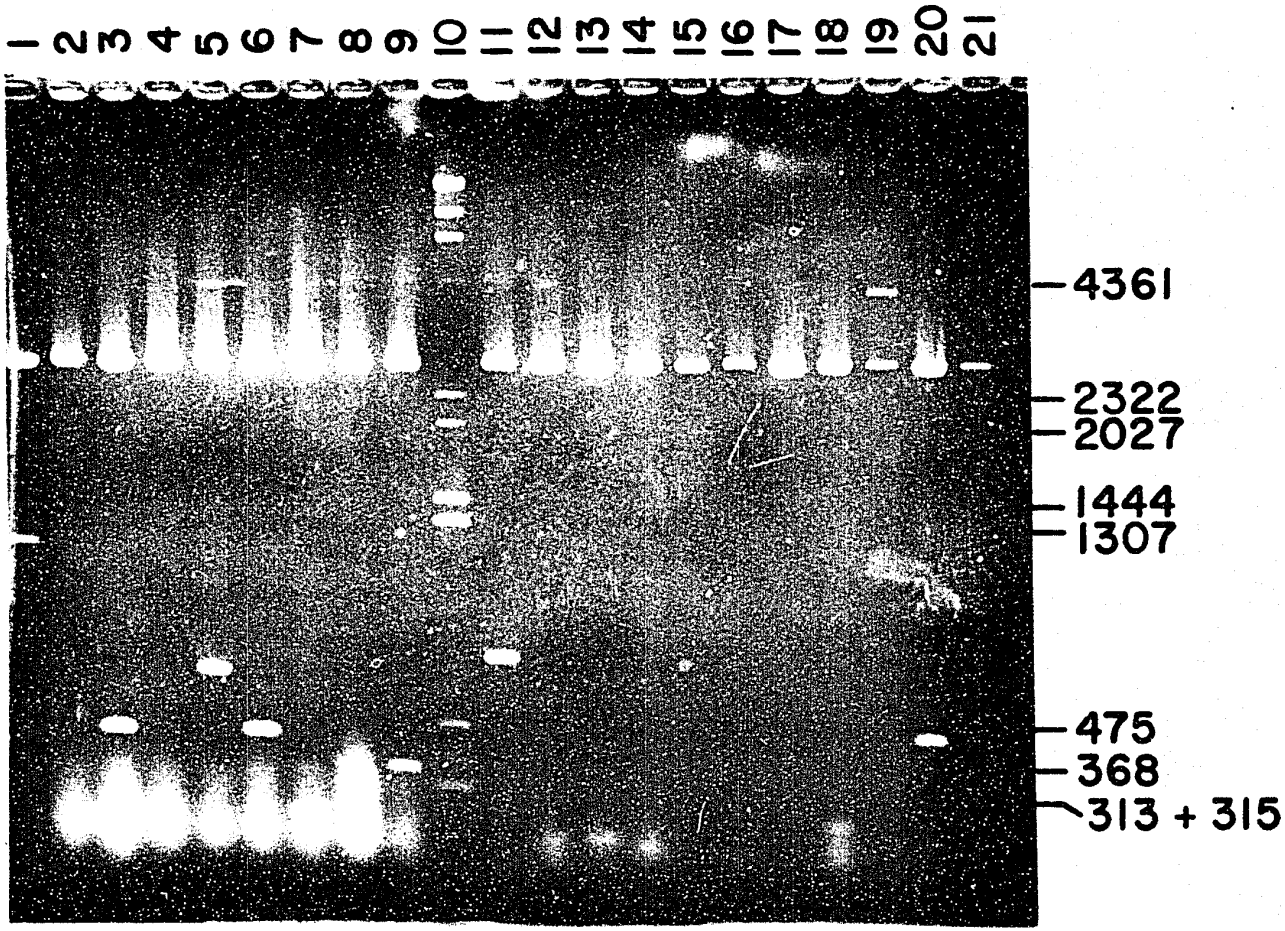
Rapid plasmid preparations were performed on cultures grown from colonies which generated positive signals after the second colony screening.

Lanes 1-9, 11-20:	Rapid plasmid preparations (Lanes 1 to 5 correspond to colonies marked in Figure 13B);
Lane 10:	Marker;
Lane 21:	pGEM 4B.

Figure 14B: Southern Analysis:
(next page)

Plasmid DNA's from hybridizing colonies were restricted with *Eco*RI, subjected to electrophoresis, transferred to nylon membranes and probed with the labelled *Sma*I fragment of pSR-XD2. Insert-containing plasmids from four separate plasmid preparations are combined in this analysis.

Lanes 1-17:	Plasmid preparations from putative positives (lanes 3 to 7 correspond to lanes 1,3,5,6,9 in Figure 14A);
Lane M:	Marker;
Lane 18:	pGEM 4B restricted with *Eco*RI;
Lane 20:	600 pg of pSR-XD2 restricted with *a Eco*RI/*Bgl*I double digest to release *env* region; positive control.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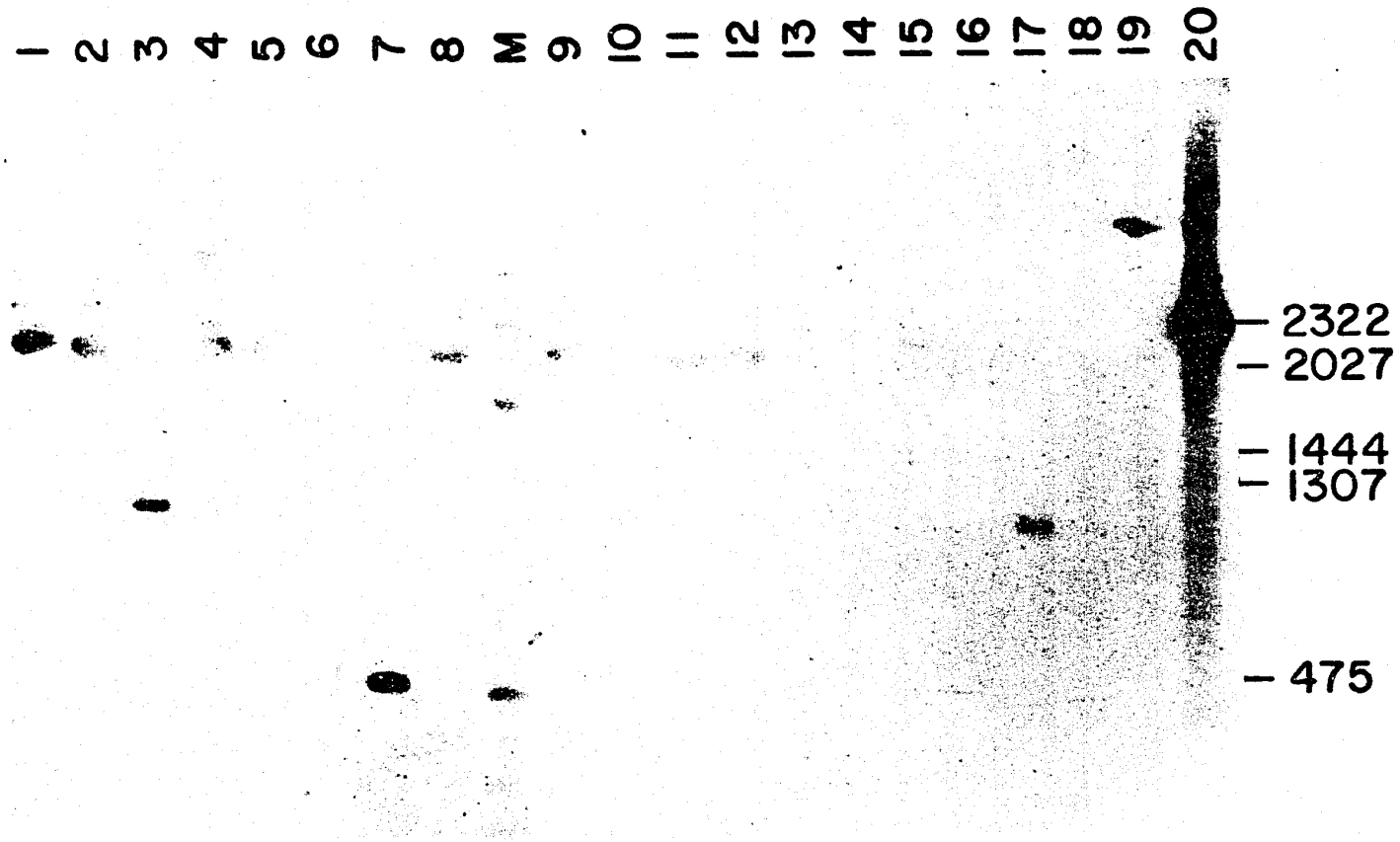
20
21
4361
2322
2027
1444
1307
475
368
313 + 315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2322
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1444
1307
475

Despite these results it was clear from the dot blots that the prepared cDNA did contain sequences homologous to RAV-1 DNA and that the *Nco*I probe contained no sequences homologous to the vector. Additionally, we were confident that our original selected positives were not simply background signals due to the presence of an insert as we had seen numerous other instances where an insert was present but did not hybridize with either of the two probe sequences.

Selection Against Insert Carrying Plasmids

One possible explanation for the results described above was that there was selection against insert-carrying plasmids and that, under some conditions, the plasmid was being lost from JM 109. To test this hypothesis, rapid plasmid preparations were performed on nine colonies of JM 109 containing recombinant plasmids that had hybridized with the *Sma*I probe. Cultures for the preparation were grown in the presence of ampicillin to maintain selection for the plasmid. The isolated plasmids were linearized with *Kpn*I in preparation for gel electrophoresis; an autoradiogram of the Southern blot after hybridization with the 700 bp *Nco*I fragment is shown in Figure 15A. The presence of an insert is indicated by a lower mobility than the linearized vector (not visible on the Southern blot). Several recombinants show strong hybridization with the probe (lanes marked 1b, 1c and 2a), while others show a weaker hybridization (lanes 2b, 2c, 1a and 4a). Two of the plasmids did not hybridize to the probe (lanes 3a and 3b); these were false positives selected from the colony hybridization. Additional investigation of these plasmids suggested that the hybridizing bands seen in lanes 1b and 2b resulted from contamination by neighbouring gel wells rather than the presence of homologous inserts (data not shown).

Each of the cell cultures utilized for the plasmid preparations was used to innoculate 200 ml of LB media, and, after overnight growth, was plated on L-agar-ampicillin plates at a low density (approx. 200 cells/plate). Rapid plasmid preparations were conducted on cultures grown from six colonies from each plate (Figure 15B). Daughter colonies from two of the three colonies that had originally contained a strongly

Figure 15A: Southern Analysis: Plasmid DNA from Positive Colonies Demonstrating Unstable Plasmids

Rapid plasmid preparations performed on colonies that had hybridized with the 1.7 kb *Sma*I fragment of pSR-XD2 (see Figure 14B for examples). The plasmids were linearized with *Kpn*I; the Southern Blot was probed with the labelled 700 bp *Nco*I fragment of pSR-XD2.

Lane M:	Marker
2a, 2b, 2c:	↘
1a,1b,1c:	→Plasmid DNA from colonies selected after first screening
3a, 3b, 4a:	↗
Lane Pos:	pGEM containing the 700 bp *NcoI* fragment of pSR-XD2.

4.3
2.7
2.0
Pos.
4a
3b
3a
1c
1b
1a
2c
2b
2a
M

Figure 15B: Restriction Analysis: Plasmid DNA Prepared from Refreshed Cultures

Plasmid preparations from six daughter colonies of each originally hybridizing colony (shown in Figure 15A) were digested with *Eco*RI plus *Bam*HI.

Top Row:

Lane 1: Marker;

Lane 2-7: Prepared from daugther colonies of 1b;

Lane 8-13: Prepared from daughter colonies of 1a.

Second Row:

Lane 1: Marker;

Lane 2-7: Prepared from daughter colonies of 2b.

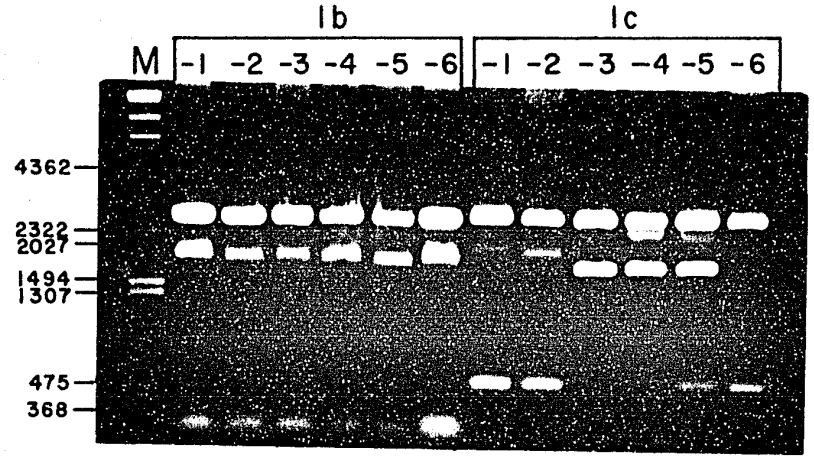
Lane 8-13: Prepared from daughter colonies of 2c.

Third Row:

Lane 1: Marker;

Lane 2-7: Prepared from daughter colonies of 4a.

Lane 8: pGEM 4B restricted with *Eco*RI.


1b
1c
M
-1 -2 -3 -4 -5 -6
-1 -2 -3 -4 -5 -6
4362
2322
2027
1494
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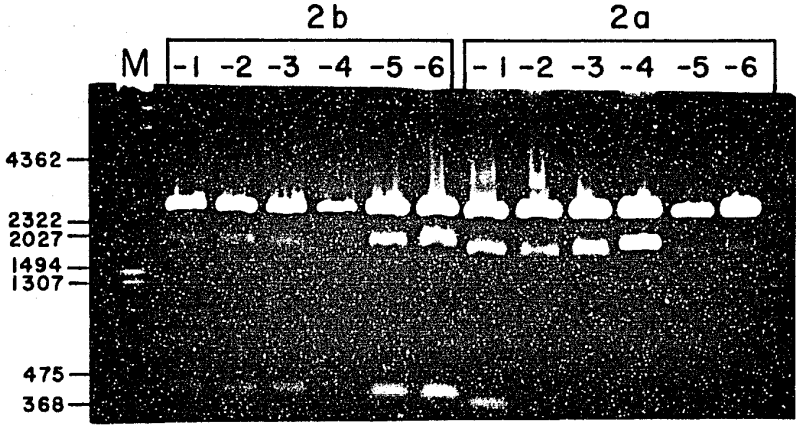
475
368


2b
2a
M
-1 -2 -3 -4 -5 -6
-1 -2 -3 -4 -5 -6
4362
2322
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1494
1307
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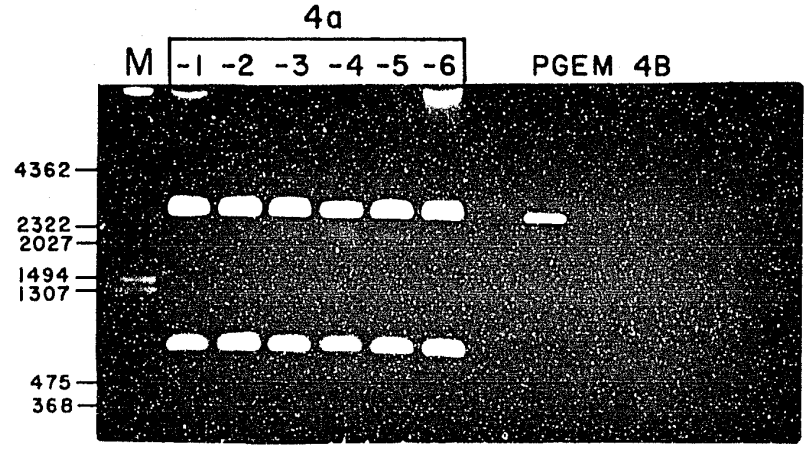
368


4a
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368

hybridizing insert experienced some insert loss. For colony 1a, two of the six selected daughter colonies appeared to have lost their insert (see lanes 1a-1 to 1a-6 in Figure 15B) while in the daughter cells prepared from colony 2a, five out of six plasmid preparations had no insert (lanes 2a-1 to 2a-6 in Figure 15B). None of the non-hybridizing inserts were lost, nor were the inserts from plasmids which had hybridized less strongly.

Several explanations for these results are possible. The trivial case is that this inconsistency resulted from experimental error in which impure colonies were selected. When this phenomenon was first observed during the initial RAV-1 work this was believed to be the answer. However, during this set of isolations, low plating densities and extremely careful selection were used to eliminate this possibility. A second, more plausible interpretation is that there was strong selection against cells with plasmids containing the insert which led to insert loss even in a *rec A*$^-$ host. This possibility is examined further in the Discussion.

RAV-2 cDNA Library

RNA Isolation and cDNA Preparation

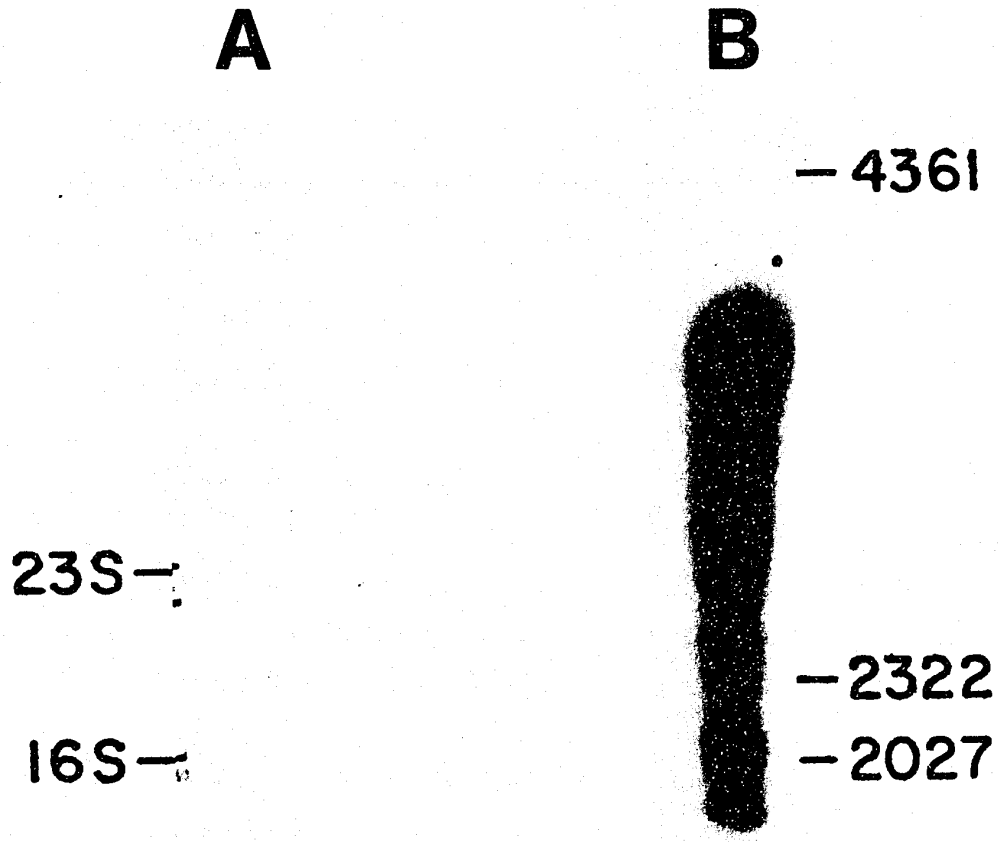
Cell culture fluid from a three week RAV-2 infection of chick embryo fibroblasts was generously provided by Dr. Lloyd Spencer (Animal Diseases Research Institute). Virus was purified and viral RNA was isolated as described in Materials and Methods. 54 μg of viral RNA was recovered as measured by spectrophotometry. The integrity of the RNA was confirmed by gel electrophoresis in the presence of formaldehyde; the size and condition of the viral RNA was as expected with a single band appearing at approximately 9 kb with a smear of degraded RNA present below the discrete band (data not shown). A Northern blot of the RNA gel was probed with the *Nco*I fragment of pSR-XD2; there was clear homology between the probe and the RNA (Figure 16A). In Figure 16A the upper band (approximately 9 kb) corresponds to the only

Figure 16A: Northern Analysis of Purified RAV-2 RNA

RAV-2 RNA prepared from chick embryo fibroblast cell culture fluid (Dr. Lloyd Spencer, ADRI) was electrophoresed on an agarose gel containing formaldehyde and the RNA was blotted onto nylon membrane. The Northern blot was hybridized with the 700 bp *Nco*I fragment of pSR-XD2.

Figure 16B: Analysis of cDNA Prepared from RAV-2 RNA

cDNA was prepared from the RNA shown in Figure 16A. An aliquot of the cDNA was electrophoresed through agarose gel, which was Southern blotted and probed with the 1.7 kb *Sma*I fragment of pSR-XD2.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band which can be seen by flourescence. It was proposed that the lower band might correspond to a different physical conformation of the RNA, to a degradation product or to a deletion event during viral growth.

RAV-2 RNA was used to prepare cDNA and the yield from a 25 μg aliquot of the viral RNA was 156 ng of cDNA. 10 ng of this cDNA was used for Southern transfer analysis and was probed with the 1.7 kb *Sma*I fragment from pSR-XD2. Figure 16B shows the size range observed; the largest cDNA fragments have a size of approximately 4000 bp. The hybridization pattern of the cDNA and the strong homology shown to the pSR-XD2 derived probe indicated that the cDNA was suitable for use in further studies.

Colony Hybridization

A portion of this cDNA was ligated into the *Sma*I site of pGEM 4B as had been previously done for the RAV-1 cDNA library. In an attempt to minimize the spontaneous deletion of plasmids and the severe selection against insert-containing plasmids, *E. coli* DH5α was transformed with the ligation mix[5]. After overnight growth at 37°C, normal colony hybridization procedures were followed using the 700 bp *Nco*I fragment as a probe. Thirty-six strongly positive colonies were identified from a total of approximately 3000 colonies; these were picked for a second screening. After the second screening, positive colonies were picked and rapid plasmid preparations were performed. Ampicillin was included with all cell cultures intended for use in the plasmid preparations in order to maintain selective pressure for the plasmid. This was essentially the same strategy as used with the RAV-1 cDNA earlier in the project but we found that the change from JM109 to DH5α as a host and the inclusion of ampicillin selection at every stage of the preparation significantly increased the retention rate of plasmids. After the second screening, plasmids hybridizing to the 700 bp

[5] We hoped that differences between this *rec*$^-$ strain and JM109 would result in the elimination of the spontaneous deletion of inserts.

*Nco*I fragment were identified in seventeen of the thirty-six positive colonies initially tested. Six additional colonies contained inserts which did not hybridize with the *Nco*I probe. Figure 17 shows a Southern blot of a series of these positives after further purification; the inserts which hybridized with the *Nco*I probe range in size from 250 bp to approximately 1100 bp.

Several additional portions of the cDNA were ligated into pGEM 4B restricted with *Sma*I and treated as described above. After second screenings, purified positive colonies were picked and their homology with the pSR-XD2 fragment was verified using rapid plasmid preparations and Southern Blots. In each case, only those recombinants which clearly contained single plasmids and which hybridized with the 700 bp *Nco*I probe most strongly were selected for further work. When the cDNA library screenings had been completed, a total of twenty-three recombinants had been identified as containing inserts which hybridized with the Rous sarcoma virus derived probe.

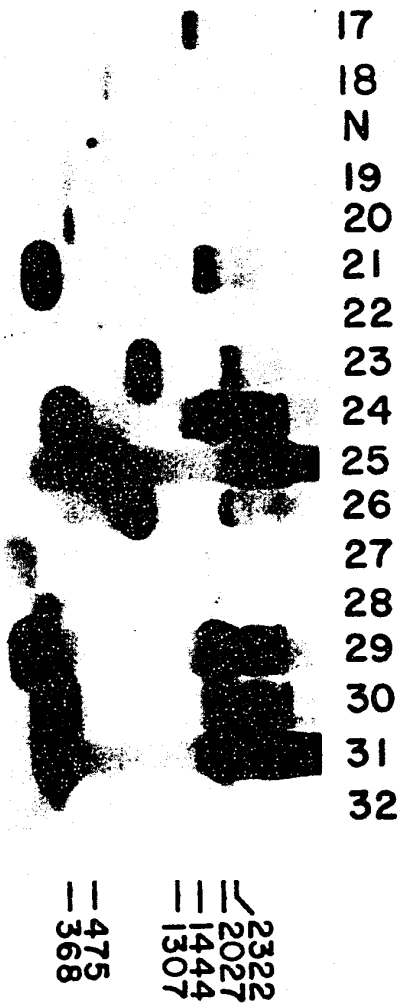
Characterization of the Inserts

From the group of plasmids identified through colony hybridizations and confirmed through Southern blots, eleven plasmids were selected for further characterization. This selection was based on insert length and strength of hybridization. As estimated by agarose gel electrophoresis, plasmid sizes ranged from 250 bp to 2900 bp. Insert sequences were determined from purified quantities of each of these plasmids. The purpose of this detailed characterization was not to obtain complete sequences for each of the selected inserts — rather the goal was to be able to compare the isolated fragment sequences to known RSV sequences. In this way it would be possible to confirm that the inserts contained RAV-2 cDNA sequences and to identify the exact coding regions that had been isolated. This information would have predictive value when later attempts to obtain expression of RAV-2 polypeptides were made.

Figure 17: Southern Analysis: RAV-2 Recombinant Plasmids from Hybridizing Colonies

Plasmid DNA's from several hybridizing colonies were restricted with *Eco*RI and *Bam*HI, subjected to electrophoresis, transferred to nylon membranes and probed with the multiprimed *Nco*I fragment of pSR-XD2. Plasmids in the lanes highlighted below were further purified, named and sequenced.

Lane 7: pGR2503-42;
Lane 9: pGR2419-12;
Lane 10: pGR2419-16;
Lane 25: pGR2419-14.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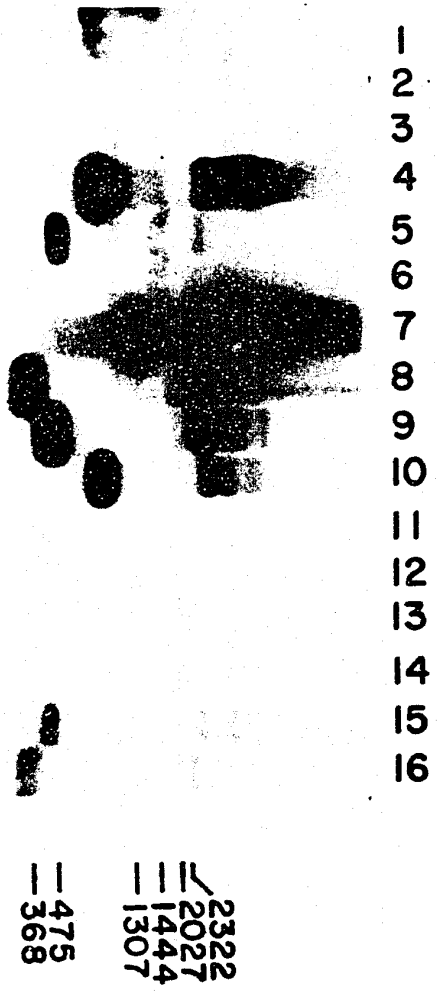
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Sequencing

The Southern Blot in Figure 17 includes four of the plasmids that were characterized by sequencing — pGR2503-42 (950 bp); pGR2419-12 (375 bp); pGR2429-14 (540 bp); pGR2419-16 (490 bp). Since the inserts were already located in the pGEM 4B multiple cloning site (MCS), sequencing was performed directly on plasmid DNA, from the Sp6 and T7 promoters. The partial sequences of three of the four clones noted above are shown in Figure 18A along with sequence from an additional clone isolated during a separate set of sceeenings, pGR2512G19, containing a 930 bp insert. These sequences are aligned with the homologous regions of Rous sarcoma virus (Prague-C strain)[6]. Of the longer inserts that had been identified, only pGR2512-G12 (a 2900 bp insert) showed no homology in the sequenced regions. Further examination with restriction analysis showed this recombinant to be a dimer of pGEM 4B with a small (200 to 250 bp) insert.

As expected, because of the probe chosen, each of the homologous sequences aligned with portions of the *env* and LTR regions in RSV. Differences in sequence result from the fact that RAV-2 has B type host specificity and the RSV sequences are from a strain with C type host specificity. As expected, all sequences begin in the LTR region. The longest sequences obtained appear to terminate approximately 200 bp upstream of the first *Nco*I site within the *env* gene, and within 75 bp of the beginning of the expressed gp37 sequence (Figure 18B). The significance of the inclusion of these sequences within the recombinants obtained is examined in detail in the Discussion. Briefly, it would appear that the sequence obtained includes potential antigenic determinants within the gp37 polypeptide. All three potential asparagine glycosylation sites, as suggested by Schwartz *et al.* (1983) are included in the recovered sequence. However, no sequences coding for the more highly glycosylated

[6] All RAV-2 sequences that were obtained can be found in Appendix B.

Alignment of Insert Sequences from Hybridizing Colonies with Rous Sarcoma Virus Sequence.

After large scale preparation and purification, sequences were obtained from both the T7 and Sp6 promoters of pGEM 4B. The alignment of these sequences with that of RSV (as per Schwartz *et al*, 1983) is shown.

The end of the *env* gene is marked as determined by Scharwtz *et al* (1983) in the Prague C strain; however, Hunter *et al* (1983) note that the carboxy-terminus of gp37 is variable in length and composition among strains.

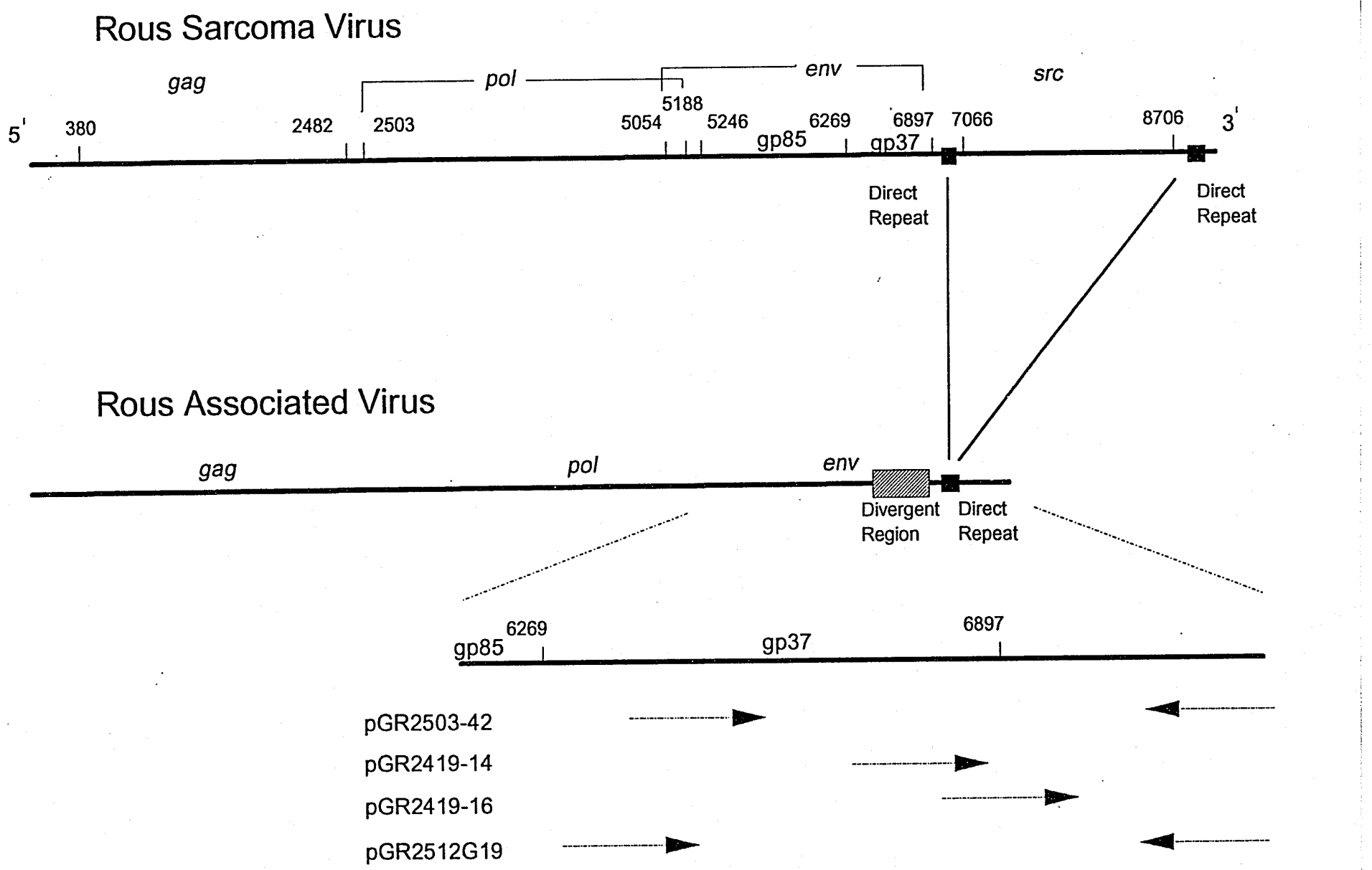
The position marked as the start of gp85 is the position of the codon for the first amino acid found in mature gp85. The splice acceptor site of *env* involved in splicing with the first portion of the sequence coding for the 62 amino acid signal peptide is found 5' to this (not shown).

Figure 18A: Schematic Positions
(opposing page)

The four sequences described in Figure 18B are placed on a generalized schematic map of Rous associated virus strains. These strains do not contain the *src* gene and are known to show considerable divergence at the carboxyl terminus of gp37 and in the 3' region, upstream from the LTR. The direct repeat which may serve as a target for recombination with c-*src* is also shown.

Figure 18B: Nucleic Acid Sequence
(next page)

Dashes indicate matching bases, asterices indicate missing bases, bases shown in the derived sequence indicate a mismatch with the published RSV sequence.


Rous Sarcoma Virus
gag
pol
env
src
5'
380
2482
2503
5188
5054
5246
6269
6897
7066
8706
3'
gp85
gp37
Direct Repeat
Direct Repeat
Rous Associated Virus
gag
pol
env
Divergent Region
Direct Repeat
6269
6897
gp85
gp37
pGR2503-42
pGR2419-14
pGR2419-16
pGR2512G19

```
                                                  | gp85 start (5246)
5210  GTCCTGGTCTTGTGTGAGGTTACGGGGGTAAGAGCTGATGTTCACTTACTCGAGCAGCCA

5270  GGGAACCTTTGGATTACATGGGCCAACCGTACAGGCCAAACGGATTTCTGCCTCTCTACA

      CAGTCAGCCACCTCCCCTTTTCAAACATGTTTGATAGGTATCCCGTCTCCTATTTCCGAA

      GGTGATTTTAAGGGATATGTTTCTGATACAAATTGCTCCACTGTGGGAACTGACCGGTTA

      GTCTTGTCAGCCAGCATTACCGGCGGCCCTGACAACAGCACCACCCTCACTTATCGAAAG

      GTTTCATGCCTGCTGTTAAAGCTGAACGTCTCCATGTGGGATGAGCCACCTGAACTGCAG

      CTGCTAGGTTCCCAGTCTCTCCCTAACGTTACTAACATTACTCAGGTCTCTGGCGTGGCC

      GGGGGATGTGTATATTTCGCCCCAAGGGCCACTGGCCTGTTTTTAGGTTGGTCTAAACAA

      GGTCTCTCGCGGTTCCTCCTCCGTCACCCCTTTACCTCCACCTCTAACTCCACGGAACCG

      TTCACGGTGGTGACAGCGGATAGACACAATCTTTTTATGGGGAGTGAGTACTGTGGTGCA

      TATGGCTACAGATTTTGGGAAATATATAACTGCTCACAGACTAGGAATACTTACCGCTGT

      GGAGACGTGGGAGGTACTGGCCTCCCTGAAACCTGGTGCAGAGGAAAAGGAGGTATATGG

      GTTAATCAATCAAAGGAAATTAATGAGACAGAGCCGTTCAGTTTTACTGCGAACTGTACT

      GGCAGTAATTTGGGTAATGTCAGCGGATGTTGCGGAGAACCAATCACGATTCTCCCACTA

      GGGGCATGGATCGACAGTACGCAAGGTAGTTTCACTAAACCAAAAGCGCTACCACCCGCA

6110  ATTTTCCTCATTTGTGGGGATCGCGCATGGCAAGGAATTCCCAGTCGTCCGGTAGGGGGC

6170  CCCTGCTATTTAGGCAAGCTTACCATGTTAGCACCCAACCATACAGATATTCTCAAAATA
                                                  |Start of gp37 (6269)
6230  CTTGCTAATTCGTCGCGGACAGGTATAAGACGTAAACGAAGCGTCTCACACCTGGATGAT

                                    pGR2512G19(5')  ------G---------
6290  ACATGCTCAGATGAAGTACAGCTTTGGGGTCCTACAGCAAGAATCTTTGCATCTATCTTA

      --------------------A-----------------------------------------
                        pGR2503-42(5') ---------------------------
6350  GCCCCGGGGGTAGCAGCTGCGCAAGCCTTAAGAGAAATTGAGAGACTAGCCTGTTGGTCC

      --------------  pGR2512G19(3')
      ---------------------------------------------A---------------
6410  GTTAAACAGGCTAACTTGACAACATCACTCCTCGGGGACTTATTGGATGATGTCACGAGT

      -----------A---------------*----G---------------*------------
6470  ATTCGACACGCGGTCCTGCAGAACCGAGCGGC TATTGACTTCTTGCTTCTAGCTCACGGC

      |NcoI site
      --- pGR2503-42(3')
6530  CATGGCTGTGAGGACGTTGCCGGAATGTGTTGTTTCAATCTGAGTGATCACAGTGAATCT

6590  ATACAGAAGAAGTTCCAGCTAATGAAGAAACATGTCAATAAGATCGGCGTGGACAGCGAC

                                                  pGR2419-14(5')--
6650  CCAATCGGAAGTTGGCTGCGAGGGATATTCGGGGGAATAGGGGAATGGGCCGTTCATCTG

      --G--------------------------C--G-C----G-A--A---T-----------
6710  CTAAAAGGACTGCTTTTGGGGCTTGTAGTTATTTTATTGCTACTGGTGTGCCTGCCTTGC

      -----G----G---A-----------------------GC---A----C--GG-----GC
```

```
6770   CTTTTACAATTTGTGTCTAGTAGTATTCGAAAGATGATTA ATAGTTCAATCAACTATCAT

                                              | end of env
       GAG-----T-AA--AT-A-----G-CTTATAAGC-- pGR2419-14(3')
6830   ACTGAATACAGGAAGATGCAGGGCGGAGCAGTCTAGAGCTCAGTTATAATAATCCTGCGA
             | start of direct repeat
       pGR2419-16(5')
         ----T---------*-----------T--------A---G-----T------------
6890   ATCGGGCTGTAACGGGGCAAGGCTTGACCGAGGGGACTATAACATGTATAGGCGAAAAGC

       ----CT---------*----G------*--------C---A---A-T--T-TCA--T----
6950   GGGGTCTCGGTTGTAACGCGCTTAGGAAGTCCCCTCGAGG TATGGCAGATATGCTCTTGC

        | end of direct repeat
       --(cont'd at base 9047)
7010   ATAGGGGGAAAAAATGTAGTCTTAATATTGTCTGTGTGCTGCAGGAGCTAAGCTGACTCT

                                                          NcoI site|
7070   GCTGGTGGCCTCGCGTACCACTGTGGCCAGGCGGTAGCTGGGACGTGCAGCCGACCACCA

7130   TGGGGAGCAGCAAGAGCAAGCCTAAGGACCCCAGCCAGCGCCGGCACAGCCTGGAGCCAC

7190   CCGACAGCACCCACCACGGGGGATTCCCAGCCTCGCAGACCCCCGACGAGACAGCAGCCC

7250   CCGACGCACACCGCAACCCCAGCCGCTCCTTCGGGACCGTGGCCACCGAGCCCAAGCTCT

       TCTGGGGCTTCAACACTTCTGACACCGTCACGTCGCCGCAGCGTGCCGGGGCACTGGCTG

       GCGGCGTCACCACTTTCGTGGCTCTCTACGACTACGAGTCCTGGACTGAAACGGACTTGT

       CCTTCAAGAAAGGAGAACGCCTGCAGATTGTCAACAACACGGAAGGTGACTGGTGGCTGG

       CTCATTCCCTCACTACAGGACAGACGGGCTACATCCCCAGTAACTATGTCGCGCCCTCAG

7550   ACTCCATCCAGGCTGAAGAGTGGTACTTTGGGAAGATCACTCGTCGGGAGTCCGAGCGGC

       TGCTGCTTAACCCCGAAAACCCCCGGGGAACCTTCTTGGTCCGGAAGAGCGAGACGGCAA

       AGGGTGCCTATTGCCTCTCCGTTTCTGACTTTGACAACGCCAAGGGGCCCAATGTGAAGC

       ACTACAAGATCTACAAGCTGTACAGCGGCGGCTTCTACATCACCTCACGCACACAGTTCG

       GCAGCCTACAGCAGCTGGTGGCCTACTACTCCAAACATGCTGATGGCTTGTGCCACCGCC

       TGGCCAACGTCTGCCCCACGTCCAAGCCCCAGACCCAGGGACTCGCCAAGGACGCGTGGG

       AAATCCCCCGGGAGTCGCTACGGCTGGAGGCGAAGCTGGGGCAGGGCTGCTTTGGAGAGG

       TCTGGATGGGGACCTGGAACGACACCACCAGAGTGGCCATAAAGACTCTGAAGCCCGGCA

       CCATGTCCCCGGAGGCCTTCCTGCAGGAAGCCCAAGTGATGAAGAAGCTCCGGCATGAGA

       AGCTGGTTCAGCTGTACGCAGTGGTGTCGGAAGAGCCCATCTACATCGTCATTGAGTACA

8150   TGAGCAAGGGGAGCCTCCTGGATTTCCTGAAGGGAGAGATGGGCAAGTACCTGCGGCTGC

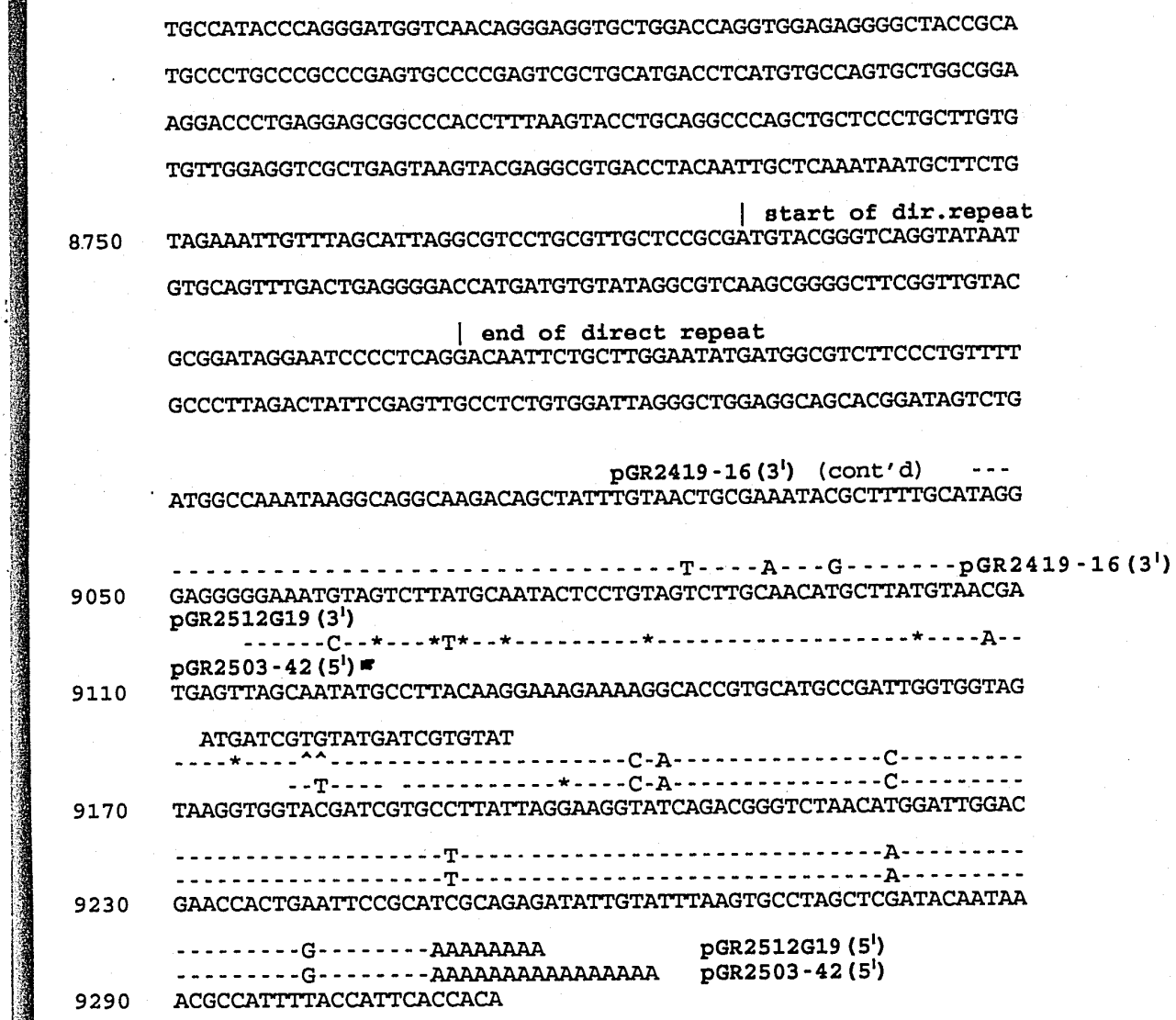
       CACAGCTCGTCGATATGGCTGCTCAGATTGCATCCGGCATGGCCTATGTGGAGAGAATGA

       AACTACGTGCACCGAGACCTGCGGGCGGCCAACATCCTGGTGGGGGAGAACCTGGTGTGCA

       AGGTGGCTGACTTCGGGCTGGCACGCCTCATCGAGGACAACGAGTACACAGCACGGCAAG

       GTGCCAAGTTCCCCATCAAGTGGACAGCCCCCGAGGCAGCCCTCTATGGCCGGTTCACCA

       TCAAGTCGGATGTCTGGTCCTTCGGCATCCTGCTGACTGAGCTGACCACCAAGGGCCGGG
```


TGCCATACCCAGGGATGGTCAACAGGGAGGTGCTGGACCAGGTGGAGAGGGGCTACCGCA
TGCCCTGCCCGCCCGAGTGCCCCGAGTCGCTGCATGACCTCATGTGCCAGTGCTGGCGGA
AGGACCCTGAGGAGCGGCCCACCTTTAAGTACCTGCAGGCCCAGCTGCTCCCTGCTTGTG
TGTTGGAGGTCGCTGAGTAAGTACGAGGCGTGACCTACAATTGCTCAAATAATGCTTCTG
| start of dir.repeat
8750 TAGAAATTGTTTAGCATTAGGCGTCCTGCGTTGCTCCGCGATGTACGGGTCAGGTATAAT
GTGCAGTTTGACTGAGGGGACCATGATGTGTATAGGCGTCAAGCGGGGCTTCGGTTGTAC
| end of direct repeat
GCGGATAGGAATCCCCTCAGGACAATTCTGCTTGGAATATGATGGCGTCTTCCCTGTTTT
GCCCTTAGACTATTCGAGTTGCCTCTGTGGATTAGGGCTGGAGGCAGCACGGATAGTCTG
pGR2419-16(3') (cont'd) ---
ATGGCCAAATAAGGCAGGCAAGACAGCTATTTGTAACTGCGAAATACGCTTTTGCATAGG
----------T----A---G-------pGR2419-16(3')
9050 GAGGGGGAAATGTAGTCTTATGCAATACTCCTGTAGTCTTGCAACATGCTTATGTAACGA
pGR2512G19(3')
------C--*---*T*--*---------*----------*----A--
pGR2503-42(5')
9110 TGAGTTAGCAATATGCCTTACAAGGAAAGAAAAGGCACCGTGCATGCCGATTGGTGGTAG
ATGATCGTGTATGATCGTGTAT
----*----^^----------C-A----------C---------
--T---- ----------*----C-A----------C---------
9170 TAAGGTGGTACGATCGTGCCTTATTAGGAAGGTATCAGACGGGTCTAACATGGATTGGAC
----------T----------A---------
----------T----------A---------
9230 GAACCACTGAATTCCGCATCGCAGAGATATTGTATTTAAGTGCCTAGCTCGATACAATAA
---------G--------AAAAAAAA pGR2512G19(5')
---------G--------AAAAAAAAAAAAAAAA pGR2503-42(5')
9290 ACGCCATTTTACCATTCACCACA

gp85 polypeptide[7] were obtained nor did any sequenced insert include the regions thought to determine host specificity (the hypervariable regions as described by Hunter *et al.* (1983) and Dorner *et al.* (1985). The importance of the inclusion of cDNA sequences encoding some suggested glycolsylated sites and the absence of others is unknown. Although *E. coli* does not have the ability to perform these post--translational modifications it is possible that a polyclonal antibody would contain antibodies to the general region even without glycosylation; this area was not sufficiently understood to draw any conclusions about the imunogenicity of the encoded polypeptide directly from this sequence

Construction of the Expression Recombinants

Preparation of the Inserts

Plasmids pGR2512G19 and pGR2503-42 each contained inserts greater than 900 bp in length and showed high sequence homology to the gp37 coding region of RSV. Inserts from these two plasmids were isolated and cloned into λgt11 for expression studies. In order to obtain expression it is critical that the insert be placed in the appropriate reading frame. Because the full sequence of each of the large inserts had not been ascertained, inserts corresponding to all three reading frames were processed. Three aliquots (1.5 μg) of each the three plasmids were separated. Each aliquot was restricted with *Bam*HI which cuts at a single site in the MCS at position 28. Then, for each plasmid, one aliquot was restricted with *Bst*XI (cuts at 2734, upstream of the MCS), *Kpn*I (cuts at position 23, just upstream of the *Sma*I insertion site) and *Eco*RI (cuts at position 1 in the MCS)[8]. These enzymes were selected based upon the absence of their restriction sites in the corresponding *env* region in other

---

[7] Schwartz *et al.* identified 14 potential glycosylation sites within this polypeptide (1983).

[8] See Appendix A for the multiple cloning site sequence.

known sequences of RSV and RAV strains. Each of the digests was run on an agarose gel and the excised insert was purified using the GeneClean system. The protruding ends on each of the digested fragments were repaired using the Klenow fragment of DNA polymerase I. The fragments restricted with *Kpn*I and *Bst*XI were treated with *Eco*RI methylase. *Eco*RI linkers were then ligated to the fragments and multiple linkers were removed by digestion with *Eco*RI. The prepared fragments were viewed on an agarose gel to provide an estimate of the remaining DNA quantity.

Figure 19 shows the predicted reading frames of excised, repaired and linked fragments for pGR2503-42. In this plasmid the sense strand for gp37 is oriented with the 5' end beginning near the Sp6 promoter. Section 1 shows the reading frame in native Rous sarcoma virus with the base corresponding to the first base of pGR2503-42 marked. Section 2 shows the sequences resulting after processing the DNA's with each of the three restriction enzymes as described above. Note that the reading frame of the *Eco*RI fragment remains the same, that of the *Kpn*I fragment is shifted by one base (+1) and that of *Bst*XI is shifted by two bases (+2) after treatment. Section 3 shows the predicted reading frame in λgt11 and the position of the *Eco*RI site. If the RSV sequence and open reading frame determined by Schwartz *et al.* (1983) is also correct for RAV-2 then the fragments excised with *Eco*RI can be determined to be in reading frame 0 after ligation into λgt11 restricted with *Eco*RI; those excised with *Kpn*I will be in reading frame +1 and those excised with *Bst*XI will be in reading frame +2. In this way, all three possible reading frames should be represented in the λgt11 recombinants. A similar route was followed for pGR2512G19.

Cloning the Insert

Portions of each of the six digests were then combined, ligated into λgt11 arms, restricted with *Eco*RI and packaged in vitro. After ligation it is important to note that the *Eco*RI fragment will be in the correct reading frame for expression from the λgt11 β-galactosidase promoter if the reading frame of RSV is conserved in RAV-2. Titres

Figure 19: Reading Frame Analysis of Recombinant λgt11

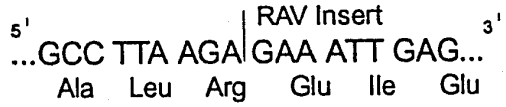
The insert fragments of pGR2503-42 resulting from three restriction digests after treatment with the Klenow fragment of DNA polymerase I and the addition of *Eco*RI linkers were ligated with λgt11.

Section 1: **Rous sarcoma virus (Schwartz *et al*, 1983).** The amino acids coded for by the open reading frame are shown under the DNA sequence. The first base of the insert sequence in pGR2503-42 is marked with a vertical line.

Section 2: **Processed insert reading frames.** The predicted 5' sequence which resulted from the processing of the insert excised from pGR2503-42. Parts a,b, and c correspond to the sequences predicted when the insert was excised with *Eco*RI, *Kpn*I and *Bst*XI respectively, along with *Bam*HI (see text for full description of processing). For each enzyme the distance from the 5' end of the modified insert to the beginning of the putative open reading frame of the RAV seqeunce is marked.

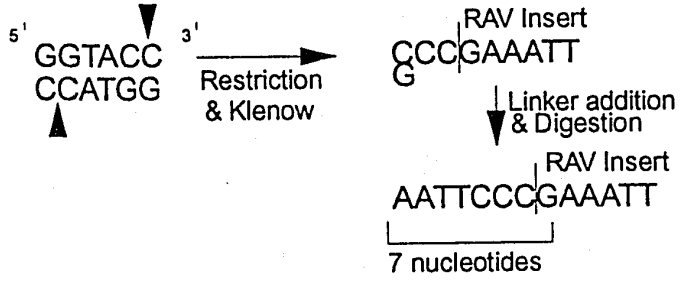
Section 3: **λgt11 (Huynh *et al*, 1985).** The open reading frame of the *lacZ* gene in λgt11 is shown by the amino acids encoded. The *Eco*RI digestion site into which the processed pGR2503-42 inserts were ligated is marked.

1. RSV Open Reading Frame

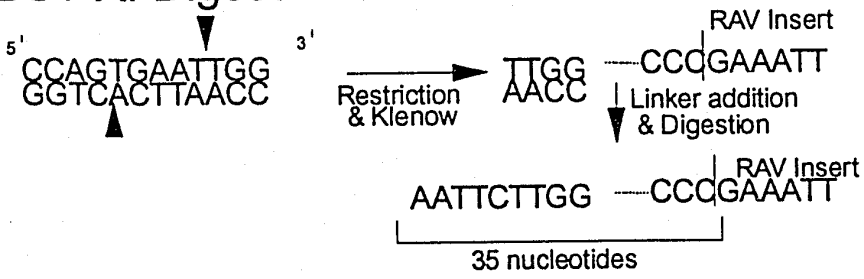


2a. Eco RI Digest

2b. Kpn I Digest



2c. BST XI Digest



3. gt11 Open Reading Frame & Insert Site

were performed on the packaged virus and from the ratio of clear to blue plaques it was calculated that recombinant phage constituted approximately 23% of the library. Dilution series established that a total of approximately 30000 recombinants were present in the packaged library. It was not possible to determine which recombinants contained inserts in the correct reading frame and orientation but if there was equal representation of each of the three reading frames in one orientation and the reading frame of the second orientation then it could be expected that approximately 5000 recombinants would ligate in the correct orientation for expression.

Plaque Hybridizations

To determine the quality of the phage library, Y1090 was infected with a portion of the library. After 5 hours growth the plaques were lifted onto HyBond-N and hybridized with the labelled 700 bp *Nco*I fragment from pSR-XD2. Approximately 20% of the plaques showed homology to the probe; this compares favourably to the 23% recombinants determined on the basis of plaque colour. However, from these studies it was not possible to estimate what percentage of these recombinants represented each of the three reading frames or two orientations.

Immunological Detection

Plaque Immunoassays

After confirmation of the status of the phage library, immunoassays were carried out. Two platings of the phage library were used. The first was a normal plating of Y1090 infected with a portion of the library and induced with IPTG after plaque formation was observed (see Materials and Methods). The primary antibody, a polyclonal antibody prepared in chicken against Rous sarcoma virus, was provided by

Dr. Lloyd Spencer of ADRI[9]. The secondary antibody was a mouse anti-chicken antibody conjugated with peroxidase. Colour detection was with DAB. Despite several efforts involving a total of approximately 15000 plaques (of which approximately 2500 could statistically be expected to be of the correct reading frame and orientation), no expression above background levels was seen from any of the immunoscreenings.

The second set of platings took advantage of the fact that it was possible to determine the presence of an insert in a plaque on the basis of its colour, when plated on X-gal/IPTG plates. Since the recombinant λgt11 was the result of directed clonings of the *env* sequence we expected that clear plaques should contain RAV-2 sequence in one of the three reading frames. In preparation for targeted immunoscreenings, a second infection of Y1090 with the phage library was prepared. After low density plating (500 plaques per plate) and growth, 40 clear plaques and 7 blue plaques were selected and grown on a second plate. No positive reactions were seen when these plates were processed through the normal immunological screening methodology using the antibodies described above. From these results I concluded that the λgt11 recombinants were not producing any polypeptide fragments which were immunogenically recognizable to the RSV antibody supplied by ADRI.

We proposed three possible technical reasons for the failure of the immunological detection.

1/ Inability of the antibody serum to detect RAV-2 antigenic determinants. This might be due to several deficiencies, including a lack of gp37 specific antibodies in the serum.

2/ Inability of the λgt11 phage to produce a recognizable epitope when detection was performed by the antibody supplied by ADRI.

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[9] I expected the RSV antibody to cross-react with RAV-2 antigens, in part because of its polyclonal nature. (Dr. Lloyd Spencer, personal communication).

3/ Inability of the recombinant λgt11 to encode any recognizable RAV-2 antigenic determinants, perhaps due to blockage of protein expression from the open reading frame.

Assessing Immunological Detection

A series of experiments was designed to test the three possibilities for the failure of detection. RSV antigens in serum were supplied by Dr. Lloyd Spencer. 5 μl of 10^{-1}, 10^{-2} and 10^{-3} dilutions of this serum were dotted on nitrocellulose membranes and processed by the normal immunodetection procedures. Detection of the antigens was clearly seen even at the 10^{-3} dilution (data not shown). This indicated that the antibody was capable of detecting at least one of the several antigenic determinants normally produced from Rous sarcoma virus. Although Rous associated virus antigenic determinants differ slightly from those produced during an RSV infection it is reasonable to expect some cross-reaction from a polyclonal antibody.

To ensure that the λgt11 library was capable of producing a recognizable antigenic determinant, Y1090 was infected with a mix of non-recombinant λgt11 and λgt10 phage. After normal growth and induction, the plates were lifted onto nitrocellulose and regular immunoscreening protocols were followed. Detection of β-galactosidase was accomplished with a 1:400 dilution of a primary antibody prepared in mouse (Promega Biotech) followed with 1:5000 and 1:7500 dilutions of rabbit anti-mouse secondary antibody conjugated with HPTG. Figure 20 shows the clear detection of the β-galactosidase-producing λgt11 phage; no λgt10 phage produced an immunological reaction. Thus, we were able to conclude that the immunoscreening methods being used were capable of detecting antigen expressed within λgt11.

The effect of λgt11 expression on normally antigenic cDNA insert sequences could not be assessed in this manner as, by definition, there was no method of verifying that an antigen-encoding cDNA sequence had been inserted in the vector. Thus, in

Figure 20: Immunoscreening of λgt11 and λgt10

Approximately 200 plaques representing a 1:1 mixture of λgt11 and λgt10 phage were plated and screened with a primary antibody for the production of β-galactosidase. Successful antigen detection is indicated by the presence of colour. Below the plaque lift filter, a titration series of β-galactosidase (which acts as a positive control) is shown with 10^{-3} through 10^{-1} dilutions from left to right.

assessing the three basic reasons for detection failure, we were able to disprove two of them. The experiments detailed above showed that the antibody could react with native antigens and that the λgt11 host was able to produce recognizable antibodies under the immunological screening conditions used. This suggests that the failure of the RAV-2/λgt11 recombinant screenings to demonstrate any immunological reaction was due to the inability of the host to produce a polypeptide specifically recognizable by the antibody. Several reasons for this are explored at length in the Discussion.

Discussion

Pseudorabies virus libraries were constructed using two methods. Inserts from a cDNA library cloned into λgt10 were subcloned into pUC8 and restriction fragments of PrV genomic DNA were cloned into pUC18. Attempts to immunologically detect clones containing pseudorabies virus envelope epitopes were unsuccessful. During the attempts at detection, two clones producing a unique 30 kD band on Western Blots were isolated.

A RAV-2 cDNA was cloned into pGEM 4B and the portion of the *env* gene encoding the gp37 protein was isolated and sequenced. Sequence analysis has confirmed published sequences and homology between this region of the *env* gene in RAV-2 and Rous sarcoma virus was demonstrated. Directed sub-cloning of the sequence in all three reading frames into λgt11 failed to result in recombinants capable of producing an antigen recognizable by an antibody to Rous sarcoma virus supplied by the Animal Diseases Research Institute.

I. Pseudorabies Virus

The cDNA and Genomic Libraries

Selected inserts isolated from the cDNA library provided by Dr. Pierre Moreau initially appeared to result in a number of immunologically positive clones when subcloned into pUC8. Furthermore, as expected when working with a polyclonal detection system there was some, but not complete, cross-hybridization between insert DNA's isolated from different clones. However, when permission was received to begin work with the pseudorabies viral DNA it was quickly ascertained that the clones had

no homology to the PrV DNA. Similarly, the pUC18 genomic library was shown to contain a high number of sequences capable of hybridizing with PrV DNA by direct colony hybridization. When immunologically positive colonies were further characterized it was found that they did not contain inserts capable of hybridizing with nick-translated PrV DNA.

Because of the lack of material available for further examination and because of the continued physical difficulties involved in working with the PrV system, a detailed examination of these misleading results was not performed and an explanation of them remains unclear. However, it is possible to draw some conclusions which narrow the range of possible interpretations for the results obtained. There are three possible causes for the failure to correctly identify clones containing epitope-encoding pseudorabies viral DNA and capable of producing antigens detectable by the supplied antibody: i) the detection methodology used was flawed; ii) the cDNA library did not contain antigen-encoding pseudorabies virus cDNA nor did the genomic library contain sequences capable of encoding PrV antigens; or iii) the antibody was not capable of recognizing proteins produced in the recombinant bacterial cells.

Detection Methodology

In the last five years a large quantity of literature detailing complex methods for detecting the production of eucaryotic proteins and polypeptides and the use of sophisticated expression vectors for maximizing protein production has been published. When compared to this literature, the methods used in the course of my studies appear simplistic. When the work was begun, there were few references to similar work in the literature but several other laboratories had had success with detection systems comparable to the one used in this portion of the project. In particular, Mayer *et al.* (1983) successfully used a polyclonal antibody, prepared in mink, to detect polypeptides produced from a genomic library of Aleutian disease virus prepared in pUC8 and amplified in *E. coli* C600. Helfman *et al.* (1983) isolated two

cDNA clones of 600 bp and 900 bp from a cDNA library prepared from chicken smooth muscle mRNA, constructed in pUC8 and amplified in *E. coli* DH-1, using a polyclonal antitropomyosin antibody prepared in rabbit. These studies suggest that the basic premise of the cloning and detection methodology was sound.

Inclusion of Suitable Pseudorabies DNA Sequences

By definition, it is not possible to conclusively determine whether the pseudorabies virus cDNA or genomic libraries contained any clones capable of producing an immunologically active antigen until such a clone was isolated. However, statistical estimates of the completeness of these libraries provide some indication of how representative each was. 10,000 recombinants resulting from the subcloning of the 500 bp to 1 kb glycerol gradient smear were screened from the cDNA library. Since the PrV genomic DNA is 140 kb it is possible that this was the equivalent of screening approximately 150 genomes, assuming equal representation of all cDNA sequences in the λgt10 cDNA library following the cloning and amplification steps. It is unlikely that this assumption is completely correct; at the time that these experiments were conducted, specific information on the timing of expression of PrV mRNA's in infected cells was not available and it was unclear if the various epitope-coding sequences would be equally represented in the library. Since I was not able to perform nucleic acid hybridizations upon the cDNA library at the time that the immunological studies were being conducted it is impossible to assess the inclusion of potential epitope-encoding cDNA sequences. However, these statistics provide a tenuous estimate of the library's potential.

More substantial conclusions can be drawn with respect to the genomic library constructed in pUC18. The manner in which the library was constructed would result in an equal distribution of all DNA sequences if the vigor of *E. coli* cells containing recombinant sequences was not compromised by the presence of the insert sequence. Nucleic acid hybridization experiments indicated that 68% of the 8000 colonies

screened, or approximately 5500 colonies, contained PrV DNA. The insert sizes of the immunologically positive clones recovered from this library varied between 700 bp and 900 bp — this would have been the equivalent of screening 25 to 35 complete pseudorabies virus genomes. Given the polyclonal nature of the antibody, we anticipated that in a screening of this sample size, DNA sequences responsible for encoding recognizable epitopes within normal host cells should exist.

Antigen Recognition Capability

Initially, immunodetection was the only method of identifying clones which contained PrV DNA. It was not until very close to the end of the work that full-length pseudorabies virus DNA was available from ADRI for manipulation outside of their laboratories. Because of this problem, and in order to obtain clones producing recognizable PrV antigens, the quality of the antibody was critical. As described in Materials and Methods, the polyclonal antibody was prepared in swine infected with pseudorabies virus. The antibody provided by ADRI was identical to that which was used in ELISA testing for screening imported pigs. Its effectiveness in this application has been well-documented but the implications of this effectiveness for immunoblotting protocols is unknown.

At the time that my work was initiated there were several reports in the literature of polyclonal antibodies being successfully used in a similar manner to that which was planned for our experiments. Notably, Helfman *et al.* (1983), Kemp *et al* (1983) and Mayer *et al.* (1983) provided examples of the use of polyclonal antibodies (produced in rabbit, human and mink, respectively) for the detection of polypeptides encoded by eucaryotic DNA in *E. coli.* Towards the end of the work literature became available which documented the possibility of using an affinity-purified polyclonal antibody (eg. Gerald *et al.*, 1986). However, reports continued to note the successful use of antiserum directly prepared from inoculated animals (eg. Peters and Baumeister, 1986; Whitehead and Rabinowitz, 1986). There was considerable evidence which

led us to believe that the polyclonal antibody prepared directly from infected swine would be sufficient for immunodetection.

However, this assumption was later shown to be incorrect for polyclonal antibody preparations prepared in swine against the pseudorabies virus. Timmins *et al.* (1986) provided the first explanation for the difficulties which appeared during my immuno-screenings. They noted that they were unsuccessful in screening a λgt11 library of sheared PrV DNA using PrV hyperimmune sera prepared in swine. It was only after screening the library with antibodies raised in mice, targeted against denatured PrV proteins eluted from SDS-PAGE gels, that fusion proteins produced by recombinant phage were recognized. They hypothesized that their method of antibody production allowed for greater immunogenicity of linear epitopes (those in which the tertiary structure has a limited effect upon antigenicity) in *E. coli*. This is important since the lack of post-translational processing of mammalian proteins in *E. coli* commonly results in different protein tertiary structures than those found in native mammalian cells. Further work by the same group (Petrovskis *et al.*, 1986) provided additional confirmation of the requirement to raise antiserum against denatured PrV proteins. Once I became aware of this work it became clear that I would be unable to achieve my goals using the antibody prepared at ADRI.

The 30 kD Proteins

Although it is now clear that it would not have been possible to detect pseudorabies antigens using the antibodies available, there is no doubt that certain clones were isolated that generated a positive immunological reaction with the antibody. In particular, the Western blots of the three recombinant clones 305, 306 and 309 produced distinctive results. The 30 kD proteins seen in the Western Blots of protein preparations from 306 and 309 (Figure 12) is considerably smaller than the 40 to 45 kD fusion protein that is predicted from the approximately 1 kb of insert DNA in these

two clones. Similar results were observed by Mayer *et al.* (1983). This group isolated a 1.6 kb fragment of Aleutian disease virus DNA from a recombinant pUC8 plasmid. This fragment would be expected to maximally encode a protein with a molecular weight of approximately 59 kD. However, they detected antigenic proteins with molecular weights of 55, 33 and 27 kD using Western Blot analysis. They suggested that these smaller proteins could be breakdown products or the result of premature termination during transcription of the larger protein. Suggestions of the latter possibility are also found in work performed by Watson *et al.* (1982) with herpes simplex virus. It is possible that my observations of the smaller than expected protein could be due to a similar phenomenon.

Throughout the course of my work with pseudorabies virus it was fairly common to see a recombinant clone, which had been immunogenically detected over several screenings, stop providing a positive reaction with the antibody. I hypothesized that this was likely due to the loss of the antigen producing insert from the clone; this seemed quite possible in early work since the C600 strain is *rec* A^+. However, it is difficult to understand why protein preparations from clone 305, isolated at the same time as 306 and 309 did not show the 30 kD band on the Western blot. Peters and Baumeister (1986) experienced a similar problem in cloning DNA from *Deinococcus radiodurans* into pUC18, transforming JM 109 and detecting protein production by Western blots. In their work, they noted that they originally isolated five clones expressing distinct protein bands. Four of the five lost this ability during further cultivation; they were only able to continue work with one of the originally positive recombinants. Although they do not provide an explanation for their observations there is a strong parallel with my observations.

The exact origin of the DNA responsible for encoding the 30 kD antigenic protein fragment remains unclear. As noted, subsequent DNA hybridization conclusively demonstrated that the DNA did not originate in the pseudorabies virus. The best hypothesis which can be presented is that the approximately 1 kb DNA inserts in clones 305, 306 and 309 represented contaminants, in either the PrV DNA prepara-

tion, or introduced during the cloning into pUC18. The polyclonal nature of the PrV antibody ensured that numerous non-PrV proteins would demonstrate an antigenic reaction. For example, even though the antibody was cleared with *E. coli* lysate prior to use, the high background levels seen on the Western Blots show the large quantities of unbound *E. coli* antibodies remaining. It is likely that the 30 kD protein fragments encoded by the contaminant DNA contained an epitope to an antibody found within the polyclonal mixture.

II. Rous Associated Virus

Immunological Analysis

Ishizaki and Vogt (1966) originally identified the viral envelope as containing the antigen(s) responsible for type and subgroup specificity. Tozawa *et al.* (1970) provided a more complete characterization and showed that although the majority of type-specific antigenicity was determined by what is now known as the gp85 protein, the gp37 protein was also capable of eliciting an immunological reaction. In serum neutralization testing it is thought that the glycosylated residues act as the antigenic determinants. There are 14 potential glycosylation sites in gp85 and 2 or 3 in gp37; most sites are utilized, with some strain-specific differences (Hunter *et al.*, 1983). The longer inserts which I isolated from the RAV-2 cDNA library and subcloned into λgt11 included all but the first 75 nucleotides of the gp37 protein coding sequence; they did not contain any of the sequence coding for gp85.

As with work on the pseudorabies virus, we felt that the literature provided sufficient examples of normally glycosylated proteins which remained antigenically active when produced from bacterial cells, given that a polyclonal antibody was used for detection. In particular, Keegan and Collett (1986) were able to identify four antigenic determinants of the G2 glycoprotein of Rift Valley Fever virus (a Phlebovirus) from

clones producing B-galactosidase fusion proteins in *E. coli*. Person *et al.* (1985) isolated the glycoprotein B (gB) gene of herpes simplex virus type 2. They cloned DNA sequences coding for a portion of the HSV-2 gB peptide into a bacterial *lacZα* expression vector and transformed *E. coli*. Successful detection of a 500 amino acid, antigenically active, polypeptide encoded by an HSV-2 derived insert was accomplished with a polyclonal antibody prepared against HSV-2.

The sequences of RAV-2 that I isolated contained two gp37 glycosylation sites (at base pairs 6429 and 6568). Both of these sites are located on the exterior of the viral envelope; the sequences found downstream of these locations code for the transmembrane and intracytoplasmic regions of gp37 (Hunter *et al,* 1983; Perez *et al.*, 1987). Although the products of this region would not be glycosylated in *E. coli*, I believed that, based upon the examples given above, their presence on the exterior of the protein in a native conformation[10] resulted in a good possibility of the production of immunogenically recognizable polypeptides.

Failure of Immunological Detection

Despite the theoretical possibility of antigen detection described above, the antibody preparation used was not able to detect any protein production from the directed subclonings of RAV-2 inserts into λgt11. I was able to show that the antibody could react with native antigens and that the λgt11 host was able to produce recognizable antigen to β-galactosidase antibodies under the immunological screening conditions used. This suggests that the inability of these screenings to demonstrate any immunological reaction was due to the inability of the recombinants to produce a polypeptide specifically recognizable by the RSV antibody.

[10] In adddition to being glycosylated, predictive analysis of the polypeptide encoded by this sequence suggests that its hydrophilic nature would result in its exposure on the exterior of the gp85-gp37 "ball and spike" in its native state.

It is possible that, unlike the experimental systems described above, recognition of the gp37 protein may be dependent upon the presence of glycosylated residues. If this were true then it would be impossible to produce recognizable antigen in *E. coli* due to the lack of post-transcriptional processing capability. Alternatively, it could be considered that the RSV antibody, prepared from chickens infected with subgroup C virus, does not contain antibodies capable of recognizing the subgroup B polypeptides. However, given the homology between the sequences of the clones I isolated and the Prague C strain of RSV in the region corresponding to the inserts, this appears to be an unlikely possibility. The implausibility of this is further reinforced by efforts which have been directed at producing subgroup specific monoclonal antibodies — a task which has proven to be extremely difficult (Lee *et al.*, 1985; Mizuno and Itohara, 1986).

A third explanation, and perhaps the most plausible, is that the polyclonal antibody preparation did not contain antibodies against the gp37 polypeptide, in either glycosylated or unglycosylated forms. The physical ball and spike conformation of gp85 and gp37 results in the presentation of gp85 to the host system while a considerable portion of gp37 is hidden in the mature virion. As well, the 3' portions of the gp37 sequence that was subcloned into λgt11 have been identified as the transmembrane and intracytoplasmic components of the disulphide complex formed by the two glycoproteins (Hunter *et al.*, 1983; Wills, *et al.*, 1984; Perez *et al.*, 1987). In this position, it is virtually impossible for normally exposed epitopes to be found within these domains. Finally, it is known that host specificity (Dorner *et al.*, 1985; Bova *et al.*, 1986) and type-specific antigenicity (Bova *et al.*, 1988) reside within the gp85 protein (although this latter attribute of gp85 was not known when my work was conducted). However, the inability of gp37 to include antigenic determinants is not certain. Incomplete formation of virus particles due to stage-specific blocking or due to cellular lysis (potentially resulting from the host immune response) prior to the completion of particle construction could result in antibodies being formed against gp37 polypeptides. Heider *et al.* (1986) note that the formation of partial proviruses, and the occurrence of developmental blockages are quite frequent. They were able to

detect significant antibodies against reverse transcriptase in serum samples prepared from chickens.

The RAV-2 Sequences

At the time this work was performed there were numerous Rous associated virus and Rous sarcoma virus DNA sequences available in the literature. Schwartz *et al.* (1983) presented the entire sequence of the Prague-C strain of RSV, using virion 35S RNA as a template for cDNA synthesis. Most other published sequences have focused upon the *src* gene and the long terminal repeat regions in Rous sarcoma virus (Hughes, 1982; Katz *et al.*, Takeya *et al.*, 1982; Takeya and Hanafusa, 1982; Onuki *et al.*, 1987) and in the identification of the hypervariable regions (Dorner *et al.*, 1985; Bova *et al.*, 1986; Bova *et al.*, 1988) across a number of avian leukosis virus strains. Much of this sequence data has been derived from similar molecular clones of viral DNA isolated using the method of Schwartz *et al.* (1977). This has led to a well-documented understanding of the LTR regions and the composition of the circular DNA intermediate found prior to integration but there is little literature on the manner in which the retroviral RNA is processed. As a result, the mechanisms of avian leukosis virus antigenicity and host specificity, in relation to the translation and processing of the *env* gene were not well understood. In addition, there was limited sequence data published for RAV-2, a subgroup B virus (Bizub *et al.*, 1984). Thus, although the primary purpose of sequencing the various clones that I isolated was for verification of their composition prior to immunoscreening, the sequence data merits some further examination.

Sequence Homology

The previously published nucleotide sequence of a subgroup B virus (Bizub *et al.*, 1984) does not provide any information upstream of base pair 6422 (per the numbering scheme of Schwartz *et al.*, 1983). Sequences obtained from pGR2512G19 extend this in the 5' direction to base 6334. It is of interest to note that the

```
                  6350
pGR2512G19   CTTTGC GTCTATCTTA GCCCCGGGG GTAGCAGCTG CACAAGCCTT
PR-C RSV     ------ A--------- --------- ---------- -G--------
rASV1441     ------ ---------- --------- ---------- ----------

             6390
pGR2512G19   AAGAGAAATT GAGAGACTAG CCTGTTGGTC CGTTAAACAG GCTAACT
PR-C RSV     ---------- ---------- ---------- ---------- -------
rASV1441     --A------- ---------- ---------- ---------- -------
```

Figure 21. Conservation of the Amino Terminus of gp37: PR-C RSV (subgroup C) — Schwartz *et al.* (1983); rASV1441 (subgroup A) — Takeya *et al.* (1982).

sequence in this region is very similar to that obtained for subgroup A and C type virus (see Figure 21). The two nucleotide differences from the sequence of PR-C RSV are silent mutations, maintaining the amino acid residue (alanine) predicted in PR-C-RSV. The difference from the rASV1441 sequence, at position 6381, predicts an arginine residue, as in PR-C RSV, instead of the lysine residue predicted for rASV1441.

The remainder of the sequence data confirms the sequence of the 3' end of RAV-2 obtained by Bizub *et al.*, 1984 with the exception of the region between nucleotides 9179 and 9181 for plasmid pGR2512G19 (see Figure 18 in Results). My isolate appears to have a 22 bp sequence inserted into this region. If the expanded region from nucleotide 9177 to 9186 (in RSV) is examined, a triple repeat of

'GTATGATCGT' can be discerned. This repeat includes the 22 bp repeat plus flanking sequences 5' and 3' to the point of insertion. The formation of the repeat appears to have been accompanied by the loss of two nucleotides at positions 9179 and 9180. No explanation of this replication is possible; because the original sequencing was limited to determining homology with Rous sarcoma virus this anomaly was not examined further.

Polyadenylation Sites

The poly(A) addition signal AAUAAA (Proudfoot and Brownlee, 1974) is found at nucleotides 9285 to 9290 in PR-C RSV (Schwartz *et al.*, 1983). This sequence is conserved in other strains of avian sarcoma and leukosis viruses (Hughes, 1982; Bizub *et al.*, 1983) and is found in the sequences determined for the RAV-2 inserts isolated in my study (plasmids pGR2503-42, pGR2419G19 are shown in Figure 18; see Appendix B for others). Schwartz *et al.* (1977) noted the presence of three potential polyadenylation sites, at nucleotides 9307, 9310 and 9312 in the extreme 3'-terminal region. However, since almost all subsequent studies have used molecularly cloned circular DNA isolated from infected cells, there has been no determination of which of these sites is actually used for polyadenylation or whether any site is consistently used. From a sample of eight clones for which the sequence I obtained included a part of the poly-A tail, five placed the polyadenylation site at the nucleotide corresponding to position 9307 (in PR-C RSV) and three placed the site at nucleotide 9310. None of the clones which I sequenced demonstrated poly-A addition beginning at position 9312. Because of the small sample size I worked with, it is not known whether the use of these sites is characteristic of RAV-2 or if the actual site of polyadenylation is dependent upon other factors. These results do indicate the use of at least two different sites for polyadenylation in RAV-2.

cDNA Quality vs Probe Composition

Despite the conclusions reached above regarding the difficulties encountered in the immunological screenings, it would have improved the comprehensiveness of the experiments if a probe for the gp85 coding region of *env* could have been used. However, the manner in which pSR-XD2 was constructed (see Appendix A) and the separation of the *env* coding region into two sites made it extremely difficult to obtain a probe of a reasonable length with homology to gp85. This problem was exacerbated by the fact that those restriction fragments which could have been isolated would have contained a high percentage of one of the hypervariable regions (Dorner *et al.*, 1985) and that the subgroup A composition of these sequences was likely to differ significantly from the subgroup B RAV-2 sequence. I felt that the reduced stringency of hybridization necessary to allow binding of such a probe might have significantly increased background levels during colony hybridizations. Work with the longer *Sma*I probe, which did extend into the gp85 region, and gave rise to high background levels of hybridization, seemed to confirm this suggestion.

I expected that given the quality of the cDNA, the *Nco*I probe should have been sufficient to isolate sequences extending into the gp85 coding region. It is difficult to understand why this was not observed. None of the eleven clones which were isolated on the basis of hybridization to the *Nco*I probe included sequences which extended into the gp85 region. It is possible that the average length of the cDNA library was not as large as predicted from testing. It is also possible that the presence of gp85 sequences diminished cellular viability to the point where they were under-represented in the population or that their presence resulted in deletions of the coding regions or loss of the plasmid. This explanation is most reasonable for the portion of the insert population which would be statistically expected to be inserted in an open reading frame since the most likely cause of this reduced viability would be the presence of the foreign, expressed protein. No evidence of such selection was observed.

In hindsight, the negative immunological results which were obtained from the gp37 sequences subcloned into λgt11 did not support the strategy of using the shorter *Nco*I probe. The complex cloning strategy necessary for the construction of a gp85 specific probe, containing no hypervariable region sequences, could have been carried out to enable more conclusive results. Assuming that cDNA sequences of the required length existed within the library, a probe with this construction would have presented a better chance of isolating them.

Future Work

Since my experimental work has been completed, numerous improvements and advances in the areas I was studying have occurred. These would have a significant impact on the manner in which unresolved questions arising from my work would be answered. In recognition of this I have first identified what work remained to be done at the time of completion of my experiments. I have followed this with an overview of the current knowledge and how this affects these results and future work in the areas discussed in this thesis.

Pseudorabies Virus

The work on pseudorabies virus was halted because of references in the literature regarding the special requirements for antibody preparations for the successful detection of antigen prepared from recombinant cells. Because of the restrictions placed on working with any pseudorabies viral material in Canada and the expertise in immunology required to prepare the antibody, it was not possible to have proceeded with this project as originally defined. However, the ability to work directly with PrV DNA, outside the biohazard controls of the Animal Diseases Research Institute could have allowed for alternative approaches. Although Petrovskis *et al.* (1986) had succeeded in using immunological detection techniques to obtain λgt11 clones producing por-

tions of three PrV glycoproteins, there were numerous other glycoproteins, thought to be antigenically active, that had not been isolated. Allen and Yeargan (1987) used monoclonal antibodies to obtain recombinant λgt11 coding for six major equine herpesvirus glycoproteins. Because of the homology between the various members of the herpesvirus family these clones could have been used as probes to isolate PrV DNA sequences coding for similar glycoproteins, from the genomic library I had constructed. By sequencing these clones and subcloning fragments into open reading frames to produce fusion proteins, the requirements for immunological detection could be positively ascertained.

Today, with the completed identification of all glycoprotein products of pseudorabies virus and the mapping and sequencing of the genes encoding these proteins, the emphasis of much recent work has shifted to the identification of the epitopes on the glycoproteins. Most antigenic activity has been attributed to the three major glycoproteins — GI (Fuchs *et al.*, 1990; Jacobs *et al.*, 1990; Van Oirscot *et al.*, 1991), GIII (Zuckerman *et al.*, 1990; Yamada *et al.*, 1991) and gp50 (Eloit *et al.*, 1990). These workers have succeeded in identifying and cloning defined epitopes from each of these proteins. Although there is no mention in the literature of the use of bacteria for the production of pseudorabies virus proteins, Inumara and Yamada (1991) have succeeded in producing a GIII virus neutralization antigen from a baculovirus expression vector. With the availability of increasingly sensitive ELISA tests (Afshar *et al.*, 1987) and the recent developments using insect cells, the relevance of attempting to produce a recognizable pseudorabies viral antigen in *E. coli* is doubtful.

Rous Associated Virus

Two paths for the continuation of the work with RAV-2 were available. The first would have been to perform the complex subclonings necessary to create a probe with homology to the gp85 region of *env* as discussed above. This probe could then

be used in nucleic acid hybridizations of the RAV-2 cDNA library which was already known to contain a high portion of Rous associated virus DNA sequence. Alternatively, a new cDNA library could have been constructed. To ensure that gp85 sequences were obtained, the primer for library creation could have been changed from oligo-dT to a synthetic primer capable of annealing with unique sequences immediately 3' to the sequence coding for the carboxy terminus of gp37. The sequences isolated from this library could be subcloned into a strong expression vector as was done in the work described in this thesis. Since the antibody was known to be capable of detecting RSV antigen, if a complete copy of *env* could be inserted into an expression vector in the correct reading frame it would provide a definitive answer regarding the ability of RSV antibody to detect unglycosylated residues.

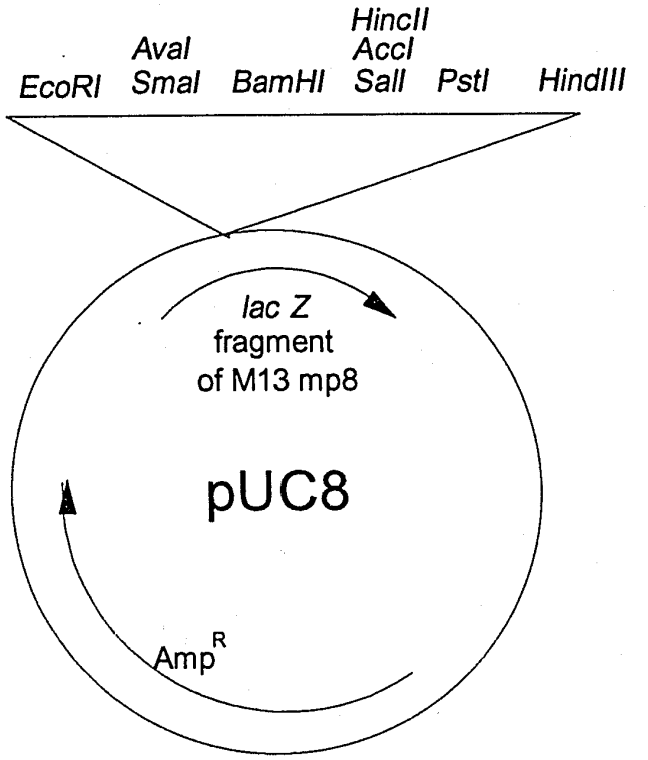
There is little recent literature on cloning and expression of the *env* gene from any of the avian leukosis viruses. Results reported by other laboratories during the course of my laboratory work provided much of the important information concerning host range specificity and antigenic activity (Onuki *et al.*, 1987; Bova *et al.*, 1988). Recent work has focused on expanding upon this information to direct the production of recombinant retroviral vectors, with targeted host ranges (Federspiel *et al.*, 1991) and to gaining an understanding of the relationship between the host range of a subgroup and its oncogenicity (Brown and Robinson, 1988; Svoboda *et al.*, 1990). The level of understanding of the *env* gene and the ease with which the avian leukosis viruses can be maintained in chick embryo fibroblast cells has made the bacterial production of *env* antigens considerably less important than when this work was initiated.

Appendix A - Cloning and Expression Vectors

The cloning and expression vectors which were used in both the pseudorabies virus and Rous associated virus experiments are described in this appendix. Restriction sites important to the work performed are shown.

Figure 22: The pUC Plasmids

pUC8 and pUC 18 plasmids were used as expression vectors in the study of the pseudorabies virus. The portion coding for β-lactamase confers ampicillin resistance to cells transformed with the plasmid. The multiple cloning site (MCS) of M13mp vectors has been inserted in the partial *lac*Z gene included in the vector. By inserting foreign DNA fragments into the *Eco*RI site of the MCS a β-galactosidase fusion protein can be produced. The location of this insertion site is towards the 5' end of the *lac*Z gene in both pUC vectors relative to its position in λgt11 (see Figure 23). (Vieira and Messing, 1982; Norrander *et al.*, 1983)


HincII
AccI
AvaI
SmaI
EcoRI
BamHI
SalI
PstI
HindIII
lac Z
fragment
of M13 mp8
pUC8
Amp^R

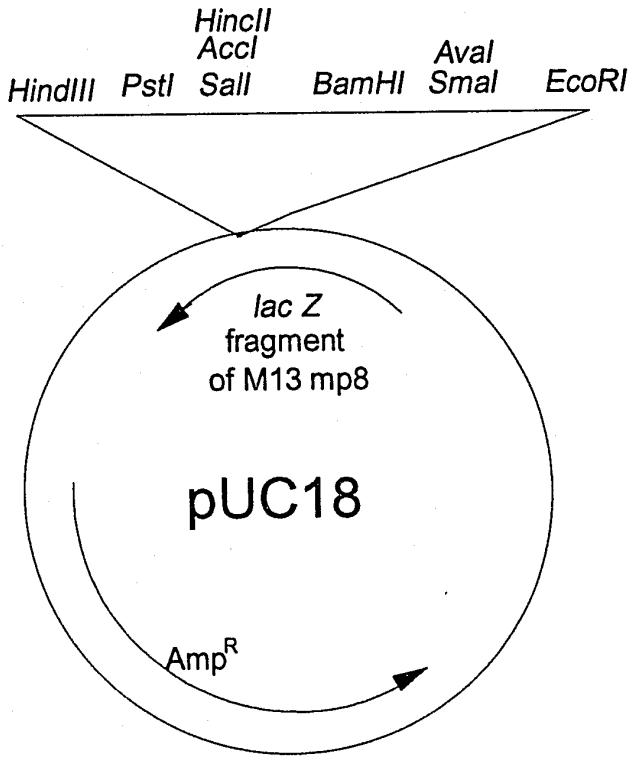

HincII
AccI
AvaI
HindIII
PstI
SalI
BamHI
SmaI
EcoRI
lac Z
fragment
of M13 mp8
pUC18
Amp^R

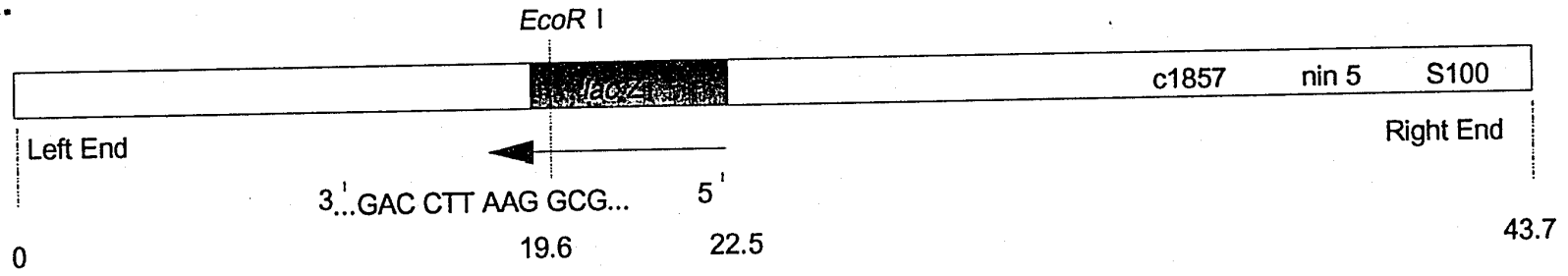
Figure 23: 23A. The Structure of λgt11

The entire *lac*Z gene is included within λgt11. The arrow below the gene indicates the direction of transcription and the nucleotide and amino acid sequence around the *Eco*RI site is shown. Positional markers are numbered in kilobases. The *Eco*RI site is found 53 bp upstream of the β-galactosidase termination codon; cloning into this site results in fusion proteins which contain almost the entire *lac*Z gene product (Young and Davies, 1983).

23B. The pGEM 4B Multiple Cloning Site

The multiple cloning site, derived from pUC19, and the locations of both the T7 and SP6 polymerase promoters are shown. The plasmid also carries the *lac*Zα-peptide coding sequence and the direction of transcription is shown. The sequence shown corresponds to the coding strand of the *lac*Zα-peptide and the T7 primer site. Sequencing reactions were performed upon inserts ligated into the *Sma*I site within the polylinker (Promega Biotech).

A.


EcoR I
lacZ
c1857
nin 5
S100
Left End
Right End
3'...GAC CTT AAG GCG...
5'
0
19.6
22.5
43.7

B.

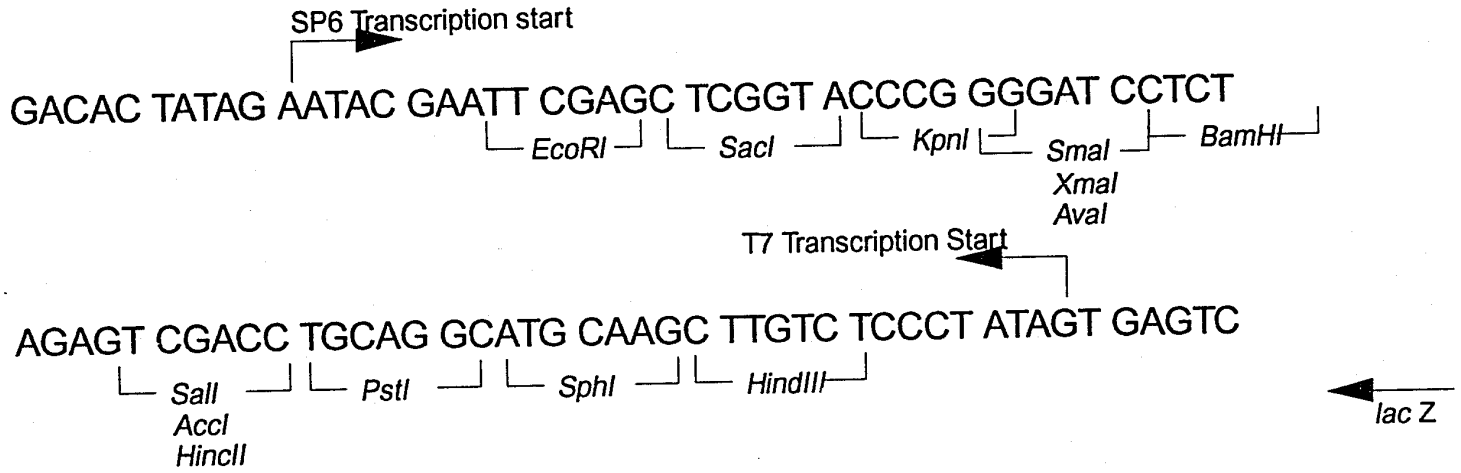
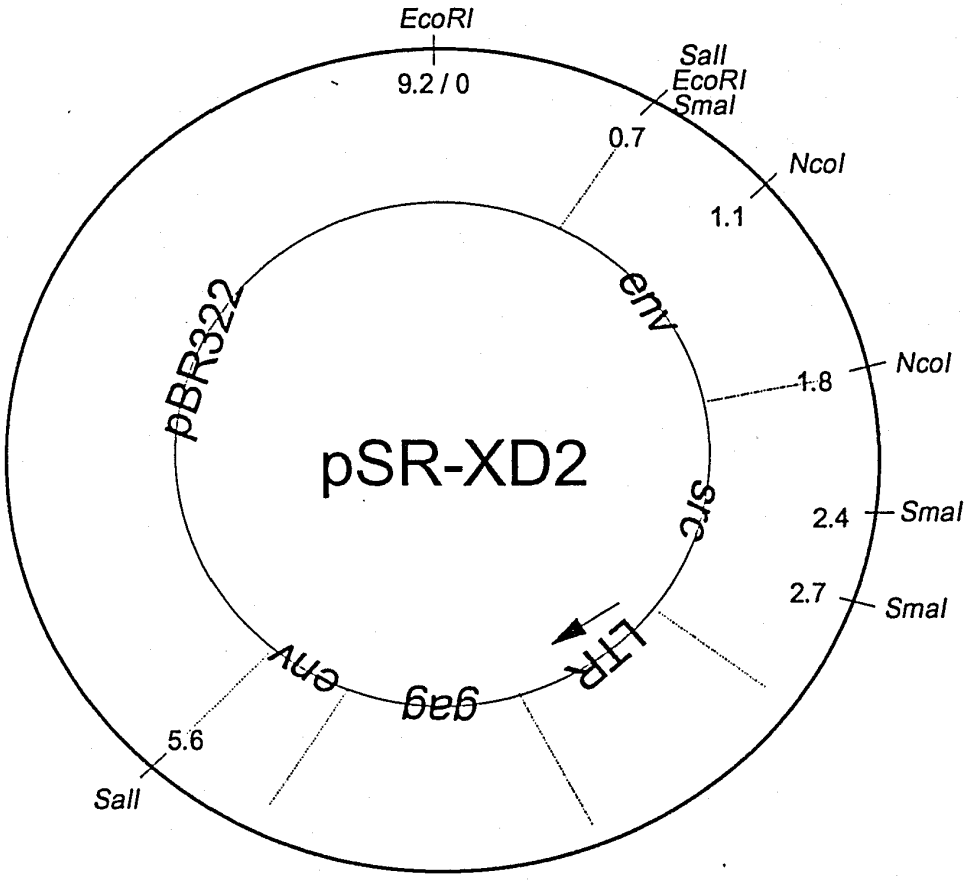

SP6 Transcription start
GACAC TATAG AATAC GAATT CGAGC TCGGT ACCCG GGGAT CCTCT
EcoRI
SacI
KpnI
SmaI
XmaI
AvaI
BamHI
T7 Transcription Start
AGAGT CGACC TGCAG GCATG CAAGC TTGTC TCCCT ATAGT GAGTC
SalI
AccI
HincII
PstI
SphI
HindIII
lac Z

Figure 24: pSR-XD2 Restriction Map and Position of the RSV Genes

pSR-XD2 was developed from a clone of unintegrated Schmidt-Ruppin A RSV DNA ligated into pBR322. The *env* region is divided almost equally by the pBR322 sequences. A 4.5 *Xho*I-*Xho*I deletion has removed most of the *gag-pol* sequences (Cross and Hanafusa, 1983).

The 700 bp *Nco*I probe used extensively in my work corresponds to the region between 1.1 and 1.8 kb on the map. The *Sma*I probe, which was occassionally used, was developed from the restriction fragment between location 0.7 and 2.4 kb.


EcoRI
9.2 / 0
SalI
EcoRI
SmaI
0.7
NcoI
1.1
NcoI
1.8
SmaI
2.4
SmaI
2.7
SalI
5.6
pBR322
env
src
LTR
gag
env
pSR-XD2

Appendix B - RAV-2 Sequences

Sequences were determined for a number of clones isolated from the RAV-2 cDNA library. Representative clones are aligned with the sequence derived for the Prague C strain of Rous sarcoma virus in Figure 18 and are not repeated here. The remainder of the sequences which could be identified as having some homology with the avian sarcoma and leukosis virus sequences are presented here. Each sequence was screened against the MicroGenie databank to determine its likely origin — the base pair numbers provided in the following sequences position each fragment on the PR-C RSV sequence as per a MicroGenie homology search, using the standard numbering of Schwartz *et al.* (1983).

pGR2419-14

Sequenced with T7 primer:

```
                    |9310
TTTTTTTTTTTTTTTTTTGGTGAATGGTCAAATGGCGTTTATTGTATCGAGCTAGGCATT

AAATACATATCTCTGCATGCGGAATTCAGTGGTTCGTCCAATCCGTGTTAGACCCGTCTG

                                                  |9170
TTGCCTTCTAATAAGGCACGATCATACAGATCATACACTTATTCACATG
```

pGR2419-16

Sequenced with T7 primer:

```
                    |9310
TTTTTTTTTTTTTTTTTTGGTGATATGGTCAAATGGCGTTTATTGTATTGAGCTAGGCATT

AAATACAATATCTCTGCAATGCGGAATTCAGTGGTTCGTCCAATCCGTGTTAGACCCGTC

                                       |9158
TGTTGCCTTCTAATAAGGACGATCATACAG
```

pGR2512G17

Sequenced with Sp6 primer:

```
|6499
GGCTATTGACTGTCTTGCTCCTAGCGTGCACGGCCATGGCTGTGAGGAATCATTGGCGTG

                                       |6581
AATGTGTTGTTTATCTGAGTGGAGTCATA
```

pGR2512G17

Sequenced with T7 primer:

```
|9307
TGAATGGTCAAATGGCGTTTATTGTATTGAGCTAGGCACTTAAATACAATATCTCTGCAAT

                |9234
GCGGAATTCAGTGG
```

pGR2512G18

When this sequence was developed it was determined that there was read-through into pGEM 4B sequences on the other side of the RAV-homologous insert. Restriction fragment analysis indicated the presence of a 2900 bp insert - apparently this resulted from a dimerization of the pGEM 4B vector during ligation.

Sequenced with Sp6 primer:

```
                    |9310
TTTTTTTTTTTTTTTTTTTGGTGAATGGTCAAATGGCGTTTATTGTATTGAGCTAGGCAC

TTAAATACAATATCTCTGCAATGCGGAATTCAGTGGTTCGTCCAATCCGTGTTAGACCCG

      |9202
TCTGTT
```

pGR2060126

The sequences which are 3' of nucleotide 9179 show homology to other avian leukosis viruses and fall within the widely divergent area found in several non-transforming strains.
Sequenced with T7 primer:

```
                  |9307
TTTTTTTTTTTTTTTGAATGGTCAAATGGCGTTTATTGTATCGAGCTAGGCACTTAAATA

CATATCTCTGCAATGCGCGAATTCAGTGGTTCGTCCAATCCGTGTTAGACCCGTCTGTTG


                                  |9179
CCTTCGTAATAAGGCAGATCATACCAGATCATACATGATCATACACTTCACTTCACATCG

GATGAGGTGTTTTTTCTTCTTATA
```

pGR20601211

As with pGR2060126, the sequences which are 3' of nucleotide 9179 show homology to other avian leukosis viruses and fall within the widely divergent area found in several non-transforming strains.
Sequenced with T7 primer:

```
                  |9307
TTTTTTTTTTTTTTTGAATGGTCAAATGGCGTTTATTGTATCGAGCTAGGCACTTAAATA

CAATATCTCTGCAATGCGGAATTCAGTGGTTCGTCCAATCCGTGTTAGACCCGTCTGTTG


                  |9179
CCTTCGTAATAAGGCACGATCATACACGATCATACACGATCATACACTTAGTTCACAATG

GCATGCAGGTGTTTTTTCTTCTTATAAGGATGTT
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RÉsumÉ

Le virus pseudorage(PrV), ainsi que les diverses souches de virus du sarcome aviaire et virus de la leucose aviaire sont des pathogènes d'importance économique. Au Canada, la pseudorage est un virus éxotique qui infecte les porcidés. La maladie est rapidement mortelle pour la plupart des espèces, bien que les porcs adultes peuvent devenir porteurs du virus. Ce virus étant exotique au Canada, tous animaux importés sont rigoureusement contrôlés pour la présence de titres élevés d'anticorps contre le virus. La leucose causée par le virus associé au virus Rous (RAV) a un grand impact sur la santé des volailles. Les différentes souches de virus de la leucose aviaire provoquent des dommages variables, mais les caractéristiques générales de l'infection incluent des réductions de production d'oeufs, ainsi que de viande. Il serait nettement bénéfique de pouvoir produire des fractions antigéniques non-infectieuses venant de ces deux virus dans des cellules bactériennes. En effet, ces protéines pourraient être utilisées dans les contrôles d'anticorps de sérum, rendant la détection des deux virus considérablement plus simple que les méthodes d'ELISA et de neutralization de virus utilisées actuellement. De plus, la production de grandes quantités de protéine *env* de RAV pourrait simplifier les études sur le mécanisme de la spécificité à une hôte, tout en offrant de l'information additionnelle sur les récepteurs cellulaires de poule.

J'ai donc essayé de produire une protéine antigénique reconnaissable, provenant des virus PrV et RAV. Des essais ont porté sur l'expression d'une telle protéine du virus pseudorage par clonage de fragments soit d'ADN complémentaire, ou d'ADN génomique, dans des plasmides, suivi par l'immunodétection de colonies avec un anticorps polyclonal porcin contre le PrV. Malgré l'obtention de plusieurs signaux positifs avec certains recombinants, une examination plus minutieuse a révélé qu'aucun d'entre eux ne contenaient de séquences d'ADN de PrV. Les méthodes courantes de hybridation ont été modofiées et améliorées durant le cours de cette étude. Subséquemment, d'autres

laboratoires ont identifié de sérieuses difficultés à l'usage d'anticorps porcins polyclonaux non-purifiés, ce qui a donc offert une explication pour mes résultats observés.

Une librairie d'ADNc construite à partir d'ARN issu de RAV-1 a été cherchée à l'aide d'une sonde contenant des séquences de *env* de pSR-XD2, une plasmide comportant presque toutes la séquence de RSV. Des transformants contenant de l'ADN homologue à RSV ont été identifiés. Durant la purification de ces clones, et les hybridations qui ont suivi, un grand nombre des plasmides identifiées comme contenant des séquences homologues à RSV ont perdu leur insertions. Des études supplémentaires ont démontré que les plasmides comportant les plus fortes homologies à une sonde RSV, et les insertions les plus longs, perdent leurs insertions avec haute fréquence.

Un deuxième clonage d'ADNc utilisant un autre hôte bactérien, et une souche différente de virus (RAV-2) a produit plusieurs clones positifs, portant des insertions de longeur allant de 250 pb à 950 pb. Ces insertions ont été séquencées et comparées aux séquences connues de RSV et RAV. Un haut degré d'homologie a été observé. Les plus longs fragments d'ADNc de RAV-2 contenaient une grande partie de la séquence de *env* gp37. En vue de contrôler l'expression d'un antigène, les insertions provenant de deux clones recombinants indépendants ont été sousclonées dans λgt11 dans chacune des trois cadres de lecture. Un anticorps polyclonal préparé contre le virus du sarcome Rous a été utilisé pour contrôler l'expression d'antigènes reconnaissables. Aucune production d'antigène n'a été détectée, malgré la démonstration par hybridation d'acides nucléiques aux plaques que le sousclonage avait introduit les séquences de RAV-2 dans λgt11 avec succès. Des explications possibles à cet égard, ainsi qu'une discussion portant sur la composition des séquences RAV-2 et leur placement sur la carte génétique de RSV sont considérées.